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FERMENTATION, CELL CULTURE AND BIOENGINEERING

Synthesis of poly(3-hydroxybutyrate) by the autotrophic CO-oxidizing bacterium *Seliberia carboxydohydrogena* Z-1062

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Abstract The present study addresses growth parameters and physiological and biochemical characteristics of the aerobic CO-oxidizing carboxydobacterium *Seliberia carboxydohydrogena* Z-1062. Poly(3-hydroxybutyrate) yields were investigated in experiments with limiting concentrations of mineral nutrients (nitrogen or sulfur or nitrogen and sulfur) in batch culture of *S. carboxydohydrogena* Z-1062 grown on gas mixtures consisting of CO_2, O_2, H_2, and CO. CO concentrations of 10, 20, and 30 % v/v did not affect polymer synthesis, whose content after 56-h cultivation under limiting concentrations of nitrogen and sulfur was 52.6–62.8 % of biomass weight at a productivity of 0.13–0.22 g/L h. The inhibitory effect of CO on cell concentration was revealed at CO concentration of 30 % v/v. That also caused a decrease in substrate (H_2 and O_2) use efficiency. Thus, this carboxydobacterium can be regarded as a potential producer of polyhydroxyalkanoates from industrial hydrogenous sources.

Keywords Carboxydobacteria · Synthesis · Poly(3-hydroxybutyrate) · Carbon monoxide

Introduction

The development and use of novel, eco-friendly materials that can be involved in biospheric cycles correspond to the concept of environmentally safe sustainable industrial production. Thermoplastic microbial polyesters (polymers of hydroxy-derived fatty acids, or polyhydroxyalkanoates, PHAs) have recently aroused much interest among microbiologists, biotechnologists, and materials scientists as analogs of non-degradable polyolefins. The main advantage of PHAs is that they are degraded in the environment without releasing toxic products, which is important because of the growing environmental concerns [1]. Large-scale production and wide application of these polymers cannot be achieved without reducing their cost. Therefore, one of the main lines of biotechnological research is broadening of the trophic potential of PHA-producing microorganisms and finding inexpensive substrates [2]. PHA synthesis can be achieved on various substrates. The best known ones are individual compounds (sugars, alcohols, organic acids), wastes of alcohol, sugar, and hydrolysis industries, olive and palm oil production, etc., [3–5], and unusual substrates, including toxic ones, such as the poorly soluble toxic octane and octanoate [6], methacrylic acid [7], sodium benzoate, and phenol [8]. Among the C_1 carbon sources potentially suitable for PHA synthesis are methane, methanol, and CO_2 [9].

A promising branch of PHA biotechnology is development of technologies for PHA synthesis without employing organic substrates, with CO_2 and H_2 used as sources of constructive and energy metabolism. Carbon dioxide from biochemical and chemical industries may be used as a carbon source. Hydrogen can be produced by various techniques: water electrolysis, which is the most energy-consuming and costly process, conversion of natural gas, and gasification of natural carbonaceous materials (brown coals, wood, plant wastes, sewage sludge, etc.) [10].

Gaseous hydrogen mixed with carbon dioxide and oxygen, although poorly soluble and explosive, has also attracted the attention of researchers studying PHAs [11, 12]. The main advantages of hydrogen-based biosynthesis

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are its high energy efficiency and environmental friendliness. The PHA yield of this process is close to 1, while alcohol- and sugar-based synthesis produces PHAs in lower yields: between 0.25 and 0.3 g/g [12, 13]. Moreover, the only side product of hydrogen oxidation reaction is water.

Until recently, PHA biosynthesis on CO_2 and H_2 as the main growth substrate has been consistently studied by two teams: the Department of Biological and Environmental Chemistry, Faculty of Humanity-Oriented Science and Engineering, Kinki University, Kayanomori, Iizuka-si (Japan) and the Laboratory of Chemoautotrophic Biosynthesis at the Institute of Biophysics SB RAS (Russia). The Japanese team scaled up P(3HB) synthesis in fermenters with high mass exchange parameters, in the culture of *Alcaligenes eutrophus*, and achieved cell biomass and polymer content of 91.3 g/L and 61.9 %, respectively [11, 14]. The research team at the Institute of Biophysics SB RAS produced P(3HB) and copolymers with different chemical structures in autotrophic cultures of the strains *Ralstonia eutropha* B5786 and *Cupriavidus eutrophus* B-10646 [12, 15].

Biotechnological research aimed at PHA synthesis on hydrogenous products of processing of carbonaceous materials such as syngas has been discussed in a number of papers [16–18]. Industrial hydrogenous gas mixtures—by-products of natural gas conversion and high-temperature gasification of low-grade coals, wood, plant wastes, etc., which contain various proportions of H_2, CO, CO_2, and CH_4—can be used to manufacture biotechnological products, including PHAs.

In recent years, more studies have been published that describe the use of well-known and new organisms to produce cell biomass and PHAs from CO_2 and H_2 [16–18]. Moreover, some of the hydrogen-oxidizing bacteria were found to be CO resistant. These are hydrogen-oxidizing bacteria of the genus *Cupriavidus* (formerly known as *Ralstonia*, *Alcaligenes*), representatives of the genera *Pseudomonas*, *Seliberia*, *Comamonas*, *Azotobacter*, *Ideonella*, *Rhodospirillum*, etc. These PHA producers may be grown on industrial hydrogenous sources, which contain not only carbon dioxide and hydrogen but also a very toxic carbon monoxide. The first bacterial strain that was described as CO resistant was *Alcaligenes eutrophus* Z1—a strain maintained in G.A. Zavarzin's collection at the Institute of Microbiology RAS (Moscow) [19]. The hydrogenase of this microorganism is not inhibited by CO [20]. Then, *R. eutropha* B5786 was studied for CO resistance and the ability to grow and synthesize PHAs in the presence of CO; those studies showed that CO did not inhibit the key enzymes involved in the PHA intracellular cycle [21]. Based on those results, for the first time in practical biotechnology, PHA synthesis was conducted on model gas mixtures in the presence of CO and on syngas produced by gasification of brown coals from the Kansk-Achinsk coal mines and hydrolysis lignin [22–24]. That research proved the feasibility of producing PHAs from hydrogenous products of processing of natural carbonaceous materials (natural gas, low-grade coals, hydrolysis lignin, etc.).

In a rather recent study, Tanaka et al. reported CO resistance of the hydrogen-oxidizing bacteria *R. eutropha* ATCC7697 and *Alcaligenes latus* ATCC29712 and their ability to synthesize PHAs in the presence of CO; the authors isolated and studied a new microorganism, *Ideonella* sp. O-1, capable of synthesizing P(3HB) in high yields in the presence of CO [17]. Do et al. described *Rhodospirillum rubrum* culture growth and P(3HB-co-3HV) synthesis on model gas mixtures containing CO and on the syngas produced by gasification of corn waste [16].

