PHA SYNTHESIS BASED ON GLYCEROL AND IMPLEMENTATION OF THE PROCESS UNDER CONDITIONS OF PILOT PRODUCTION

Tatiana Volova[a,b*], Aleksey Demidenko[a,b], Evgeniy Kiselev[a,b], Sergey Baranovskiy[a], Natalia Zhila[a,b]

[a]Siberian Federal University, 79 Svobodnyi Ave., Krasnoyarsk, 660041, Russian Federation
[b]Institute of Biophysics SB RAS, Federal Research Center “Krasnoyarsk Science Center SB RAS”, Akademgorodok, Krasnoyarsk, 660036, Russian Federation

*Corresponding author: Tatiana Volova
e-mail: volova45@mail.ru

Abstract

The synthesis and properties of polyhydroxyalkanoates (PHA) of different composition synthesized by *Cupriavidus eutrophus* B-10646 using glycerol as a carbon substrate were studied. In fed-batch culture in a 30-l fermenter the suitability of glycerol of various purification degrees for the synthesis of poly(3-hydroxybutyrate) P(3HB) with a 99.5, 99.7, and 82.07% content of the main substance was demonstrated. Purified glycerol (99.7%) was used for a 100-L pilot scale fermentation. The total biomass and P(3HB) yields were 110 and 85.8 g/L respectively after 60 h in fed-batch fermentation. An average productivity of P(3HB) was 1.33 g/L h The physical and chemical properties of P(3HB) synthesized on glycerol have changed: the degree of crystallinity and molecular weight decreased, but the temperature characteristics remain unchanged.

Keywords: glycerol, polyhydroxyalkanoates, synthesis, productivity, properties

50 ## 1. Introduction

51 The development of new technologies aimed at integrated waste processing and 52 reproduction of target products, including the production of environmentally friendly energy 53 carriers and materials, is consistent with the concept of environmentally sound sustainable 54 industrial development. Microorganisms are a source for obtaining a variety of food, fodder, 55 medical and technical products. Polyhydroxyalkanoates (PHAs) are a valuable product of 56 biotechnology; they have a wide range of valuable properties, including biocompatibility and 57 biodegradability in biological media, and are promising for use in various fields [2, 9, 10, 20, 21, 58 23, 25, 35, 40, 44]. To a large extent (up to 45-48%), the cost of PHAs is determined by the cost 59 of carbon raw materials [11], therefore, one of the most high-priority areas of research is the 60 development of technologies involving accessible substrates. Potential raw materials for PHA 61 synthesis are various substrates with different degrees of reduction, energy content and cost, 62 including individual compounds (carbon dioxide and hydrogen, sugars, alcohols, organic acids), 63 by-products of alcohol, sugar and hydrolysis industries, as well as production of olive, soybean 64 and palm oil, etc. [1, 14, 18, 22, 27, 37, 38]. The type of raw material used to produce PHAs is 65 determined based on the physiological and biochemical properties of the producers and the 66 economic feasibility of the chosen strategy, taking into account the field of application of the 67 finished product. One of the promising substrates for large-scale production of PHAs is glycerol, 68 the scale of production of which is currently increasing. This is due to the growing production of 69 biodiesel as an alternative renewable energy source [15, 32]. Biodiesel production has increased 70 dramatically from 500,000 gallons in 1999 to 450 million gallons in 2007. Glycerol is a by- 71 product, amounting to about 10%, when biodiesel is produced by transesterification of animal 72 and vegetable fats and oils (rapeseed, mustard, soybean, palm) [14]. In industrial glycerol grades, 73 the water content varies between 5.3 and 14.2%; methanol content — 0.001-1.7%; NaCl — 74 traces — 5.5%; K_2SO_4 — 0.8-6.6% [28]. Glycerol has been explored as a possible carbon source 75 for the fermentative production of hydrogen, succinic acid, glycolipid biosurfactants, citric acid,

