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Cell growth and accumulation of polyhydroxyalkanoates from CO_2 and H_2 of a hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646

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HIGHLIGHTS

- PHA synthesis by *Cupriavidus eutrophus* B-10646 was studied in autotrophic culture.
- Conditions of the synthesis of PHAs with different composition have been found.
- Physicochemical properties of different PHA specimens have been studied.

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ABSTRACT

Synthesis of polyhydroxyalkanoates (PHAs) by a new strain of *Cupriavidus* – *Cupriavidus eutrophus* B-10646 – was investigated under autotrophic growth conditions. Under chemostat, at the specific flow rate $D = 0.1\ h^{-1}$, on sole carbon substrate (CO_2), with nitrogen, sulfur, phosphorus, potassium, and manganese used as growth limiting elements, the highest poly(3-hydroxybutyrate) [P(3HB)] yields were obtained under nitrogen deficiency. In batch autotrophic culture, in the fermenter with oxygen mass transfer coefficient $0.460\ h^{-1}$, P(3HB) yields reached 85% of dry cell weight (DCW) and DCW reached 50 g/l. Concentrations of supplementary PHA precursor substrates (valerate, hexanoate, γ-butyrolactone) and culture conditions were varied to produce, for the first time under autotrophic growth conditions, PHA ter- and tetra-polymers with widely varying major fractions of 3-hydroxybutyrate, 4-hydroxybutyrate, 3-hydroxyvalerate, and 3-hydroxyhexanoate monomer units. Investigation of the high-purity PHA specimens showed significant differences in their physicochemical and physicomechanical properties.

1. Introduction

The wide use of synthetic polymers poses a global environmental problem: their consumption rate is many times as high as the replenishment rate of carbon-based fossil fuels, causing a serious imbalance in the carbon cycle and leading to the pollution of the biosphere. A real solution is to impart degradability under natural conditions to synthetic materials or to use novel materials, manufactured from plant biomass and products of microbial synthesis (Chanprateep, 2010).

Polyhydroxyalkanoates (PHAs), along with lactic acid based polymers, are increasingly recognized as materials of the 21st century. PHAs may be potentially produced by microorganisms from numerous substrates, which can be reduced to different degrees and vary in energy content and cost. Manufacturing of PHAs on a larger scale requires lower-cost polymers and technologies that would enable stable tailor-made PHA production (Chen, 2010).

Bacterial strains of the genus *Cupriavidus* are among the well-known PHA producers of hydrogen-oxidizing bacteria. The taxonomic position and the name of this taxon have been changed several times: *Wautersia*,*Ralstonia*,*Alcaligenes*,*Hydrogenomonas* (Vandamme and Coenye, 2004; Tindall, 2008). The ability of hydrogen-oxidizing bacteria to synthesize PHA under autotrophic conditions, without organic media, with CO_2 as a source of constructive metabolism and H_2 as a source of energy metabolism, makes them good candidates for commercial production of PHAs (Tanaka et al.,

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1995; Ishizaki et al., 2001; Khosravi-Darani et al., 2013). The fact that some of the hydrogen-oxidizing bacterial species are tolerant to CO suggests the possibility of cultivating them using such feedstocks as industrial hydrogen (hydrogen-containing products of natural gas and coal conversion, wood residues, hydrolysis lignin, etc.) (Volova et al., 2002; Tanaka et al., 2011).

There are, however, rather few published studies reporting PHA synthesis under autotrophic growth conditions, using a mixture of CO_2, O_2 and H_2 gases. Among the research teams that have been investigating CO_2 and H_2 as growth substrates for biosynthesis of targeted products, including PHAs with different composition are the Institute of Biophysics SB RAS, Russia (Volova, 2009) and Kyushu University, Japan. The research team headed by Professor A. Ishizaki (Japan) investigated production of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus* culture in fermenters with different mass exchange parameters (Tanaka and Ishizaki, 1994; Tanaka et al., 1995; Sugimoto et al., 1999; Ishizaki et al., 2001).

Poly(3-hydroxybutyrate), however, has high crystallinity (75 ± 5%), and the products based on this material have rather low mechanical strength and are prone to "physical aging" (Laycock et al., 2013). On the other hand, synthesis of PHA copolymers, which are more readily processable materials, is a very complicated technological task. For bacteria to synthesize PHA copolymers, the culture medium must be supplemented with C-substrates in the concentrations that, on the one hand, would enable the production of the targeted monomer units (valerate, hexanoate, octanoate, etc.) and, on the other, would not significantly inhibit or suppress the growth of the PHA producing strains employed (Volova et al., 2008; Zhao and Chen, 2007; Bhubalan et al., 2010). PHA copolymers are usually produced using recombinant strains or mixed substrates and strains tolerant to them.

Synthesis of PHA copolymers described in the literature was only performed on organic carbon sources (sugars, fatty acids, palm oil, and complex substrates); moreover, the majority of the PHA producing strains employed in those studies were genetically modified ones. For instance, a recombinant strain, *Aeromonas hydrophila* 4AK4, was used to produce a P(3HB/3HV/3HHx) terpolymer from lauric acid and sodium valerate (Zhang et al., 2009) and from mixtures of dodecanoic acid and propionic acid (Bhubalan et al., 2010). *Alcaligenes* sp. A-04 synthesized P(3HB/3HV/4HB) copolymers on the medium containing fructose, valeric acid, and sodium 4-hydroxybutyrate (Chanprateep and Kulpreecha, 2006). Hexanoate copolymers synthesized by a culture of *Defluviicoccus vanus* were produced from a mixture of acetate and propionate (Dai et al., 2008); and the recombinant strain *C. necator* synthesized PHB4 from mixtures of crude palm kernel oil (Bhubalan et al., 2010).