Aerobic CO-oxidizing bacteria, the so-called carboxydobacteria, are capable of using CO oxidation reaction as a source of energy and CO_2 produced by this reaction as a substrate for constructive metabolism, which is assimilated via the reductive pentose phosphate cycle. On the $CO_2 + O_2 + H_2$ gas mixtures, some of the strains of these microorganisms can grow at high rates, similar to those of true hydrogen bacteria, producing large biomass [19]. In the 1980s, German scientists investigated the growth of carboxydobacteria in batch culture and examined enzymatic oxidation of CO [25]. Russian researchers studied physiological and biochemical properties, including CO oxidation activity, of several strains of carboxydobacteria maintained in the Collection of Chemolithoautotrophic Cultures at the Institute of Microbiology RAS: *Seliberia carboxydohydrogena* Z-1062, *Pseudomonas gazotropha* Z-1156, *Comamonas compransoris* Z-1155 [19, 26]. *S. carboxydohydrogena* Z-1062 was used to grow the first continuous culture of carboxydobacteria and to study growth kinetics and composition of intracellular molecules as related to the composition and proportions of components in the gas mixture and CO concentration [27, 28].

CO should rather be considered as an external inhibitor, since, as we found previously, its oxidation rate is low (30–50 μM CO/min g protein) [12]. Although carboxydobacteria have an enzymatic system for CO oxidation, during cultivation of cells in the presence of CO_2 and H_2, CO concentration in the gaseous phase would have inevitably increased if the mixture composition had not been adjusted by adding the gases consumed by the culture: CO_2, O_2, and H_2.

It is well known that in the majority of microorganisms, inhibitors cause more dramatic consequences than limiting concentrations of growth substrates or non-optimal values of the medium pH or temperature. CO, as a strong respiratory poison, usually inactivates iron-bearing enzymes in the electron transfer chain. Our previous studies showed, however, that in experiments with CO-resistant

carboxydobacteria, an increase in CO concentration caused a decrease in specific growth rate of the cells but enhanced the activity of hydrogenase (the key enzyme of hydrogen metabolism), increased cytochrome concentrations, and caused the cell membrane apparatus to become more complex [28]. At the same time, the energy substrate (hydrogen) consumption increased considerably (by a factor of 1.5–2), and the protein-synthesizing activity of ribosomes remained high. That is, CO adversely affected the consumption of the energy substrate but did not inhibit synthesis of nitrogen-bearing cell macromolecules.

Results of the initial evaluation of carboxydobacteria as potential PHA producers were reported in a number of studies [29, 30], which showed that the main factors inducing P(3HB) accumulation in *S. carboxydohydrogena* Z-1062 cells were mineral (sulfur, nitrogen, phosphorus) deficiencies in the medium and that the highest intracellular polymer content in the continuous culture deficient in these minerals reached 28 %, with the specific growth rate of 0.05–0.12 h^{-1}. In the batch culture on the $CO_2 + O_2 + H_2$ gas mixtures, in the absence of CO, the P(3HB) yield was higher (40–45 %) [31]. The authors of those studies also reported that in autotrophic batch culture of Z-1062, supplementation of the medium with alkanoic acid salts induced synthesis of PHA terpolymers, which, in addition to the major molar fraction of 3-hydroxybutyrate (74–83 mol%), contained 3-hydroxyvalerate (1.0–3.2 mol%) and 3-hydroxyhexanoate (15.0–25.0 mol%); the total polymer yield was, however, rather low (3.3–8.7 %).

The purpose of this research was to study P(3HB) synthesis by autotrophic *S. carboxydohydrogena* Z-1062 culture from the gaseous substrate in the presence of CO, to increase productivity of the process.

Materials and methods

Bacterial strain, media and growth conditions

Seliberia carboxydohydrogena Z-1062 is registered in the Russian Collection of Industrial Microorganisms (VKPM). The strain was kindly donated to the Laboratory of Chemoautotrophic Biosynthesis at the Institute of Biophysics SB RAS by G.A. Zavarzin, Full Member of RAS. *S. carboxydohydrogena* Z-1062 was grown in continuous culture and thoroughly studied.

Cells were grown under batch cultivation conditions in a laboratory installation constructed at the Institute of Biophysics SB RAS. The installation was explosion proof and hermetically sealed; it contained gas and liquid closed loops, a fermenter, a gasholder, compressors, a gas feeder, gas analyzers, optical density sensors, temperature sensors, pH meters, etc (Fig. 1). The fermenter was cylinder shaped, 10 L in volume, equipped with a turbine-type mixer at 1000 rpm. The volume coefficient of mass transfer for oxygen (KLa) was 460 h^{-1}. The liquid loop contained a system of sensors and a peristaltic pump to maintain medium flow through the culture vessel. Readings of gas analyzers,

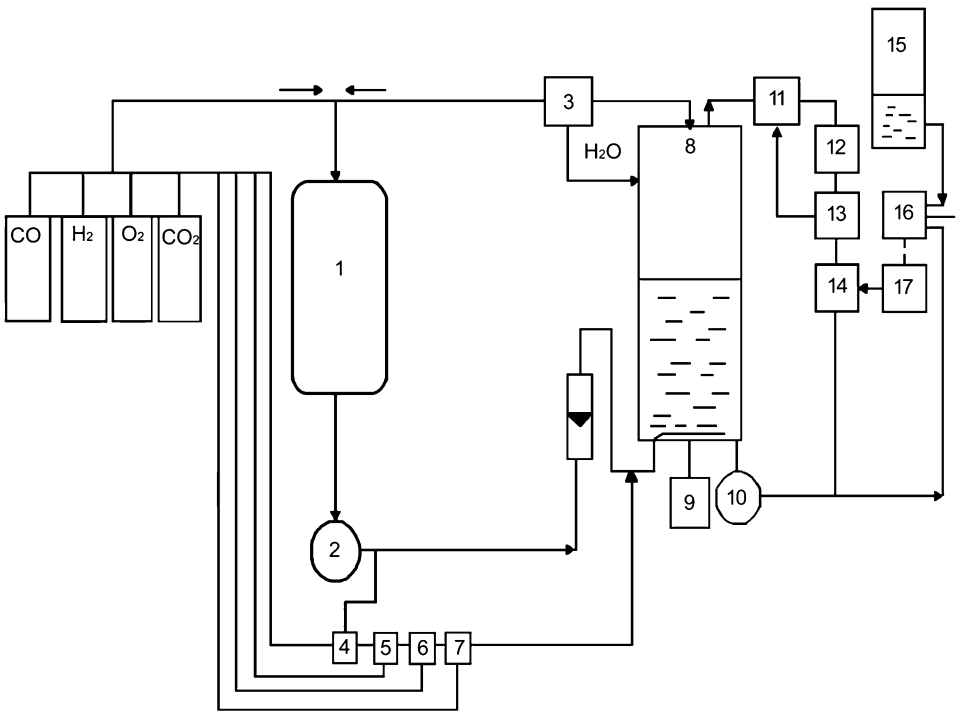

Fig. 1 A schematic diagram for continuous cultivation of bacteria: *1* gasholder, *2* compressor, *3* moisture separator, *4–7* gas analyzers (H_2, O_2, CO_2, CO), *8* fermenter, *9* engine of agitator, *10* pump, *11* condenser, *12–14* sensors of temperature, pH, optical density, *15* a nutrient medium vessel, *16*, *17* dispensers

thermometers, and pressure gauges were recorded by the secondary devices. Gas analyzer cases were hermetically sealed and purged with nitrogen. The main growth substrate was the gas mixture, which was prepared in a 50-L metal gasholder. A recycled-gas closed-circuit culture system was used. Specified proportions of growth substrates and the carbon (CO_2) and energy source (H_2) were mixed with O_2 and CO in a 50-L gasholder. A diaphragm compressor was used to continuously pump the gas mixture through the culture, at 8–12 L/min. The compressor structure and materials were also explosion proof.