76 and single cell oils [19, 26, 31], as well as for PHA synthesis [12]. The analysis of publications
77 (Table 1) showed the prospective viability of glycerol and the ability of representatives of
78 various taxa to utilize it for PHA synthesis. This side carbon compound could be the ideal source
79 for industrial production of PHAs. However, if glucose is used as carbon source, it is
80 metabolized to pyruvate via the Entner-Doudoroff pathway (2-keto-3-deoxy-6-phosphogluconate
81 pathway), and pyruvate can be converted by a dehydrogenase to acetyl-CoA, the central
82 intermediate of the cellular metabolism, and the starting compound for the P(3HB) synthesis,
83 then glycerol can be metabolized to pyruvate as well, but via the inter-mediate compound
84 glyceraldehyde-3-phosphate [19, 36].

85 Representatives of the genus *Cupriavidus* (formerly *Ralstonia*) are characterized by the
86 highest yield of PHAs on various substrates. Studies were conducted to implement the synthesis
87 of PHAs of various chemical compositions in autotrophic and heterotrophic conditions on gas
88 mixtures of hydrogen and CO_2, synthesis gas, sugars and other organic substrates [44-48]. The
89 possibility of synthesizing poly-3-hydroxybutyrate on glycerol was shown in 1990 in the culture
90 of *Alcaligenes eutrophus* Z-1 (later renamed to Ralstonia eutropha) [43]. Systematic studies of
91 this substrate were deployed in the late 90's — early 2000's and became more active in the last
92 decade. To date, the synthesis of PHAs (mainly P(3HB) but also copolymers P(3HB/3HV),
93 P(3HB/4HB)) has been studied under different cultivation conditions on a mineral salt medium
94 containing glycerol by natural strains of various taxa: *Methylobacterium rhodesianum* [5],
95 *Methylobacterium extorquens* [39], *Cupriavidus necator* [28], *Paracoccus denitrificans* [28],
96 *Pseudomonas oleovorans* [3], *Pseudomonas corrugate* [3], *Burkholderia cepacia* [49],
97 *Caldimonas manganoxidans* [17], as well as mutant microorganisms, for example, *Cupriavidus*
98 *necator* DSM 545 [7, 8], *Pandoraea* sp. prp25 [13] and recombinant strains *Ralstonia eutropha*
99 KNK-DCD1 [42], *E.coli CT106* [30] (Table 1). Processes were described that were implemented
100 in shake flask cultures and fed-batch cultures in fermenters from 2.0 to 10-15 L; as well as a
101 scaled process of P(3HB) synthesis by *Burkholderia cepacia* ATCC 17759 at a culture volume of

102 200 L [49]. The achieved productivity levels by the yield of bacterial biomass and the yield of
103 polymers vary considerably, from 2–10 to 40–65 g/L and from 20–40 to 60–70%, respectively.
104 The production parameters of the processes are significantly influenced by the type of glycerol
105 used and the content of the main substance and impurities in it, the presence of which (chlorides,
106 sulfates, methanol) inhibits bacterial growth and PHA synthesis, and also reduces its molecular
107 weight [4, 7, 16, 28, 36, 42]. In general, the analysis of publications indicates an undoubted
108 prospective viability of glycerol as a substrate for PHA production. It is obvious that involving
109 new strains and improving the technological stages of the process will contribute to the
110 improvement of PHA production.

111 The present work demonstrates the kinetic and production indices of the *Cupriavidus*
112 *eutrophus* B-10646 culture in cultivation on purified and crude glycerol, the properties of
113 synthesized PHAs, and the results of the technology scaling in pilot production.

114 **2. Material and methods**

115 *2.1. Bacterial strains*

116 The strains used in this study were *Ralstonia eutropha* B 5786; *R.eutropha* B 8562 and
117 *Cupriavidus eutrophus* B-10646, registered in the Russian Collection of Industrial
118 Microorganisms (RCIM). Chemolithoorganotrophic bacteria of the genus *Cupriavidus* (formerly
119 known as *Ralstonia*) are regarded as very promising PHA producers, as these bacteria are
120 capable of synthesizing PHAs in very high yields (80-90% of cell dry weight) from various
121 substrates [44].