It is important to note that among *Cupriavidus* (*Ralstonia*) species there are strains capable of synthesizing both poly(3-hydroxybutyrate) and PHA copolymers containing short-chain-length monomers (3-hydroxybutyrate and 3-hydroxyvalerate) and medium-chain-length monomers (3-hydroxyhexanoate and 3-hydroxyoctanoate). We were the first to produce PHA copolymers consisting of short- (3HB and 3HV) and medium-chain-length (3HHx) monomer units from mixed carbon substrate, with salts of alkanoic acids added to the culture medium of the autotrophic culture of *Ralstonia eutropha* B5786, a wild-type strain (Volova et al., 1998). A similar result, confirming synthesis of 3-hydroxybutyrate/3-hydroxyhexanoate copolymers by the wild-type strain, *R. eutropha* H-16, was reported by Green et al. (2002). However, except for the studies performed at the Institute of Biophysics SB RAS, synthesis of PHA copolymers by *Cupriavidus* species has only been achieved under heterotrophic conditions, with sugars or organic acids, palm oil, biodiesel production wastes, and other feedstocks used as the main growth C-substrate.

The present study investigated a strain of hydrogen-oxidizing bacterium, *Cupriavidus eutrophus* B-10646, in autotrophic culture and its ability to synthesize P(3HB) and PHA copolymers with different chemical structure from CO_2 as the main carbon substrate.

2. Methods

2.1. Bacterial strain, media and growth conditions

The strain used in this study was *C. eutrophus* B-10646, registered in the Russian National Collection of Industrial Microorganisms. The strain has a broad organotrophic potential and can use as carbon sources different substances; it is tolerant to concentrations of a number of organic C-substrates (valerate, hexanoate, γ-butyrolactone) reaching 3–5 g/l in the culture medium and is able to use them to synthesize PHA copolymers containing short- and medium-chain-length monomer units (Volova and Shishatskaya, 2012).

Cells were grown on Schlegel's mineral medium (Schlegel et al., 1961): $Na_2HPO_4{\cdot}H_2O$ – 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) and a medium matching the elemental composition of microbial cells, which contained $MgSO_4$, H_3PO_4, KOH, NaOH, $CO(NH_2)_2$ in amounts corresponding to the chemical composition of cells (Volova, 2009). Thus, the elements were added separately, and their concentrations varied widely (from single digits to 100 g/l and greater), depending on cell concentration (estimated by dry cell weight, DCW) in the culture. When NH_4Cl was replaced by urea, $CO(NH_2)_2$, 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 contains 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).

The main growth substrate was the gas mixture of CO_2:O_2:H_2 = 1:2:7 by volume, which was prepared in a 50-l metal gas holder. A recycled-gas closed-circuit culture system was used. When flasks were used as fermentation vessels, the gas mixture was fed at a small overpressure from the gas holder; when the fermentation system was used, the gas mixture was continuously pumped through the culture at a rate of 10–12 l/min, with a diaphragm-type compressor.

Cells were grown in the batch and continuous cultures. The batch culture was performed in an Innova® 44 constant temperature incubator shaker ("New Brunswick Scientific", USA), which provided a leak-proof gas circuit and enabled gas supply to the 0.5–3.0-l flasks. The scale-up of the process in the batch or continuous chemostat mode was carried out in a 10-l explosion-proof laboratory bioreactor equipped with a turbine-type mixer at 1000 rpm, constructed in the Institute of Biophysics SB RAS. The volume coefficient of mass transfer for oxygen (KLa) was 460 h^{-1}. The cultivation and substrate feeding were conducted as follows: the gas mixture was continuously pumped through the culture with a diaphragm-type compressor; concentrated major and trace mineral elements were fed into the fermenter periodically, as cell concentration increased. Nitrogen was fed to the culture with a peristaltic pump.

Synthesis of PHA copolymers was achieved as follows: after the first stage of the culture was completed and nitrogen supply was discontinued (30–35 h), the culture medium was supplemented with precursor substrates (γ-butyrolactone; propionic, valeric, and caproic acids in the form of potassium salts). Concentrations of these substrates in the culture medium were controlled using chromatographic analysis of the culture medium samples, which was done after preliminary extraction with chloroform from acidified samples.

2.2. Monitoring the process parameters

Under autotrophic conditions, gas substrate concentration was determined continuously, using such gas analyzers as MKG-14, TP-11200, and OA-2209 7811 for O_2, H_2, and CO_2, respectively, 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.

During the course of cultivation, culture samples were taken for analysis every 4 h: 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 by 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.