The initial proportions of CO_2, O_2, and H_2 in the control were 1:1:8 v/v, respectively. When 10–30 % v/v CO was added to the gas mixture, H_2 concentration decreased accordingly. The addition of 10 % CO reduced H_2 concentration to 70 % v/v; at 20 % CO, H_2 concentration was 60 % v/v; and at 30 % CO, H_2 concentration was 50 % v/v. O_2 and CO_2 concentrations were 10 and 10 % v/v, respectively, and they did not change. At 10 % CO, its concentration in the solution was 2 mg/L, at 20 %—4 mg/L, and at 30 %—6 mg/L.

The fermenter was 30 % filled with the culture medium. Cells were grown on Schlegel's mineral medium: $Na_2HPO_4 \cdot H_2O$—9.1; KH_2PO_4—1.5; $MgSO_4 \cdot H_2O$—0.2; $Fe_3C_6H_5O_7 \cdot 7H_2O$—0.025; NH_4Cl—1.0 (g/L). At cell concentrations in the fermenter higher than 10 g/L, we used urea as a nitrogen source, as additions of the necessary amounts of NH_4Cl would have inevitably caused accumulation of Cl^- ions in the culture medium. That would have acidified the medium and pH would have needed to be adjusted. Because of the use of urea, no pH adjustment was needed. A solution of iron citrate (5 g/L), which was used as a source of iron, was added to reach a concentration of 5 ml/L. Hoagland's trace element solution was used: 3 ml of standard solution per 1 L of the medium. The standard solution contained H_3BO_3—0.288; $CoCl_2 \cdot 6H_2O$—0.030; $CuSO_4 \cdot 5H_2O$—0.08; $MnCl_2 \cdot 4H_2O$—0.008; $ZnSO_4 \cdot 7H_2O$—0.176; $NaMoO_4 \cdot 2H_2O$—0.050; $NiCl_2$—0.008 (g/L).

To achieve P(3HB) synthesis, cells were grown in fed-batch culture at pH 7.0 and temperature 30 °C during 56 h. As cell concentration increased, nitrogen and minerals were periodically added to the fermenter. To achieve maximum P(3HB) accumulation, cells were cultured in the batch mode, under a limiting concentration of one of the major elements, which, under such conditions, constituted 50 % of the initial concentrations: 0.5 g/L of NH_4Cl or $CO(NH_2)_2$ and 0.1 g/L of $MgSO_4$.

Monitoring the process parameters

PHA biosynthesis was evaluated based on cell concentration, polymer yield, the amount of the main growth substrate used, and process duration and productivity. Conventional methods were used to determine kinetic and

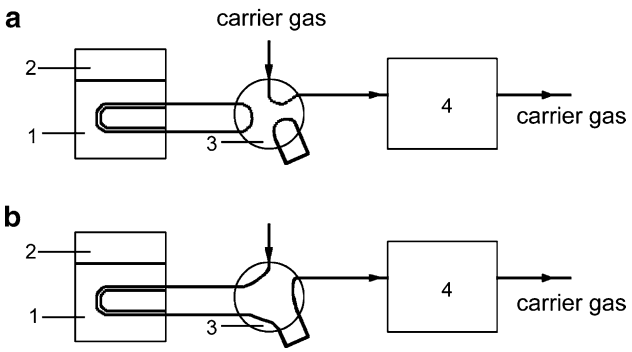

Fig. 2 A schematic diagram of gas analysis: *1* gas tube, *2* bioreactor, *3* six-way gas cock, *4* chromatograph; **a** sampling (in the diffusion mode); carrier gas; **b** sample analysis

production parameters of the culture. The cell biomass yield (X, g/L), the gas flow rate (G_r, g/h), the yield coefficient of the culture (Y, g biomass/g), and the specific growth rate (μ, h^{-1}) were calculated. In experiments with NH_4Cl as a nitrogen source, we measured ammonium ion concentration using a photometric method, with Nessler's reagent. Urea in the medium was measured using a Urea 450 kit (Erba Lachema, Czech Republic).

Under autotrophic conditions, gas substrate concentration was determined continuously, using gas analyzers, and in culture samples—using a "Maestro 7820A" gas chromatograph, with a katharometer detector (Interlab, Russia). The rate of gas consumption by the culture was determined monometrically. We used a chromatograph to determine gases dissolved in the culture medium (H_2, O_2, CO, and CO_2). A semipermeable silastic tube submerged in the culture was a sampler and a metering loop (Fig. 2).

The loop operated in the pulse mode: the tube purged with the carrier gas was excluded from the flow and functioned in the diffusion mode; then the tube was included in the carrier gas flow, which transported the gas mixture from the loop to the chromatograph detector. Preliminary experiments were conducted to determine the time when gas concentrations in the sampling tube and in the culture came into equilibrium. Complete analysis of the five-component gas mixture took about 5 min. The relative error was 1 %. The accuracy and reproducibility of determining dissolved gases was verified for oxygen by the method of gas chromatography and using the iodometric (Winkler) procedure. The largest deviation of the dissolved oxygen values measured by these methods was 0.08 mg/L [12].

During the course of cultivation, culture samples were taken for analysis: cell concentration in the culture was determined based on the weight of the cell samples dried at 105 °C for 24 h (DCW) per 1 L. Cell concentration in the culture was monitored every hour by converting the optical absorbance at 440 nm of culture broth to dry cell weight using a standard curve prepared previously. PHA

biosynthesis was evaluated based on cell concentration, polymer yield, the amount of the main growth substrate used, the process duration and productivity.

Biochemical analyses

The content of protein in cells was determined by the Lowry method. The content of nucleic acids in cells was determined by Spirin's method [32]. The main methods of analysis of lipids and fatty acids were described in detail elsewhere [33]. Briefly, the lipids were extracted from wet biomass according to the Folch procedure with the chloroform–methanol mixture (2:1 v/v). In the resulting extract, PHAs were separated from the lipids by precipitation with a double volume of hexane. The lipid extract was dried in a rotary evaporator and treated by methanolysis to produce fatty acid methyl ethers (FAMEs). Methanolysis of fatty acids was carried out for 2 h in the mixture of methanol and sulfuric acid (50:1 v/v) at 90 °C. FAMEs were analyzed on a GC-MS 7890/5975C (Agilent Technologies, U.S.).

Analysis of PHA structure and properties

Intracellular PHA content at different time points was determined by analyzing samples of dry cell biomass. To analyze PHA composition, the polymer was extracted with chloroform, and then the monomer composition was investigated by chromatography of fatty acid methyl esters on an Agilent Technologies 7890A chromatograph-mass spectrometer (U.S.). Molecular weight and molecular weight distribution of PHAs were examined using a gel permeation chromatograph ("Agilent Technologies" 1260 Infinity, U.S.) with a refractive index detector using an Agilent PLgel Mixed-C column. Chloroform was the eluent, at a flow rate of 1.0 mL/min at 40 °C. Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). X-ray structure analysis and determination of crystallinity of PHAs were performed using a D8 ADVANCE X-ray diffractometer ("Bruker, AXS", Germany).