122 *2.2. Media*

123 Schlegel's mineral medium was used as a basic solution for growing cells: $Na_2HPO_4 \cdot H_2O$
124 – 9.1; KH_2PO_4 – 1.5; $MgSO_4 \cdot H_2O$ – 0.2; $Fe_3C_6H_5O_7 \cdot 7H_2O$ – 0.025; $CO(NH_2)_2$ - 1.0 (g/L).
125 Nitrogen was provided in the form of urea, and, thus, no pH adjustment was needed. The pH
126 level of the culture medium was stabilized at 7.0±0.1. A solution of iron citrate (5 g/L), which
127 was used as a source of iron, was added to reach a concentration of 5 ml/L. Hoagland's trace

128 element solution was used: 3 ml of standard solution per 1 L of the medium. The standard
129 solution contains H_3BO_3 – 0.288; $CoCl_2{\cdot}6H_2O$ – 0.030; $CuSO_4{\cdot}5H_2O$ – 0.08; $MnCl_2{\cdot}4H_2O$ –
130 0.008; $ZnSO_4{\cdot}7H_2O$ – 0.176; $NaMoO_4{\cdot}2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g/L).

131 Synthesis of PHA copolymers [P(3HB/4HB) or P(3HB/3HV)] was achieved as follows:
132 after 10 h of cultivation, nitrogen supply was discontinued, and the culture medium was
133 supplemented with precursor substratesε(-caprolactone, and propionic or valeric acids in the
134 form of potassium salts).

135 The main carbon substrate was glycerol of various grades, which was sterilized by
136 membrane filtration using Opticap XL300 Millipore Express SHC filters (U.S.):

137 Glycerol purified (Corporate Oleon, Sweden): glycerol - 99.3; chloride - 0.0001; salts
138 (NH4) - 0.005; Fe - 0.0005; Ar - 0.00004; moisture - 0.09; fatty acid and ester - 0.25 (% mass);
139 max heavy metal - 0.00005 ug/g (Glycerol I). Glycerol refinery B.V (Duth glycerol refinery,
140 Netherlands): glycerol - 99.7; chloride <0.001; moisture - 0.09; fatty acid and ester - 1.0; sulfate
141 <0.002; organic total inpurities - 0.5-1.0; organic individual inpurities - 0.1 (% mass); heavy
142 metal <5 (ug/g) (Glycerol II). Glycerol crude (M.V.R. PINAConsultoria Tecnica, Brazil):
143 glycerol - 82.07; chloride - 4.35; mong - 0.13; methanol - 0.13; ash - 6.59; moisture - 9.88 (%
144 mass); pH 5.8 (Glycerol III).

145 Due to the significant increase in biomass during the process in the cultivator, substrate
146 flows were supplied to the culture to provide the cells with the necessary substrates in
147 accordance with the dynamics of the cells biomass increase in the culture. The feeding substrates
148 were the solutions which were fed into the culture using a multichannel dosing pump: glycerol,
149 urea (60 g/l), $MgSO_4$ (30 g/l) + trace elements solution. Variation of the rates of substrate
150 feeding to the culture is provided by the concentrations of the medium components (glycerol 5-
151 10 g/L, nitrogen 0.1-0.2 g/L at the first stage; phosphorus 20-40 mg/L; sulfur, potassium,
152 magnesium 10 mg/L for each).

153 *2.3. Cultivation of bacteria*

154 To cultivate bacteria in shake flask culture, an Innova 44 constant temperature incubator
155 shaker (New Brunswick Scientific, U.S.) was used. Inoculum was prepared by resuspending the
156 museum culture maintained on agar medium. Museum culture was grown in 1.0-2.0 L glass
157 flasks half-filled with saline liquid medium, with the initial concentration of glycerol from 5 to
158 10 g/L.

159 Growth kinetics of bacterial cells was studied in automated laboratory fermentors
160 (Bioengineering AG, Switzerland), with a 30-L and 150 L fermentation vessel and the working
161 volume of the culture from 18 to 100 L, under strictly aseptic conditions.