2.3. Analysis of PHA structure

Intracellular PHA content at different time points was determined by analyzing samples of dry cell biomass. Intracellular PHA content and composition of extracted polymer samples were analyzed by a GC–MS (6890/5975C, "Agilent Technologies", USA). Both lyophilized cells and extracted PHAs were subjected to methanolysis in the presence of sulfuric acid, and PHA was extracted and methyl esterified at 100 °C for 4 h. Benzoic acid was used as an internal standard to determine total intracellular PHA (Braunegg et al., 1978; Brandl et al., 1988). Monomer units were identified in the extracted and purified PHA samples based on their retention times and mass spectra.

^{1}H NMR spectra of copolymers were recorded at room temperature in $CDCl_3$ on a BRUKER AVANCE III 600 spectrometer operating at 600.13 MHz.

2.4. Analysis of PHA properties

Molecular weight and molecular-weight distribution of PHAs were examined using a gel permeation chromatograph ("Agilent Technologies" 1260 Infinity, USA) with a refractive index detector using an Agilent PLgel Mixed-C column. Chloroform was the eluent. Calibration was made using polystyrene standards (Agilent, USA). Molecular weights (weight average, M_w, and number average, M_n) and polydispersity ($Đ = M_w/M_n$) were determined.

Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning calorimeter (METTLER TOLEDO, Switzerland). The specimens were heated to temperatures from 25 to 350 °C, at 5 °C min^{-1}. The thermograms were analyzed using the STARe v11.0 software.

In order to determine the crystallinity of the PHAs, films were prepared from a 2% polymer solution. X-ray structure analysis and determination of crystallinity of PHAs were performed using a D8 ADVANCE X-ray diffractometer ("Bruker, AXS", Germany). To determine the degree of crystallinity (C_x), spectra were collected with a Vantec high-speed detector, exposure time 300′, to measure intensity at point. The operating mode of the instrument was 40 kV × 40 mA.

3. Results and discussion

3.1. Synthesis of poly(3-hydroxybutyrate) [P(3HB)] in autotrophic continuous culture of C. eutrophus B-10646

The influence of conditions of *C. eutrophus* B-10646 mineral nutrition on PHA synthesis was investigated in continuous chemostat culture, which enables limitation of cell growth under steady-state conditions. The amounts of the elements fed into the system and their current concentrations in the culture were determined by the culture medium flow rate and deficiency of one element in the medium, with excessive levels of the other elements flowing into the system. By varying the concentration of the limiting element, we created the conditions for cell multiplication at different specific growth rates determined by the inflow of a limiting element. The highest intracellular PHA contents in autotrophic chemostat culture on a single carbon substrate (CO_2) were obtained at a specific growth rate of the cells (μ) of about 0.1 h^{-1}, determined by the supply of the mineral medium components that limited cell growth (Table 1). The specific growth rate of the cells was similarly decreased when limited by the deficiency of nitrogen or potassium (40–50% of the cell's physiological requirements), sulfur and phosphorus (20%), or magnesium (10%). The highest PHA content (about 40%) was obtained under nitrogen deficiency; 20–25% under sulfur and phosphorus deficiency; and the lowest (about 15%) PHA content was recorded under potassium or magnesium deficiency. Chemical analysis showed that the polymer synthesized was poly(3-hydroxybutyrate) [P(3HB)] (over 99 mol%) with a minor fraction of 3-hydroxyvalerate (3HV) (0.3–0.9 mol%) (Table 2). At even lower levels of limiting elements and slower flow rates, polymer content did not rise significantly, but the risk of culture contamination increased. So, experiments with the flow chemostat culture showed that, similarly to the previously described PHA producer, *R. eutopha* B5786, the study strain synthesized the highest yields of poly(3-hydroxybutyrate) on a single carbon source (CO_2) under nitrogen deficiency (Volova et al., 2002).

The change in the direction of constructive metabolism influenced the effectiveness of substrate utilization (Table 1). At the highest polymer content, in some experiments with the limited growth of cells, hydrogen-based yield coefficient (Y_{H2}) varied from 0.76 to 0.85, and it was lower than in the experiment with the cells grown on complete nutrient medium (1.10–1.20).

An advantage of the chemostat is the possibility of polymer synthesis for indefinitely long periods of time and with no strict sterility measures; its disadvantage is low cell concentration in the culture (no higher than 5%) and low intracellular polymer content (no higher than 40%). Moreover, the process involves the use of the continuously operating unit for the separation of low-density culture medium, which consumes very much power.

Table 1
Characterization of autotrophic chemostat culture of *C. eutrophus* B-10646 under growth limitation by the deficiency of mineral elements.

Element	Continuous supply of the element to the culture medium (% of required amount)	Specific growth rate μ (h^{-1})	Hydrogen-based yield coefficient, Y_{H2} (g/g)	Cell concentration DCW (g/l)	Intracellular P(3HB) content, (% of DCW)
N	80	0.30	1.15	4.3	9.3
	50	0.11	0.85	3.2	39.5
K	80	0.28	1.06	4.9	4.9
	50	0.12	0.76	3.8	14.7
P	40	0.29	0.98	4.2	5.8
	20	0.10	0.70	3.6	19.7
S	40	0.24	1.12	5.0	12.4
	20	0.09	0.84	3.9	24.5
Mg	50	0.27	1.20	4.6	2.6
	10	0.10	0.78	3.1	13.9

Table 2
Characterization of PHA copolymers synthesized by bacterium *C. eutrophus* B-10646 from CO_2 supplemented with one precursor substrate (valerate or hexanoate, or γ-butyrolactone).