Results

Accumulation of P(3HB) by the carboxydobacterium *S. carboxydohydrogena* Z-1062 cultivated under autotrophic conditions is induced by limiting concentrations of nitrogen or sulfur in the culture medium, as suggested by results of a previous study [30]. Unfavorable growth conditions, including non-optimal pH or the temperature of the medium and oxygen, hydrogen or CO_2 deficiency or excess, did not affect the constructive metabolism of *S. carboxydohydrogena* Z-1062 cells. No changes occurred in proportions of the main carbohydrate or lipid macromolecules (protein and nucleic acids) and storage ones. PHA synthesis was not affected either [27, 34]. A previous study also showed that the inhibitory effect of CO was exhibited by changes in consumption of gas substrate components: an increase in consumption of energy substrate (H_2) and a decrease in substrate use efficiency. The evidence of the inhibitory effect of CO on constructive metabolism of the cells was a change in the state of their membranes: more cyclic fatty acids were formed and the FA composition became more saturated [28].

The effect of two-factor limitation of carboxydobacterium growth on P(3HB) synthesis

Kinetic and production parameters of *S. carboxydohydrogena* Z-1062 culture were studied in experiments with cell growth limited by nitrogen or sulfur deficiency and in experiments with limiting concentrations of both elements. Nitrogen and sulfur concentrations in the medium reached 50 % of the amounts needed for normal physiological function of the cells. The composition of the gas mixture and CO amount were determined by the μ/Sco relationship previously found for this strain. CO_2 and O_2 concentrations were maintained constant, 10 and 20 % v/v, respectively. CO concentrations used in different experiments were 10, 20, and 30 % v/v; hydrogen concentrations were 50, 60, and 70 % v/v. Thus, proportions of gases in the $CO{:}CO_2{:}O_2{:}H_2$ mixture were varied between 1:1:1:7 and 3:1:1:5 v/v. As the cells consumed the gases, the composition of the gas mixture was adjusted to keep the proportions of the components steady by feeding definite amounts of gases into the gasholder.

Changes in cell biomass and P(3HB) concentration under limiting concentrations of nitrogen, sulfur or nitrogen + sulfur and different compositions of the gas mixture are shown in Figs. 3 and 4; the composition of cell macromolecules and hydrogen-based yield coefficient are given in Table 1. Figure 5 shows SEM images of *S. carboxydohydrogena* Z-1062 with different intracellular polymer contents. As polymer content increased, more granules were observed: their number varied between 2 and 3 at 25–30 % P(3HB) and between 12 and 15 at 60–62 % P(3HB). The granules were of different sizes, between 0.3 and 0.8 μm in diameter. As the intracellular polymer content increased, the cells became larger due to impairment of cell division. We previously observed a similar effect in *R. eutropha* B5786 culture [35].

It can be seen from the data in Fig. 3 that 56-h cultivation of *S. carboxydohydrogena* Z-1062 on complete saline medium, with no CO in the gas mixture, resulted in accumulation of cell biomass of 16.7 g/L and P(3HB) yield of

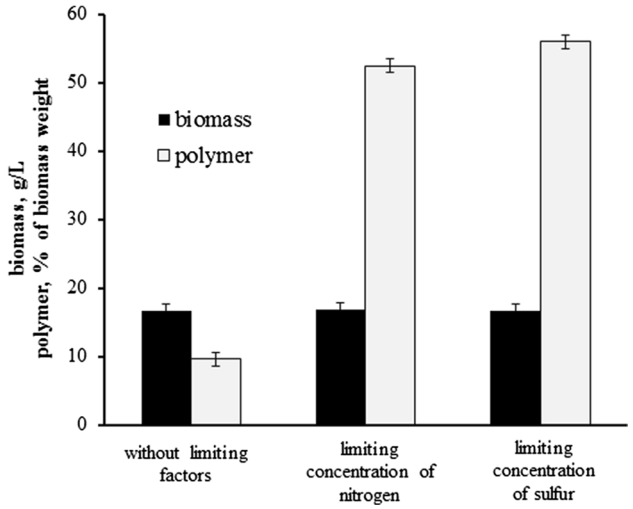

Fig. 3 Biomass and polymer yields of *S. carboxydohydrogena* Z-1062 grown without CO under limiting concentrations of nitrogen and sulfur

9.6 % of biomass weight, with biomass and polymer productivities of 0.3 and 0.03 g/L h, respectively (Table 2).

Cultivation under similar conditions but with limiting nitrogen and sulfur concentrations (50 % of the requirement) yielded a comparable cell concentration, but polymer content reached 52.4–56.0 % of biomass weight when the culture approached steady state (Fig. 3). Polymer productivity increased several fold, reaching 0.16–0.17 g/L h (Table 2).

In the autotrophic culture of carboxydobacterium cells whose growth was limited by two factors (nitrogen concentration and CO), after 56 h of cultivation, when the culture reached steady state, polymer content in cells exposed to different CO concentrations was comparable to that in the culture limited by nitrogen deficiency but without CO in the gas mixture (Fig. 4a), varying between 52.4 and 58.0 % of dry weight. Polymer and biomass productivities were 0.33–0.34 and 0.18–0.19 g/L h, respectively (Table 2). When CO concentration was increased to 30 % v/v, cell concentration decreased. It was the reason for the decrease of polymer and biomass productivities to 0.23 and 0.13 g/L h, respectively (Table 2). An increase in the intracellular polymer concentration did not affect the content of total lipids. Specific growth rate changed as follows: during the first 5–10 h of cultivation, it was about 0.35 h^{-1}, and that was comparable with μ in the experiment without CO. Then, as the culture developed, μ decreased, dropping to 0.25 h^{-1} by the end of the experiment. The slower cell growth affected the contents of nucleic acids: at 30 % CO, the total RNA + DNA decreased to 6.8 % relative to 9.3–8.8 % at 10 and 20 % CO, which did not inhibit cell growth (Table 1).

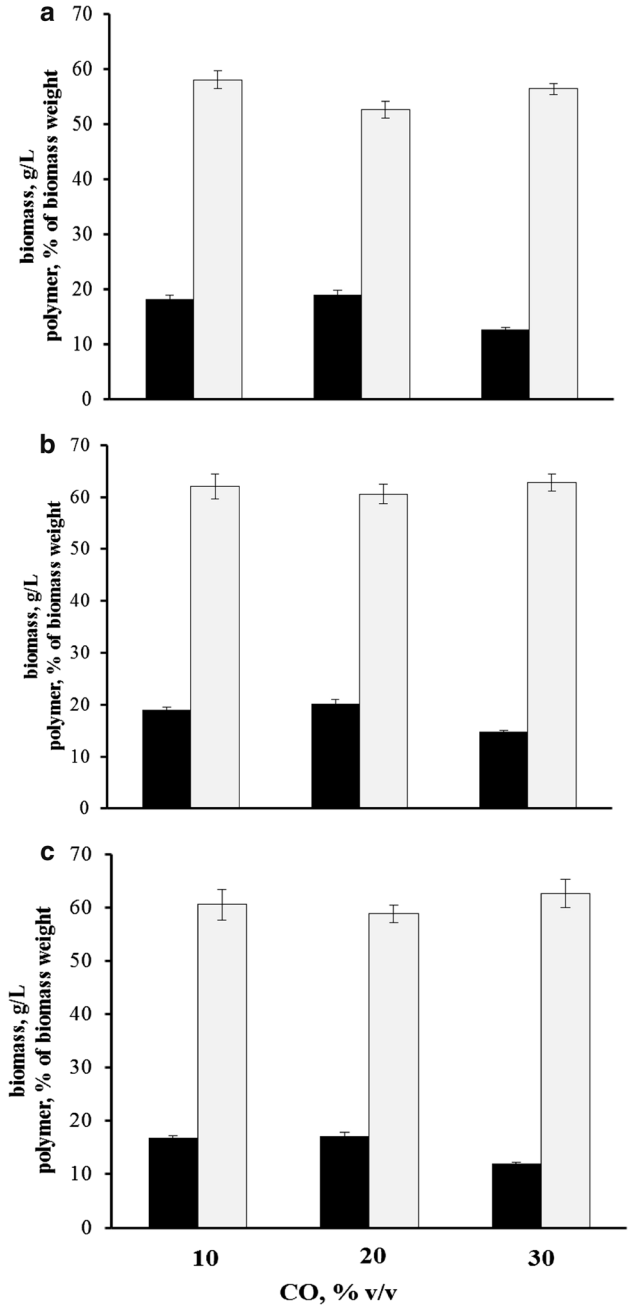

Fig. 4 Biomass and polymer yields of *S. carboxydohydrogena* Z-1062 grown at 10, 20, 30 % v/v CO concentrations under limiting concentrations of nitrogen (**a**), sulfur (**b**) and nitrogen + sulfur (**c**)