162 Fermenters were equipped with systems for monitoring pH, level, foam level,
163 temperature, pressure, and dissolved oxygen. The control of fermenters was carried out with the
164 help of BioScadaLab software in automatic mode. To supply the feeding substrates, the
165 fermenters were equipped with Bioengineering Peripex peristaltic pumps. The concentration of
166 dissolved oxygen was maintained at DO 30%. The air supply control was carried out in a
167 cascade mode (DO-air flow-mixer revolutions). During cultivation in a 30-liter fermenter, the
168 amount of air supplied per cultivation process varied from 0 to 5.5 Nl/min, the speed varied from
169 500 to 1000 rpm. During cultivation in a 150-liter fermenter, the amount of air supplied per
170 cultivation process varied from 10 to 5.5 Nl/min, the speed varied from 300 to 750 rpm.

171 A two-stage process was used. In the first stage, cells were grown under nitrogen
172 deficiency: the amount of nitrogen supplied in this stage was 60 mg/g cell biomass synthesized
173 (i.e. 50% of the cell's physiological requirements – 120 mg/g); the cells were cultured in
174 complete mineral medium and with glycerol flux regulated in accordance with the requirements
175 of the cells. In the second stage, cells were cultured in nitrogen-free medium; the other
176 parameters were the same as in the first stage. The temperature of the culture medium was
177 30 ± 0.5^{o}C and pH was 7.0 ± 0.1.

178 *2.4. Monitoring process parameters*

179 During the cultivation samples of culture medium were taken for analysis every 4-5 h (in

180 fermentors) or every 8-10 h (in flasks); cell concentration in the culture medium was determined
181 based on the weight of the cell samples dried at 105 °C for 24 h (DCW); the general biomass
182 (X_{total}) and catalytically active biomass (X_c) (X_c = X_{total}–PHA), g/L were distinguished. Cell
183 concentration in the culture medium was monitored every hour by converting the optical
184 absorbance at 440 nm of culture broth to dry cell weight by using a standard curve prepared
185 previously.

186 Glycerol concentration was determined using the method based on the oxidation of
187 glycerol by sodium periodate in a sulfuric acid solution to formaldehyde and determination by a
188 colorimetric method with chromotropic acid [29]. Nitrogen concentration in the culture medium
189 was analyzed at different time points, using a photometric method, with Nessler's reagent.

190 The criteria for evaluating the process of PHA biosynthesis were as follows:
191 concentration of cell biomass in culture, polymer yield, expenditure of the main growth
192 substrate, duration and productivity of the process. For this, the kinetic and production
193 parameters of the culture were determined by conventional methods. The biomass yield (X, g/L),
194 the catalytic biomass yeild (X_c=X–PHA, g/L), the yield coefficient of the polymer (Y, g PHA/g
195 substrate), the specific growth rate (μ, h^{-1}), the productivity (P, g/L h) were calculated.

196 Specific growth rate of the culture (μ, h^{-1}) was determined using the following equation:

197 $$\mu = dX_c\ \mathrm{dt} * 1/X_c,$$

198 where X_c is catalytic biomass, g/L; t - duration of cultivation, h.

199 Specific rate of polymer synthesis (μ_β, h^{-1}) was determined using the following formula:

200 $$\mu\beta = dPHA\ \mathrm{dt} * 1/Xc$$

201 where PHA are initial and final intracellular polymer concentrations, g/L.

202 The yield coefficient of the polymer, *Y*, g/g, was calculated using the following formula:

203 $$Y = \Delta PHA/S,$$

204 where *P* is initial and final polymer content, g and *S* is consumed substrate, g

205 PHA samples were extracted from bacterial biomass with chloroform and precipitated in
206 hexane. The optimized extraction procedure enabled the production of medically pure specimens
207 that contained no organic impurities (proteins, carbohydrates or lipids, including fatty acids).