Carbon source	PHA composition (mol%)					T_m	T_d	C_x	M_w (kDa)	M_n (kDa)	$Đ$
	3HB	4HB	3HV	3HHx	3HO						
CO_2	99.1	0	0.9	0	0	179.0	294.8	76	922	367	2.51
CO_2 + 3 g/l γ-butyrolactone (20 h)	89.5	10.0	0.5	0	0	153.9	286.4	50	473	144	3.27
CO_2 + 5 g/l γ-butyrolactone (10 h)	78.9	20.3	0.4	0.4	0	167.8	288.0	38	605	144	4.19
CO_2 + 5 g/l γ-butyrolactone (30 h)	62.3	37.1	0.3	0.3	0	170.5	287.0	36	537	156	3.44
CO_2 + 5 g/l γ-butyrolactone (20 h)	48.2	51.3	0.3	0.2	0	169.4	290.7	38	837	209	4.00
CO_2 + 3 g/l valerate (10 h)	93.4	0	6.5	0.1	0	176.2	281.1	67	1184	489	2.42
CO_2 + 3 g/l valerate (30 h)	89.5	0	10.5	0	0	173.2	283.5	59	695	220	3.15
CO_2 + 3 g/l valerate (20 h)	78.9	0	21.1	0	0	175.1	282.9	57	1132	378	2.99
CO_2 + 5 g/l valerate (10 h)	62.6	0	37.4	0	0	179.6	284.4	51	1336	500	2.67
CO_2 + 5 g/l valerate (30 h)	35.6	0	64.3	0.1	0	153.0	249.0	53	1111	318	3.49
CO_2 + 5 g/l valerate (20 h)	14.9	0	85.1	0	0	ND	ND	50	1120	359	3.11
CO_2 + 3 g/l hexanoate (20 h)	94.1	0	0.9	5.0	0	169.4	278.2	64	527	199	2.64
CO_2 + 5 g/l hexanoate (10 h)	88.5	0	1.0	10.5	0	173.8	246.2	ND	647	197	3.30
CO_2 + 5 g/l hexanoate (30 h)	84.0	0	1.0	15.00	0	174.8	283.8	60	924	225	4.10
CO_2 + 5 g/l hexanoate (20 h)	79.3	0	0.7	20.00	0	164	256	42	421	127	3.02

T_m – melting point; T_d – thermal degradation temperature; C_x – crystallinity; M_w – weight average molecular weight; M_n – number average molecular weight; $Đ$ – polydispersity; ND – not determined.

3.2. Synthesis of poly(3-hydroxybutyrate) [P(3HB)] in autotrophic batch culture of C. eutrophus B-10646

Due to their specific growth physiology and constructive metabolism, in the middle of the linear growth phase, hydrogen-oxidizing bacteria stop synthesizing protein and begin accumulating PHAs even on complete nutrient medium (Schlegel et al., 1961). Based on this fact, we investigated the cell growth and polymer yields in autotrophic batch culture of *C. eutrophus* B-10646 with different inflowing amounts of nitrogen in a 10-l fermenter, with the working volume 3 l (Fig. 1). The inoculum had been prepared in flasks on a shaker, for 30–35 h. In the one-stage process, with the amount of the nitrogen supplied to the culture corresponding to the physiological requirement of the cells (120 mg/g cells), for 70 h of cultivation, the intracellular P(3HB) content had reached 55% of dry cell weight and cell concentration 30 g/l (Fig. 1 a). The second cultivation mode consisted of two stages: in the first, the cells were grown on complete nutrient medium with continuous inflow of nitrogen in the amounts corresponding to the physiological requirement of cells (120 mg/g cells); in the second, the cells were grown on nitrogen-free medium. Under these conditions, the polymer yield reached 80% of dry cell weight, but the duration of the process was increased to 80–85 h (Fig. 1b).

The third cultivation mode also consisted of two stages. Cells were grown under limited nitrogen supply in the first stage and with no nitrogen in the second. The best results were obtained with nitrogen supply amounting to 50% of that required by cells in the first stage (60 mg/g cells) (Fig. 1c). By the end of the experiment (70 h), intracellular polymer content had reached 85% of dry cell weight and cell concentration 48 g/l. Thus, the full cycle of autotrophic batch cultivation of bacterial cells in the fermenter, taking into account the inoculum preparation stage, lasted about 100–105 h.

Consumption of the gaseous substrate by the culture accumulating P(3HB) varied significantly throughout the process. In the first stage, when the culture was growing and accumulating the polymer, specific rates of consumption of gas mixture components by the culture were 0.035–0.05 mol/(g h) for hydrogen, 0.17–0.18 mol/(g h) for oxygen, and 0.11–0.14 mol/(g h) for carbon dioxide (Fig. 2). The average yield coefficients of the culture were 3.0 g/mol on H_2, 1.2 g/mol on O_2, and 1.7 g/mol on CO_2. In the second stage, when the cells were actively accumulating the polymer, the rates of consumption of the carbon substrate (CO_2), the energy substrate (H_2), and oxygen were significantly lower, due to a dramatic decrease in the cell growth rate and an almost complete cessation of cell growth at the end of the stage. Yield coefficients of the culture were 2.3; 0.8; 1.3 g/mol, respectively. The average yield coefficients of the culture for the whole process were 2.6 g/mol on H_2; 1.0 g/mol on O_2; and 1.5 g/mol on CO_2.