It is an important fact that the stoichiometry of consumption of gas components, which quantifies the relationship between the initial substrate and the product, was generally the same for the parameters of the cultures with and without CO. CO concentration did not affect either the consumption of the gaseous substrate or the stoichiometry of gas (CO_2:O_2:H_2) consumption, which at 10 and 20 % CO was 1:2.5:6.0. At 30 % CO, however, gas consumption

Table 1 Parameters of *S. carboxydohydrogena* Z-1062 autotrophic culture synthesizing P(3HB) in the presence of CO, under limiting concentrations of mineral elements (cultivation time—56 h)

Element limiting cell growth	CO (% v/v)	Hydrogen-based yield coefficient (Y_{H2} (g/g))	Protein (% of cell weight)	RNA + DNA (% of cell weight)	Lipids (% of cell weight)	Lipid saturation index
–	0	1.3 ± 0.1	60.2 ± 5.7	10.7 ± 0.8	7.9 ± 0.5	0.63 ± 0.0
N	0	1.15 ± 0.0	28.3 ± 3.1	8.7 ± 0.4	7.4 ± 0.4	0.69 ± 0.0
N	10	1.15 ± 0.1	25.8 ± 2.7	9.3 ± 1.1	6.9 ± 0.5	0.68 ± 0.0
	20	1.0 ± 0.0	26.0 ± 1.6	8.8 ± 0.7	6.8 ± 0.5	1.49 ± 0.1
	30	0.7 ± 0.0	25.1 ± 2.1	6.8 ± 0.3	6.8 ± 0.4	1.98 ± 0.1
S	10	1.05 ± 0.1	18.7 ± 1.1	8.8 ± 0.9	7.6 ± 0.6	0.71 ± 0.0
	20	1.0 ± 0.1	19.3 ± 2.1	9.7 ± 0.5	8.0 ± 0.5	1.46 ± 0.0
	30	0.6 ± 0.0	19.8 ± 1.8	6.2 ± 0.3	8.1 ± 0.3	2.06 ± 0.1
N + S	10	1.07 ± 0.1	20.8 ± 1.6	9.1 ± 0.4	7.6 ± 0.4	0.69 ± 0.0
	20	0.98 ± 0.1	20.4 ± 2.3	10.0 ± 1.0	7.3 ± 0.5	1.57 ± 0.1
	30	0.6 ± 0.0	20.2 ± 1.8	6.5 ± 0.4	6.9 ± 0.5	2.09 ± 0.1

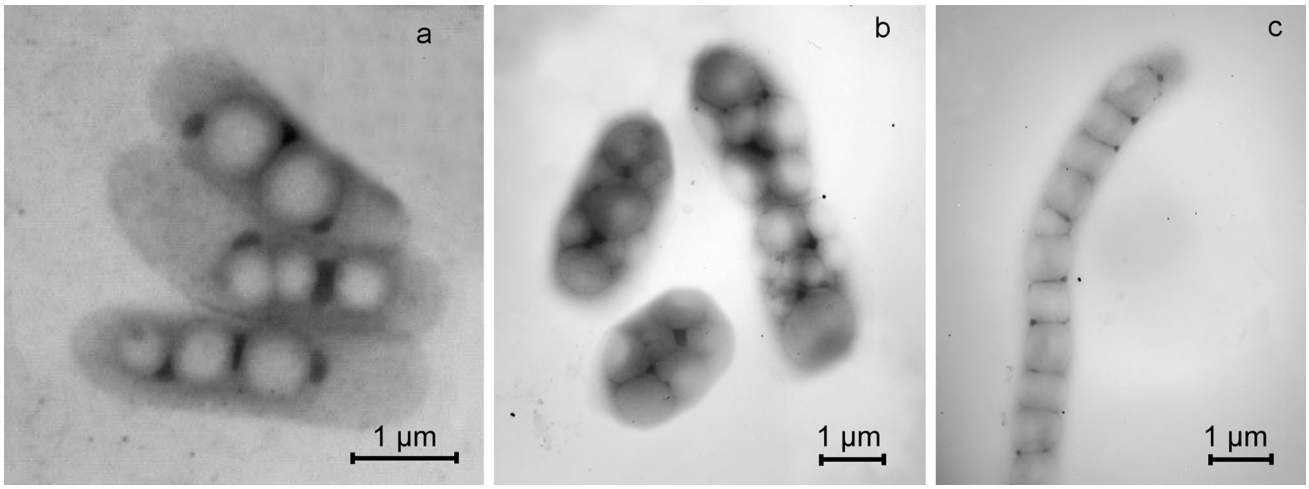

Fig. 5 SEM images of *S. carboxydohydrogena* Z-1062 cells. **a** Cells containing 25–30 wt% P(3HB), **b** cells, containing 58–63 wt% P(3HB), **c** a fragment of a giant *S. carboxydohydrogena* Z-1062 cell

increased due to consumption of O_2 and H_2, with a different stoichiometry: 1:4.2:12.0; hydrogen energy efficiency was noticeably decreased. The consumption of the gas mixture by the cells cultivated on syngas containing 10–20 % v/v CO averaged 12.6 L/g cell biomass over the entire cultivation period. The hydrogen-based yield coefficient varied between 1.0 and 1.15 g/g and was comparable with the control value. At 30 % CO in the gas mixture, Y_{H2} dropped to 0.7 g/g.

In experiments with limiting sulfur concentrations (Fig. 4b), the P(3HB) yield was higher, reaching 61–63 % of biomass weight, but other parameters of the *S. carboxydohydrogena* Z-1062 culture were similar to those in the experiment with nitrogen deficiency (Table 1). Polymer productivity at CO concentrations of 10 and 20 % v/v was 0.21–0.22 g/L h. Polymer productivity at CO concentration of 30 % v/v declined to 0.17 g/L h (Table 2) because of the biomass yield decrease. When cell growth was limited by both nitrogen and sulfur deficiencies and CO concentration was varied between 10 and 30 %, results were similar to those obtained in experiments with limiting nitrogen concentrations and CO inhibition (Fig. 4c; Tables 1, 2), i.e., growth parameters of the culture, consumption of the gaseous substrate, substrate use efficiency, and intracellular macromolecule composition were comparable to the parameters obtained in experiments with nitrogen deficiency.