208 *2.5. Analysis of PHA*

209 Intracellular polymer content at different time points was determined by analyzing
210 samples of dry cell biomass. Intracellular PHA content and composition of extracted polymer
211 samples were analyzed by a GC-MS (6890/5975C, Agilent Technologies, U.S.). Both
212 lyophilized cells and extracted polymer were subjected to methanolysis in the presence of
213 sulfuric acid, and polymer was extracted and methyl esterified at 100°C for 3 h. Benzoic acid
214 was used as an internal standard to determine total intracellular PHA.

215 ^{1}H NMR spectra of the polymer were recorded at room temperature in $CDCl_3$ on a Bruker
216 AVANCE III 600 spectrometer (Germany) operating at 600.13 MHz.

217 Molecular weights and molecular weight distributions of P3HB/DEG were examined
218 using a gel permeation chromatograph (Agilent Technologies 1260 Infinity, U.S.) with a
219 refractive index detector, using an Agilent PLgel Mixed-C column. Chloroform was the eluent.
220 Calibration was made using polystyrene standards (Fluka, Switzerland, Germany). Molecular
221 weights (weight average, Mw, and number average, Mn) and polydispersity (Đ = Mw/Mn) were
222 determined.

223 Thermal analysis of PHA specimens was performed using a DSC-1 differential scanning
224 calorimeter (METTLER TOLEDO, Switzerland). The specimens were heated at a rate of 5
225 °C/min to 200 °C, then cooled to -20 °C, held for 20 minutes and re-heated to 320 °C. Glass
226 transition temperature (T_g), crystallization temperature (T_c), melting point (T_{melt}) and thermal
227 degradation temperature (T_{degr}) were determined from peaks in thermograms using the "StarE"
228 software.

229 In order to determine the crystallinity of the PHAs, three film samples 2 cm in diameter
230 and 0.15 mm thick were prepared from a 2% polymer solution in chloroform. The samples had a

231 circular shape because during measurement the sample spins in a direction perpendicular to the
232 surface. X-ray structure analysis and determination of crystallinity of PHAs were performed
233 employing a D8ADVANCE X-ray powder diffractometer equipped with a VANTEC fast linear
234 detector, using CuKa radiation (‘Bruker, AXS’, Germany). The scan step was 0.016°,
235 measurement time in each step 114 s, and scanning range from 5° to 60° (from 48° to 60° there
236 only was a uniformly decreasing background); the registered parameter was intensity of X-rays
237 scattered by the sample; 55°/0.016° = 3438 times. The degree of crystallinity was calculated as a
238 ratio of the total area of crystalline peaks to the total area of the radiograph (the crystalline +
239 amorphous components). Measurement accuracy: point measurement accuracy ± 0.4 PPS, with
240 the lowest intensity 1.5 PPS and the highest intensity 32 PPS; the error in determination of the
241 degree of crystallinity, which was calculated based on multiple measurements, was 2% or less.

242 *2.6. Statistics*

243 Statistical analysis of the results was performed by conventional methods, using the
244 standard software package of Microsoft Excel. Arithmetic means and standard deviations were
245 found. The statistical significance of results was determined using Student’s test (significance
246 level: $P \leq 0.05$).

247 **3. Results and discussion**

248 *3.1. Selection of a productive strain in shake flask culture*

249 Glycerol II was used for selection of the most productive strain capable to synthesize
250 PHA on glycerol and to study bacteria growth and polymer accumulation. Bacteria were grown
251 in a batch culture in 1.0 L flasks using reduced urea content (0.5 g/L) and an initial glycerol
252 concentration of 5 and 10 g/L. The utilization of glycerol began after the lag phase which lasted
253 from 20 to 30-40 h, regardless of the initial concentration of glycerol in the medium. A higher
254 biomass yield after 80 h was obtained in the culture of *C. eutrophus* B-10646 (2.0 g/L) compared
255 to *R. eutropha* B 5786 (0.8 g/L) and *R. eutropha* B 8562 (1.1 g/L). The yields of the polymer
256 also differed and amounted to 57, 45 and 38%, respectively (Fig. 1). To adapt the culture to