Thus, the two-stage batch cultivation with cells grown under nitrogen deficiency in the first stage and without nitrogen in the second resulted in polymer yields and cell concentrations reaching 85% and 50 g/l. Both parameters were better than those we obtained previously, using the autotrophic culture of *R. eutropha* B5786 (Volova, 2009). These results are in good agreement with the data obtained by Tanaka and Ishizaki (1994), in a study of autotrophic culture of *A. eutrophus*, and cell concentrations are comparable to those obtained in a fermenter with similar mass exchange parameters. Higher cell concentrations (reaching 91.3 g/l) with poly(3-hydroxybutyrate) yields below 70% were only obtained in fermenters with very high mass exchange parameters (KLa) – 2970 h^{-1} (Tanaka et al., 1995).

3.3. Synthesis of poly(3-hydroxybutyrate) [P(3HB)] in chemostat batch culture of C. eutrophus B-10646

Analysis of the results of cultivating *C. eutrophus* B-10646 in different PHA synthesis modes suggested the use of autotrophic chemostat-batch process (Fig. 3). In the first stage, cells were grown under flow chemostat conditions, and the amount of nitrogen (a limiting element under chemostat conditions) supplied to the medium was 100 mg/g cells. The resulting inoculum had cell concentration and intracellular polymer content 4 ± 1 g/l and 8 ± 2%, respectively, and it took only a few hours (not more than 10) to reach these levels. Then, in the second stage, the flow was stopped, and nitrogen supply was limited to 60 mg/g cells. After 35 h, nitrogen supply was stopped. In this stage, intracellular polymer content was 47 ± 2% and cell concentration 15 ± 1 g/l. In the third stage, cultivation was conducted in nitrogen-free medium, for 35 h. By the end of the experiment (80 h), intracellular polymer content reached 80 ± 5% and cell concentration 45 ± 5 g/l. So, this method reduced the duration of the stage of inoculum preparation and enabled attaining high polymer yields and cell concentrations in the process whose total duration did not exceed 80 h.

Thus, such parameters of PHA synthesis as cell concentration and polymer yield were optimized using dry cell weight measurements to control microbial growth kinetics and chromatographic detection to monitor intracellular PHA content. It is noteworthy to mention that these parameters may be enhanced more