These experiments showed that *S. carboxydohydrogena* Z-1062 was able to synthesize P(3HB) in rather high yields (reaching 63 % of cell dry weight) in autotrophic culture in

Table 2 Biomass and polymer productivities of *S. carboxydohydrogena* Z-1062 autotrophic culture in the presence of CO, under limiting concentrations of mineral elements

Productivity (g/L h)	Culture conditions											
	Without limiting factors and CO	Limiting concentration of nitrogen				Limiting concentration of sulfur				Limiting concentration of nitrogen and sulfur			
		CO concentration (% v/v)				CO concentration (% v/v)				CO concentration (% v/v)			
		0	10	20	30	0	10	20	30	10	20	30	
Biomass productivity	0.30 ± 0.01	0.30 ± 0.01	0.33 ± 0.02	0.34 ± 0.02	0.23 ± 0.01	0.30 ± 0.01	0.34 ± 0.01	0.36 ± 0.02	0.26 ± 0.01	0.30 ± 0.02	0.31 ± 0.01	0.21 ± 0.01	
Polymer productivity	0.03 ± 0.00	0.16 ± 0.00	0.19 ± 0.01	0.18 ± 0.01	0.13 ± 0.01	0.17 ± 0.00	0.21 ± 0.01	0.22 ± 0.01	0.17 ± 0.00	0.18 ± 0.01	0.18 ± 0.01	0.13 ± 0.00	

Table 3 Fatty acid composition of lipids in *S. carboxydohydrogena* Z-1062 cells grown under nitrogen deficiency at different CO concentrations

Fatty acid	CO (0 % v/v)	CO (10 % v/v)	CO (20 % v/v)	CO (30 % v/v)
14:0	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.0	0.4 ± 0.1
16:1ω7	33.2 ± 2.4	32.9 ± 2.7	21.6 ± 1.3	17.3 ± 1.2
c-17:0[a]	1.7 ± 0.1	2.1 ± 0.1	9.7 ± 1.7	10.3 ± 0.6
16:0	37.0 ± 3.1	35.4 ± 1.9	46.6 ± 2.8	51.6 ± 3.9
18:1ω7	24.7 ± 1.7	25.4 ± 1.8	17.3 ± 0.9	15.0 ± 1.7
18:0	1.1 ± 0.1	1.7 ± 0.1	1.7 ± 0.3	2.1 ± 0.3
c-19:0[a]	0.1 ± 0.0	0.3 ± 0.0	1.0 ± 0.1	1.3 ± 0.1
Others[b]	1.8 ± 0.1	1.9 ± 0.1	1.8 ± 0.2	2.0 ± 0.2
SAT/UNSAT	0.69 ± 0.0	0.68 ± 0.0	1.49 ± 0.1	1.98 ± 0.1

SAT saturated fatty acids including straight, cyclo-chain, *UNSAT* unsaturated fatty acids

[a] Cyclopropane fatty acid

[b] 14:1ω5, 15:0, 15:1ω6, 16:1ω5, 17:0

the presence of CO and at limiting concentrations of mineral elements, even in a fermenter with low mass exchange parameters.

The protective response of the cells to the effect of CO may to a certain extent be attributed to the state and permeability of the cell wall and cytoplasmic membrane of prokaryotes, which are largely determined by the fatty acid (FA) composition of lipids. As shown in Table 1, intracellular total lipid content in the carboxydobacterium was about 7–8 % whatever CO concentration was used. However, the saturation level of membrane lipids varied because of changes in synthesis and proportions of saturated and unsaturated FAs and cyclopropane acids. While in the absence of CO in the gas mixture, the saturation index of cell lipids was 0.63–0.69, at CO concentrations raised from 20 to 30 %, this parameter increased by a factor of 2.1–3.3: unsaturated fatty acids decreased but saturated acids and C17- and C19-cyclopropane acids increased (Table 3).

PHA characterization

Analysis of the chemical composition of the polymer synthesized by *S. carboxydohydrogena* Z-1062 in the presence of CO showed that it was a copolymer containing a homopolymer of 3-hydroxybutyric acid as a major fraction (over 99 mol%) and traces of hydroxyvaleric acid (0.24–0.48 mol%). PHA specimens synthesized in the presence of CO had high molecular weight, which was not influenced by the concentration of the inhibitor (Table 4). Thus, CO did not affect intracellular polymerization of monomers of 3-hydroxybutyric acid. Temperature parameters of the

Table 4 Characterization of PHA specimens synthesized by *S. carboxydohydrogena* Z-1062 on gas mixtures in the presence of CO

CO (% v/v)	Weight average molecular weight (kDa)	Degree of crystallinity (%)	Melting point (°C)	Thermal degradation temperature (°C)
0	600	76	172	188
10	532	72	168	198
20	620	68	170	203
30	720	70	167	195

polymer and its degree of crystallinity were also comparable with the corresponding parameters of the polymers synthesized on the CO_2:O_2:H_2 gas mixture that did not contain CO.

Discussion

The purpose of the present work was to study synthesis of P(3HB) by the autotrophic culture of aerobic CO-oxidizing carboxydobacterium *Seliberia carboxydohydrogena* Z-1062. This study makes a contribution to the search for new and inexpensive substrates for synthesis of degradable polymers of microbial origin, the so-called polyhydroxyalkanoates (PHAs), which are in great demand now [36].

The choice of the substrate for PHA production is based on physiological and biochemical properties of PHA producers and economic practicability of the strategy employed, taking into account the potential area of PHA application. As PHAs are promising materials for medicine, pharmaceutics, food industry, agriculture, and municipal services, the scale of their annual production may vary between several hundred kilograms (for medicine and pharmaceutics) and several hundred thousand tons. Therefore, quality and cost requirements vary.

The bacterial strain *Seliberia carboxydohydrogena* Z-1062 used in this study is capable of growing and producing PHA on gas mixtures containing CO_2, O_2, H_2, and CO. We studied the effects of the two-factor influence on the autotrophic batch culture of *S. carboxydohydrogena* Z-1062 (growth-limiting concentrations of nitrogen or/and sulfur and inhibition by carbon monoxide). CO in the gas mixture reached 10, 20, and 30 % v/v in different experiments. P(3HB) concentration in cell biomass was not affected by CO concentration; after 56 h of cultivation, it reached 52.6–58.0 % in the experiment with nitrogen deficiency, 60.6–62.8 % under sulfur deficiency, and 58.9–62.7 % under nitrogen and sulfur deficiencies. P(3HB) productivity reached 0.13–0.22 g/L h. That was considerably higher than the P(3HB) productivity achieved in previous studies [30], when the yield of P(3HB) synthesized by *S. carboxydohydrogena* Z-1062 cells was 27–28 % of the biomass weight, but polymer productivity was no more than 0.1 g/L h. P(3HB) yields were similar to those achieved in studies of CO-resistant strains of hydrogen-oxidizing bacteria *Ralstonia eutropha* B5786 and *Cupriavidus eutrophus* 10646 [15, 21] and *Ideonella* sp. O-1 at CO concentrations 5 and 20 % v/v [17]. Moreover, they were much higher than intracellular P(3HB) in *Rhodospirillum rubrum* cells, which accumulated 37 % P(3HB) as a major fraction and 3-hydroxyvalerate as a minor one when grown on model gas mixtures in the presence of CO and on syngas produced by corn waste gasification [16].