257 glycerol, successive reseedings were performed. As the bacteria adapted to glycerol, the lag-
258 phase became shorter until its complete elimination, while the productivity increased with regard
259 to the cell biomass and polymer yield. The yields of cell biomass after 60 h were from 5.1 to 7.2
260 g/L depending on strains, which is comparable to the values on sugars. The polymer
261 concentration in the cells ranged from 57 to 71% (Fig. 1). The analysis showed that the
262 synthesized polymer was poly-3-hydroxybutyrate [P(3HB)]. This is higher than the indices
263 obtained under similar conditions in cultures of many natural strains: *C. necator* IPT 026, *Ps.*
264 *oleovorans* NRRLB-14682, *Ps. corrugate* 388, *Paracoccus* sp. LL1 [3, 6, 24], and somewhat
265 lower only in cultures of individual mutant and recombinant producers [7, 42] (Table 1). The
266 strain *Cupriavidus eutrophus* B-10646 which was selected for further research and scaling of the
267 process was recognized as the most productive strain.

268 *3.2. Synthesis of PHA copolymers by C. eutrophus B-10646 on glycerol as the main C-*
269 *substrate*

270 The most valuable producers of PHA are strains capable to synthesize, in addition to
271 highly crystalline P(3HB), copolymers containing monomers other than 3HB. The inclusion of
272 different monomers depends on many factors: the physiological and biochemical specificity of
273 the strains, the substrate specificity of PHA synthase, the resistance to the inhibitory effect of the
274 substrates which are precursors of the desired monomers, the conditions of PHA accumulation
275 on the mixed carbon substrates. The strain *C. eutrophus* B-10646 is characterized by the ability
276 to synthesize PHA copolymers with a different set of monomers when sugars, acetate, fatty
277 acids, mixtures of H_2 and CO_2 and precursor substrates (valeric acid, hexanoic acid, γ-
278 butyrolactone, etc.) are used as the main C-substrate [44]. The accumulation of PHA copolymers
279 in the culture of *C. eutrophus* B-10646 grown on glycerol and potassium propionate, potassium
280 valerate and ε-caprolactone (precursors of 3-hydroxyvalerate and 4-hydroxybutyrate monomers,
281 respectively) was studied.

282 The bacteria were grown in a batch culture in flasks on Glycerol II. During the period of
283 the most active PHA synthesis (10 hours from the start of cultivation), a precursor substrate was
284 added to the culture at a concentration 1.0 g/L. As a precursor of 3-hydroxyvalerate monomers,
285 potassium propionate or potassium valerate was used; for the synthesis of 4-hydroxybutyrate
286 monomers, ε-caprolactone was used. The use of these substrates had different effects on the cell
287 biomass and PHA yields and the ratio of monomers. Varying the amount of added potassium
288 valerate, potassium propionate orε -caprolactone made it possible to synthesize a series of PHA
289 copolymers of different composition (Fig. 2).

290 10 hours after adding potassium valerate to the culture in a quantity of 1.0 g/L, the
291 inclusion of 3-hydroxyvalerate (3HV) was at a level of 23.4 mol.% (Fig. 2) and polymer content
292 was about 40% of dry biomass weight. By the end of the process (60 h), while the intracellular
293 content of the polymer increased to 70% of dry biomass weight, the monomer content of 3HV
294 remained practically unchanged. 10 hours after adding 1.0 g/L of potassium propionate as a
295 precursor of 3HB monomers, the inclusion of 3HB monomers in the polymer was about 14
296 mol.%, the polymer content was 57.9% (Fig. 2). At the end of the experiment, the polymer
297 content increased to 81.9%, the content of 3HB monomers — to 23.1 mol.%. One addition of ε-
298 caprolactone in the amount of 1.0 g/L, as a precursor of 4-hydroxybutyrate (4HB) monomers per
299 10 hours of the process led to the incorporation of 4HB into the polymer at a level of 5 mol.%
300 and remained unchanged until the end of the cultivation process (Fig. 2). The biomass yield and
301 polymer content was about 4.8 g/L and 71.2% of dry biomass weight. With two additions to the
302 bacterial culture of potassium valerate or potassium propionate at a concentration of 1.0 g/L (Fig.
303 2), inhibition of bacterial growth was observed, as a result of which the biomass yield decreased
304 to 3.1 g/L and 3.8 g/L, and there was an increase in the inclusion of 3HV monomers to 35.7 and
305 28.5 mol%, respectively. The subsequent addition of ε-caprolactone resulted in an increase of
306 4HB content to 9.8 mol%. Thus, using glycerol as the main C-substrate in the culture of *C.*
307 *eutrophus* B-10646, it is possible to achieve a productive synthesis of not only homopolymer