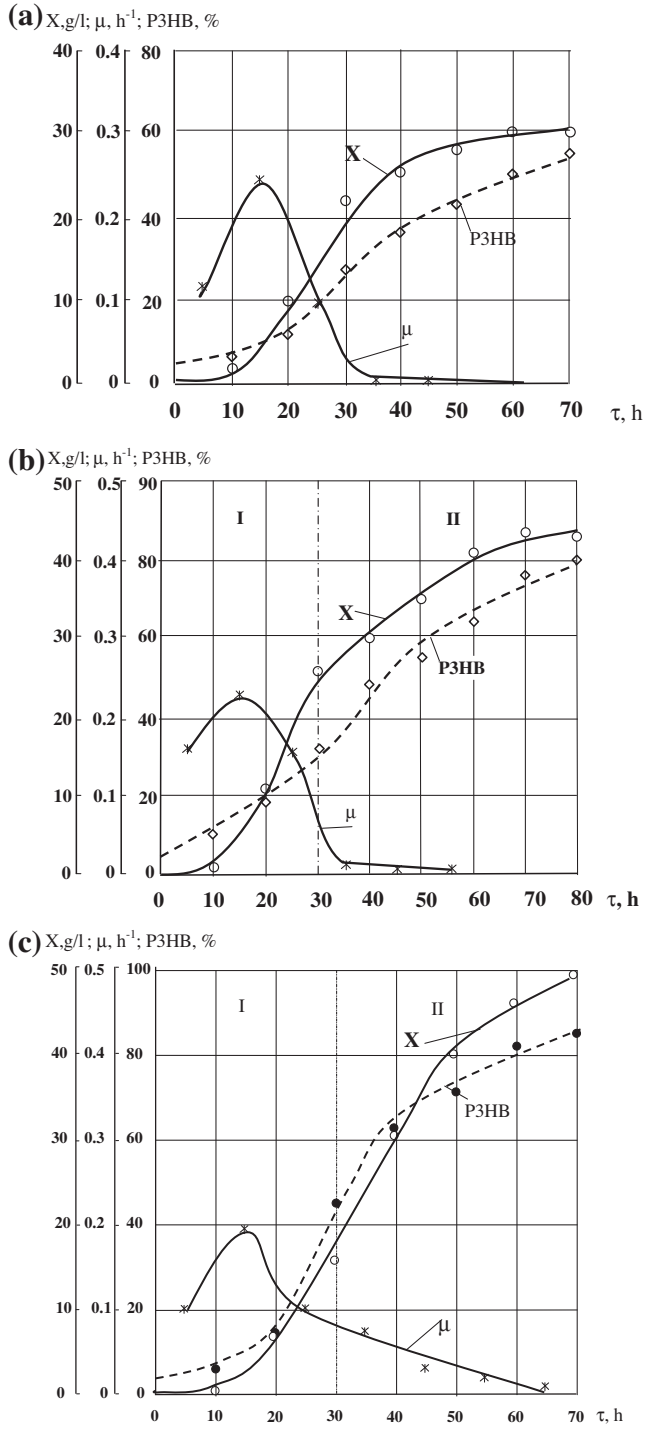

Fig. 1. Parameters of *C. eutrophus* B-10646 batch autotrophic culture: (a) – a single-stage process on complete medium; (b) – a two-stage process: Stage I (I) on complete medium and Stage II (II) on nitrogen-free medium; (c) – a two-stage process: Stage I (I) at 50% nitrogen supply and Stage II (II) on nitrogen-free medium; *X* – dry cell weight (g/l); μ – cell specific growth rate, h^{-1}; P(3HB) – intracellular polymer concentration (% of dry matter).

effectively by using new methods of monitoring real time process kinetics (Kedia et al., 2013).

3.4. Synthesis of PHA copolymers from mixed carbon substrate in C. eutrophus B-10646 culture

In order to achieve biosynthesis of PHA copolymers, one needs, first, to supplement the culture medium with additional carbon

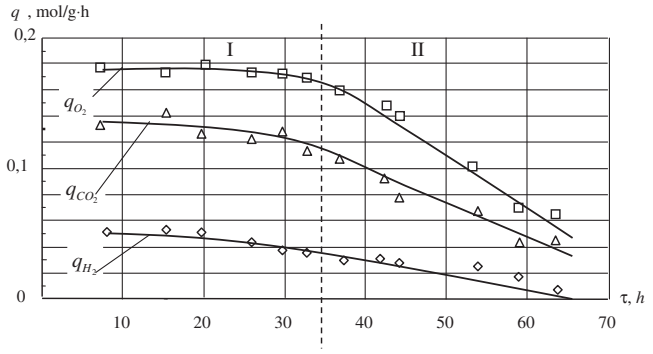

Fig. 2. Specific rate of consumption of gaseous substrate components by *C. eutrophus* B-10646 cells cultured under autotrophic growth conditions, in the mode of P3HB synthesis.

substrates (valerate, hexanoate, butyrolactone, etc.); for *C. eutrophus* B-10646, the amounts of these precursor substrates added to the culture medium reach 3–5 g/l. Second, as a substrate of endogenous respiration, PHA is metabolized intracellularly. Polymer carbon chains are broken down, and monomers containing more than 4 carbon atoms get involved in PHA resynthesis as shorter monomers, mainly 3-hydroxybutyrate. As different monomer units are synthesized and incorporated into PHAs with different rates, the duration of cultivation after the addition of the second precursor C-substrate should be regulated, or else the fraction of the second monomer in the PHA would be decreased (Volova et al., 2008; Bhubalan et al., 2010).

The validation of the conditions necessary for the synthesis of PHA copolymers was based on the view of the structure and mechanisms of substrate specificity of PHA synthases. It was previously believed that PHA synthases are highly substrate specific, and, thus, natural microorganisms can accumulate either short- or medium-chain-length PHAs. A detailed comparative study of two strains of *R. eutropha*, B5786 and H-16, cultivated under autotrophic growth conditions, was reported by Volova et al. (1998, 2008), who managed to produce a number of PHA copolymers with different chemical structure, consisting of both short- and medium-chain-length monomer units. These data suggest a broader substrate specificity, and differences between the *in vivo* and *in vitro* reactions may be accounted for by either the microenvironment of synthases in the cell or the duration of observation of the reaction of some PHA synthases (Kozhevnikov et al., 2010).

As specific growth rates of *C. eutrophus* B-10646 cells had been found to depend on the amount of the precursor substrate added to the culture medium (valerate, hexanoate, γ-butyrolactone, and a combination of butyrolactone and propionate), PHA copolymers with different composition were prepared by varying the amounts of the added precursor substrates and the duration of cultivation following their addition (Tables 2 and 3). Supplementation of the culture medium of the cells growing on the gaseous mixture of $CO_2 + O_2 + H_2$ that contained 5% by volume CO_2 as a carbon source with γ-butyrolactone, a precursor substrate, resulted in the synthesis of different copolymers of 3-hydroxybutyric and 4-hydroxybutyric acids (Table 2). Precursor substrates were added to the 30–35-h culture, immediately after the nitrogen supply was stopped.

By varying γ-butyrolactone concentration (3–5 g/l) and the duration of cultivation after the addition of this substrate (10–30 h), we obtained copolymers containing different molar fractions of 3HB and 4HB (from 10.0% to 51.3%) and minor molar fractions of 3-hydroxyvalerate (0.3–0.5%) and 3-hydroxyhexanoate (0–0.4%).