Inhibition of *S. carboxydohydrogena* Z-1062 growth, accompanied by a decrease in nucleic acid concentrations, occurred at 30 % v/v CO. At the same time, although the fast-growing bacterium *Ideonella* sp. O-1 continued growing even at 70 % v/v CO, its growth rate decreased at 20 % v/v CO in the gas mixture, and, thus, cultivation lasted 12 h longer than under 5 % v/v CO.

Tanaka et al. [17] described a new CO-tolerant hydrogen-oxidizing bacterium, *Ideonella* sp. O-1. The strain is tolerant to O_2 concentrations up to 30 % v/v and is able to grow at CO concentrations reaching 70 % v/v. However, at CO concentration of 20 % v/v, cell growth rate is dramatically decreased. The authors showed that, in contrast to the growth of *Ideonella* sp. O-1, hydrogen-oxidizing bacteria *R. eutropha* ATCC 17697, *R. eutropha* ATCC 17699, and *A. latus* ATCC 29712 were seriously inhibited at CO concentration of 5 % v/v. At H_2, CO_2, O_2, and CO concentrations in the mixture equal to 70, 10, 10, and 10 % v/v, respectively, after 40 h of cultivation, cell concentration of *Ideonella* sp. O-1 reached 6.57 g/L, with polymer content of 4.52 g/L, i.e., P(3HB) concentration in the biomass reached 74 %, and polymer productivity was 0.113 g/L h. These values are comparable with the results obtained in this study, with P(3HB) productivity reaching 0.13–0.22 g/L h under limiting concentrations of nitrogen, sulfur or nitrogen + sulfur, respectively. Moreover, different CO concentrations in the gaseous mixture—10, 20, or 30 % v/v—did not produce any substantial effect on polymer yield. In contrast to *Ideonella* sp. O-1, the growth rate of *Seliberia carboxydohydrogena* Z-1062 was not decreased at 10 and 20 % v/v CO. Tanaka et al. [17] also showed that in the recycled-gas closed-circuit culture system, CO_2, O_2, and H_2 concentrations decreased during cultivation, while CO concentration increased, because the rates of CO oxidation by *Ideonella* sp. O-1 cells were much lower than the rates with which they consumed other gases.

Our results are in good agreement with the data reported by Tanaka et al. [17], showing that in the presence of CO_2 and H_2, CO oxidation rate is low and CO is accumulated in the system; thus, CO should be regarded as an external

inhibitor. Experiments with *S. carboxydohydrogena* Z-1062 demonstrated that the presence of 30 % v/v CO in the gas mixture caused changes in the stoichiometry of gas consumption and led to increased consumption of H_2 and O_2 by the cells and decreased substrate use efficiency. The hydrogen-based yield coefficient was 0.6–0.7 g/g, and that value was lower than in the control (without CO) or in the experiment with 10–20 % v/v CO (0.98–1.15 g/g).

PHA synthesis by *Rhodospirillum rubrum* on the gaseous substrate in the presence of CO was studied by Do et al. [16]. The authors showed that bacterium *Rhodospirillum rubrum* synthesized PHA using synthesis gas produced by gasification of waste corn, but polymer yield and biomass and polymer productivities were very low. The maximal biomass and polymer productivities at CO concentration of 17 % v/v were 17 and 59.2 mg/L day, or 0.0071 and 0.0025 g/L h. These values are several orders of magnitude lower than the corresponding values for *S. carboxydohydrogena* Z-1062.

The present study showed that while CO did not damage synthesis of total lipids and their content in *S. carboxydohydrogena* Z-1062 cells, it did change the composition of fatty acids of membrane lipids. At 20 and 30 % v/v CO, the proportion of unsaturated fatty acids increased while the proportions of saturated and cyclopropane acids increased, with the saturation index increasing by a factor of 2.1–3.3 relative to the saturation index in experiments with 10 % CO or without CO. Such redistribution in the FA composition of microorganisms influences fluidity and permeability of the membrane, making it more rigid and resistant to unfavorable effects and decreasing its permeability to inhibitory substrates.

Analysis of the polymer synthesized at CO concentrations varied between 10 and 30 % v/v showed that it was a copolymer containing monomers of 3-hydroxybutyrate as a major fraction (over 99 mol%) and monomers of 3-hydroxyvalerate (0.24–0.48 mol%) as a minor one. CO did not produce any adverse effects on molecular weight, temperature parameters, and degree of crystallinity of the polymer.

Conclusion

In this study, we investigated the growth of aerobic CO-oxidizing carboxydobacterium *Seliberia carboxydohydrogena* Z-1062 under batch cultivation conditions on the gas mixtures containing CO_2, O_2, H_2, and CO and PHA synthesis by this strain under growth-limiting concentrations of mineral nutrients (nitrogen, sulfur or both nitrogen and sulfur). CO concentrations of 10, 20, and 30 % v/v did not affect polymer synthesis, and its intracellular content reached 52.6–62.8 % of biomass weight after 56-h cultivation under limiting concentrations of nitrogen and/or sulfur. PHA productivity reached 0.13–0.22 g/L h. *S. carboxydohydrogena* Z-1062 growth rate and cell concentration were adversely affected by 30 % v/v CO: the O_2 and H_2 use efficiency decreased and the lipid saturation index increased. The polymer synthesized by *S. carboxydohydrogena* Z-1062 was a copolymer with poly-3-hydroxybutyrate as a major component (over 99 mol%) and 3-hydroxyvalerate as a minor one (0.24–0.48 mol%). Owing to the high CO resistance of *S. carboxydohydrogena* Z-1062 and its ability to produce polymer yields of about 60 % when grown at CO concentrations between 10 and 30 % v/v, this carboxydobacterium can be regarded as a potential producer of degradable PHAs from industrial hydrogenous sources fabricated by conversion of natural gas and high-temperature gasification of natural carbonaceous materials, including wastes.

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References

1. Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. Prog Polym Sci 25:1503–1555
2. Choi J, Lee SY (1999) Factors affecting the economics of polyhydroxyalkanoate production by bacterial fermentation. Appl Microbiol Biotechnol 51:13–21
3. Sudesh K, Bhubalan K, Chuah JA, Kek YK, Kamilah H, Sridewi N, Lee YF (2011) Synthesis of polyhydroxyalkanoates from palm oil and some new applications. Appl Microbiol Biotechnol 89:1373–1386. doi:10.1007/s00253-011-3098-5
4. Arcos-Hernandez MV, Pratt S, Laycock B, Johansson P, Werker A, Lant PA (2013) Waste activated sludge as biomass for production of commercial-grade polyhydroxyalkanoates (PHA). Waste Biomass Valorization 4:117–127. doi:10.1007/s12649-012-9165-2
5. Nikodinovic-Runic J, Guzik M, Kenny ST, Babu R, Werker A, O'Connor KE (2013) Carbon-rich wastes as feedstocks for biodegradable polymer (polyhydroxyalkanoates) production using bacteria. Adv Appl Microbiol 84:141–200. doi:10.1016/B978-0-12-407673-0.00004-7
6. Durner R, Witholt B, Egli T (2000) Accumulation of poly[(R)-3-hydroxyalkanoates] in *Pseudomonas oleovorans* during growth with octanoate in continuous culture at different dilution rates. Appl Environ Microbiol 66:3408–3422
7. Lee IY, Kim CH, Yeon BK, Hong WK, Choi ES, Rhee SK, Park YH, Sung DH, Baek WH (1997) High production of D-β-hydroxyisobutyric acid from methacrylic acid by *Candida rugosa* and its mutant. Bioprocess Eng 16:247–252
8. Maskow T, Babel W (2000) Calorimetrically recognized maximum yield of poly-3-hydroxybutyrate (PHB) continuously synthesized from toxic substrates. J Biotechnol 77:247–253
9. Khosravi-Darani K, Mokhtari ZB, Amai T, Tanaka K (2013) Microbial production of poly(hydroxybutyrate) from C_1 carbon sources. Appl Microbiol Biotechnol 97:1407–1424. doi:10.1007/s00253-012-4649-0