Cultivation of *C. eutrophus* B-10646 on mixed carbon substrate containing CO_2 and potassium valerate (3–5 g/l) with the duration of cultivation after the addition of the precursor substrate varied

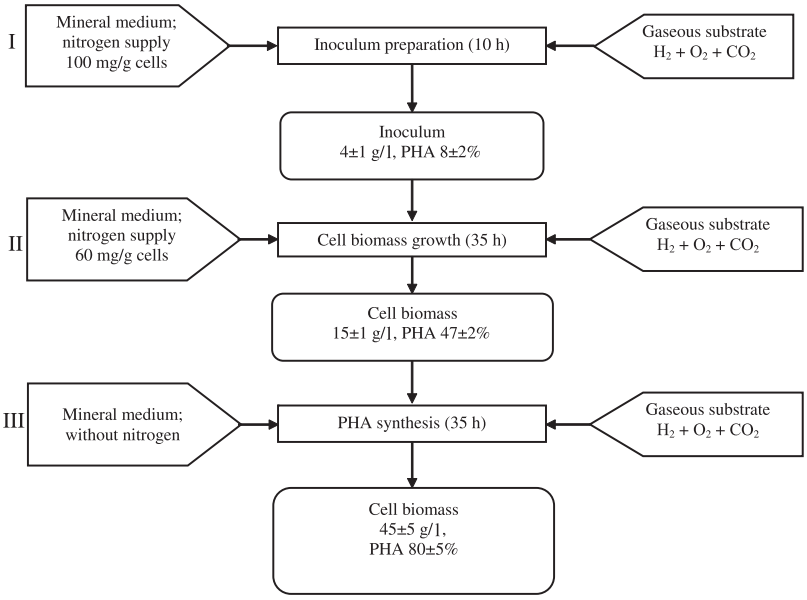

Fig. 3. A flow-chart of chemostat batch autotrophic culture of *C. eutrophus* B-10646.

Table 3
Characterization of PHA copolymers synthesized by bacterium *C. eutrophus* B-10646 from CO_2 supplemented with two precursor substrates (γ-butyrolactone + propionate or hexanoate + valerate).

Carbon source	PHA composition (mol%)					T_m	T_d	C_x	M_w (kDa)	M_n (kDa)	Đ
	3HB	4HB	3HV	3HHx	3HO						
CO2	99.1	0	0.9	0	0	179.0	294.8	76	922	367	2.51
CO_2 + 2 g/l propionate + 5 g/l γ-butyrolactone (10 h)	59.5	34.5	5.4	0.6	0	161.3	295.3	21	439	148	2.96
CO_2 + 3 g/l propionate + 5 g/l γ-butyrolactone (20 h)	25.3	62.5	12.2	0	0	163.5	295.2	18	508	147	3.44
CO_2 + 3 g/l propionate + 3 g/l γ-butyrolactone (10 h)	56.2	23.6	20.2	0	0	171.4	280.5	22	645	174	3.71
CO_2 + 3 g/l propionate + 4 g/l γ-butyrolactone (10 h)	48.3	34.8	16.9	0	0	171.8	283.8	8	475	134	3.53
CO_2 + 2 g/l propionate + 5 g/l γ-butyrolactone (35 h)	55.3	31.3	13.3	0.1	0	167.8	283.3	21	756	183	4.13
CO_2 + 5 g/l valerate + 5 g/l hexanoate (20 h)	73.1	0	24.9	2.0	0	173.4	268.8	54	537	150	3.58
CO_2 + 3 g/l valerate + 3 g/l hexanoate (25 h)	75.9	0	13.0	11.1	0	166.0	265.0	35	999	333	3.00
CO_2 + 3 g/l valerate + 3 g/l hexanoate (30 h)	84.9	0	1.3	12.7	1.1	167.8	221.0	65	288	132	2.17
CO_2 + 5 g/l valerate + 5 g/l hexanoate (35 h)	67.3	0	7.6	25.1	0	164.0	263.0	40	970	277	3.50

T_m – melting point; T_d – thermal degradation temperature; C_x – crystallinity; M_w – weight average molecular weight; M_n – number average molecular weight; Đ – polydispersity; ND – not determined.

from 10 to 30 h yielded copolymers with major fractions of 3-hydroxybutyric and 3-hydroxyvaleric acids. By controlling the inflow of valerate and the duration of the cultivation following the addition of this substrate, polymers with different molar fractions of 3HB and 3HV were synthesized: from 14.9% to 93.4% and from 6.5% to 85.1%, respectively.

Synthesis of copolymers containing 3-hydroxyhexanoate is a more complex task as this precursor substrate is more toxic for cells. The addition of potassium hexanoate to the culture medium (3–5 g/l) with the duration of cultivation after the addition of the precursor substrate varied from 10 to 30 h resulted in the formation of PHAs that mainly contained 3-hydroxybutyrate (79.3–94.1 mol%), with the 3HHx fraction making up 5–20 mol% and a minor fraction of 3-hydroxyvalerate (0.7–1.0 mol%).

Synthesis of PHAs containing three or more components has become a research subject quite recently, and there are rather few published studies addressing it.

Table 3 gives results of *C. eutrophus* B-10646 cultivation on the gaseous mixture ($CO_2 + O_2 + H_2$) with the culture medium supplemented with two precursor substrates: potassium propionate and γ-butyrolactone or potassium valerate and potassium hexanoate (Table 3). As a result, *C. eutrophus* B10646 cells cultured autotrophically synthesized PHAs with different compositions, which zcontained major molar fractions of 3-hydroxybutyrate (25.3–84.9%), 4-hydroxybutyrate (23.6–62.5%), 3-hydroxyvalerate (1.3–24.9%), and 3-hydroxyhexanoate (2.0–25.1%).