10. Koller M, Atlić A, Dias M, Reiterer A, Braunegg G (2010) Microbial PHA production from waste raw materials. In: Chen G-Q, Steinbüchel A (eds) Plastics from bacteria—natural functions and applications. Springer, Heidelberg, pp 86–114
11. Tanaka K, Ishizaki A, Kanamuru T, Kawano T (1995) Production of poly(D-3-hydroxybutyrate) rom CO_2, H_2, and O_2 by high cell density autotrophic cultivation of *Alcaligenes eutrophus*. Biotechnol Bioeng 45:268–275
12. Volova TG (2009) Hydrogen-based biosynthesis. Nova Science Publisher Inc., New York
13. Lee SY (1996) Bacterial polyhydroxyalkanoates. Biotechnol Bioeng 49:1–14
14. Ishizaki A, Tanaka K, Taga N (2001) Microbial production of poly-D-3-hydroxybutyrate from CO_2. Appl Microbiol Biotechnol 57:6–12. doi:10.1007/s002530100775
15. Volova T, Kiselev E, Shishatskaya E, Zhila N, Boyandin A, Syrvacheva D, Vinogradova O, Kalacheva G, Vasiliev A, Peterson I (2013) Cell growth and PHA accumulation from CO_2 and H_2 of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646. Bioresour Technol 146:215–222. doi:10.1016/j.biortech.2013.07.070
16. Do YS, Smeenk J, Broer KM, Kisting CJ, Brown R, Heindel TJ, Bobik TA, DiSpirito AA (2007) Growth of *Rhodospirillum rubrum* on synthesis gas: conversion of CO to H_2 and poly-β-hydroxyalkanoate. Biotechnol Bioeng 97:279–286. doi:10.1002/bit.21226
17. Tanaka K, Miyawaki K, Yamaguchi A, Khosravi-Darani K, Matsusaki H (2011) Cell growth and P(3HB) accumulation from CO_2 of a carbon monoxide-tolerant hydrogen-oxidizing bacterium, *Ideonella* sp. O-1. Appl Microbial Biotechnol 92:1161–1169. doi:10.1007/s00253-011-3420-2
18. Przybylski D, Rohwerder T, Dilßner C, Maskow T, Harms H, Müller RH (2015) Exploiting mixtures of H_2, CO_2, and O_2 or improved production of methacrylate precursor 2-hydroxyisobutyric acid by engineered *Cupriavidus necator* strains. Appl Microbiol Biotechnol 99:2131–2145. doi:10.1007/s00253-014-6266-6
19. Zavarzin GA (1978) Hydrogen bacteria and carboxydobacteria. Nauka, Moscow **(In Russian)**
20. Gruzinsky IV, Gogotov IN, Bechina EM, Semenov YV (1977) Dehydrogenase activity of hydrogen-oxidizing bacteria *Alcaligenes eutrophus*. Microbiology (Mikrobiologiya) 46:625–630
21. Volova TG, Kalacheva GS, Altukhova OV (2002) Autotorophic synthesis of polyhydroxyalkanoates by the bacteria *Ralstonia eutropha* in the presence of carbon monoxide. Appl Microbiol Biotechnol 58:675–678. doi:10.1007/s00253-002-0941-8
22. Volova TG, Yangolov OV, Kahanov YG, Konovalov NM (1996) Sposob kultivirovaniya vodorodokislyayuschih bacterii (A technique of cultivation of hydrogen-oxidizing bacteria). RF Patent No 2051962. **(In Russian)**
23. Volova TG, Kalacheva GS, Gitelson II, Kuznecov BN, Schipko AM, Shabanov VF (2003) Sposob polucheniya polimera—β-oksimaslyanoi kisloti (A technique of producing a polymer—β-oxybutyric acid). RF Patent No 2207375. **(In Russian)**
24. Volova TG, Shishatskaya EI (2012) Shtamm bakterii VKPM B-10646—produtsent poligidroksialkanoatov I sposob ikh polucheniya (Bacterial strain VKPM B-10646—a producer of polyhydroxyalkanoates and a method of their production). RF Patent No. 2439143. **(In Russian)**
25. Meyer O, Schlegel HG (1983) Biology of aerobic carbon monoxide-oxidizing bacteria. Ann Rev Microbiol 37:277–310
26. Volova TG, Terskov IA, Trubachev IN, Sidko FY (1982) Growth and biochemical composition of carboxydobacteria. Izvestia Akademii Nauk SSSR. Seriya biologicheskaya 923–931. **(In Russian)**
27. Volova TG, Kalacheva GS, Kasaeva GE, Tereshkova GM (1986) The influence of the conditions of the gas supply on the growth and metabolism of carboxydobacteria. Microbiology (Mikrobiologiya) 55:938–943 **(In Russian)**
28. Volova TG, Guseinov OA, Kalacheva GS, Medvedeva SE, Puzyr AP (1993) Effect of monoxide on metabolism and ultrastructure af carboxydobacteria. World J Microbiol Biotechnol 9:160–163
29. Volova TG, Kalacheva GS, Trubachev IN, Filippova VK (1988) Study of the physiological and biochemical characteristics of carboxydobacteria under nutrient limitation. Microbiology (Mikrobiologiya) 57:61–64 **(In Russian)**
30. Volova TG, Kalacheva GS, Konstantinova VM (1994) Accumulation of polyoxybutyrate by aerobic CO-oxidizing bacteria *Seliberia carboxydohydrogena*. Microbiology (Mikrobiologiya) 63:211–216 **(In Russian)**
31. Volova TG, Kalacheva GS, Plotnikov VF (1998) Biosynthesis of heteropolymeric polyhydroxyalkanoates by chemolithoautotrophic bacteria. Microbiology (Mikrobiologiya) 67:420–424
32. Spirin AS (1958) Spectrophotometric determination of the total amount of nucleic acids. Biochemistry (Biokimiya) 23:656–660 **(In Russian)**
33. Kalacheva GS, Volova TG (2007) Fatty acid composition of *Wautersia eutropha* lipids under conditions of active polyhydroxyalkanoates synthesis. Microbiology (Mikrobiologiya) 76:608–614. doi:10.1134/s00262611707050049
34. Volova TG, Kalacheva GS, Kasaeva GE, Tereshkova GM (1987) The influence of the physical and chemical conditions of the medium on the growth and biochemical composition of carboxydobacteria. Microbiology (Mikrobiologiya) 56:973–978 **(In Russian)**
35. Volova TG, Zhila NO, Kalacheva GS, Brigham CJ, Sinskey AJ (2013) Effects of intracellular poly(3-hydroxybutyrate) reserves on physiological-biochemical properties and growth of *Ralstonia eutropha*. Res Microbiol 164:164–171. doi:10.1016/j.resmic.2012.10.008
36. Chen G-Q (2009) A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. Chem Soc Res 38:2434–2446. doi:10.1039/b812677c