Tables 2 and 3 show that the highest molar fractions of 3-hydroxyvalerate, 3-hydroxyhexanoate, and 4-hydroxybutyrate in the PHAs were reached 20–25 h after the addition of the corresponding precursor substrates, but as the cultivation continued, they decreased. This should be taken into account if the purpose of the process is to obtain high major fractions of monomer units.

Comparison of the results obtained in this study with the literature data showed that synthesis of PHA terpolymers by *Cupriavidus* species had been only described for cells grown under heterotrophic conditions. For example, production of a P(3HB/3HV/3HHx) terpolymer with the highest molar fraction of 3-hydroxyhexanoate (28.3%, with 3HV 23.8%) was reported by Zhang et al. (2009). Rather high molar fractions of 3HHx (15%) were obtained by Zhao and Chen (2007), but the fraction of 3HV was rather low (1.2%). Terpolymer P(3HB/3HV/4HB) containing 3HV 6.7–9.8% and 4HB 26.2–30.6% was synthesized by a mutant strain, *Cupriavidus necator* DSM 545 (Cavalheiro et al., 2012). A higher molar fraction of 3HV (23%) was obtained when a natural strain, *Cupriavidus* sp. *USMAA2-4*, was grown on oleic acid, but the 4HB fraction made up 28% (Aziz et al., 2012). Chanprateep and Kulpreecha (2006) used

a wild-type strain, *Alcaligenes* sp. A-04, and obtained the highest 3HV molar fraction (40%), with the 4HB fraction reaching 59%; they also obtained a terpolymer with 3HV and 4HB molar fractions constituting 3% and 93%, respectively.

Some *Cupriavidus* strains are able to synthesize copolymers with minor fractions of 3-hydroxyvalerate even from a single carbon source (Volova et al., 2006), with 3HV fractions sometimes reaching 8% (García et al., 2013).

Thus, optimization of the cultivation conditions and carbon nutrition and the search for new PHA producers will provide a basis for producing PHAs with widely varying chemical compositions.

In this study, PHA heteropolymers were synthesized by bacterial cells of the genus *Cupriavidus* grown on the $CO_2 + O_2 + H_2$ gaseous mixture with CO_2 as the main carbon substrate and organic precursor substrates added in small amounts.

3.5. Properties of PHA copolymers

Results of comparative investigations of polymer properties using X-ray structure analysis, differential scanning calorimetry, and gel permeation chromatography are given in Tables 2 and 3. No clear relationship has been found between molecular mass of the polymers and their chemical structure, i.e. molar fractions of their monomer units. The polymers containing major fractions of 3HB, 3HV, and 4HB, however, generally showed lower values of weight average and number average molecular weight (439–756 kDa and 134–183 kDa). At the same time, PHAs that contained major fractions of only two monomers (3HB and 4HB or 3HB and 3HV, or 3HB and 3HHx) showed M_w and M_n values reaching 837 and 209; 1336 and 500; 924 and 225 kDa.

The decrease in the molecular weight of a PHA caused a decrease in its melting point and thermal degradation temperature. The incorporation of 4HB, 3HV or 3HHx into the poly(3-hydroxybutyrate) chain influenced the temperature parameters of the polymers, too; the general trend was for the temperature parameters to decrease with an increase in the molar fractions of these monomer units (Tables 2 and 3). The difference between the melting point and the thermal degradation temperature stayed within a range of 100–120 °C, i.e. the polymers remained thermally stable.

The degree of crystallinity of polymers (C_x) was definitely found to be related to the composition and molar fractions of monomer units of PHAs. The degree of crystallinity of PHA copolymers is significantly lower than that of homogenous P(3HB) (75 ± 5%). The influence of the monomer units on the ratio of crystalline to amorphous phase in copolymers containing major fractions of two monomer units increased from 3-hydroxyvalerate to 3-hydroxyhexanoate to 4-hydroxybutyrate. The properties of PHAs containing major fractions of three monomer units (3HB/4HB/3HV or 3HB/3HV/3HHx) are generally similar to those of two-component PHAs; as the fractions of 4-hydroxybutyrate, 3-hydroxyvalerate and/or 3-hydroxyhexanoate are increased, their temperature characteristics (melting point and thermal degradation temperature) become 10–15 °C lower than those of P(3HB). The degree of crystallinity decreases to 35–40% in 3HB/3HV/3HHx and to 8–22% in 3HB/4HB/3HV.

Thus, by cultivating *C. eutrophus* B-10646 in the two-stage batch mode, without essentially changing the process, with varied conditions of carbon nutrition of the cells grown using the $CO_2/O_2/H_2$ gas mixture as the main growth substrate, we employed the methods for production of PHAs with different molar fractions of monomer units and synthesized the polymers that had significantly different physicochemical properties.

4. Conclusion

A new strain of *Cupriavidus* – *Cupriavidus eutrophus* B-10646, capable of synthesizing high PHA yields (up to 85%) under autotrophic growth conditions, was investigated in this study. Conditions enabling production of PHA tri- and tetra-polymers with different major fractions of 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) monomer units from mixed carbon substrate (CO_2 and PHA monomer precursors – valerate, hexanoate, γ-butyrolactone) were tested and improved. The resulting polymers differed significantly in their temperature properties and degree of crystallinity.

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