308 P(3HB), but also of copolymer PHAs with different monomer ratios. These results are
309 comparable with existing publications [8, 16, 34].

310 *3.3. Effect of glycerol concentration on bacterial growth and PHA synthesis*

311 Cultivation of *C.eutrophus* B-10646 bacteria by varying the concentration of purified
312 glycerol within wide limits made it possible to determine the limits of physiological action of
313 this substrate for the strain and the kinetic constants. The limits of physiological action of
314 glycerol for the studied strain are very wide, varying in the range 0.5–60.0 g/L. The presence of a
315 wide plateau (from 1 to 30 g/L) was revealed; the zones of limitation and inhibition of bacterial
316 growth by glycerol were, 0.1-3.0 and 30-60 g/L, respectively. The obtained dependence of the
317 specific growth rate (μ) on the substrate concentration (S) is described by the Andrews equation,
318 which is a modified Monod equation. Using the graphical analysis method of Linuiver-Burke
319 (1/μ:1/S) and the Dickson's method (1/μ:S), kinetic constants for this strain (saturation constant
320 (*Ks*) and inhibition constant (*Ki*), μ_{max}) were calculated. It was determined that for the strain *C.*
321 *eutrophus* B-10646 the limits of the physiological action of glycerol were 1-30 g/l; K_s and K_i
322 were, respectively, 0.36 g/L (0.004 mol/L) and 62.0 g/L (0.673 mol/L); $\mu_{max} = 0.085\ h^{-1}$.

323 *3.4. Study of the growth and synthesis of PHA by C. eutrophus B-10646 on purified and*
324 *crude glycerol*

325 The content of the main substance (glycerol) in purified glycerol is more than 95-99%. In
326 unpurified (crude) glycerol, depending on the raw material and the technology used, the content
327 of glycerol proper is 80-85%, the rest is impurities, including free fatty acids (FFA) and methyl
328 esters of FFA, alcohols, as well as water and salts, which, as a rule, inhibit the microorganisms
329 responsible for PHA production.

330 Two grades of purified glycerol with a 99.3 (Glycerol I) and 99.7% (Glycerol II) content
331 of the base material and unpurified, crude glycerin (82.07%) were studied. Cultivation of *C.*
332 *eutrophus* B-10646 bacteria was carried out in a 30-liter fermenter with a starting cell
333 concentration in inoculate of 1.0-1.5 g/L, 25 g/L glycerol and 18 L working volume of the

334 culture. The process was performed in a two-stage culture (30 hours) on saline medium where
335 the growth of cells was limited by nitrogen deficiency at the first stage for 30-32 h and in the
336 nitrogen-free medium — at the second stage (24-30 h). The current concentration of glycerol in
337 the culture was maintained at a level of 5-10 g/L, when it was dosed into the culture with a
338 peristaltic pump dispenser. The results of PHA synthesis in a fermenter on three glycerol sources
339 are presented in Fig. 3.

340 The results of the production indices evaluation of the *C.eutrophus* B-10646 culture on
341 purified glycerol (Glycerol I and Glycerol II) were close with regard to the yield of cell biomass
342 and polymer yield. When glycerol purified and glycerol refinery B.V. were used, the yield of
343 total biomass reached 70 g/L at maximum, and the polymer content in cells was 72-75%. The
344 analysis of the obtained results revealed some differences in the kinetic parameters during the
345 cultivation of bacteria. As shown in Fig. 3, the specific growth rate of the total and active cell
346 biomass and consumption of purified Glycerol I varied regularly in the course of the experiment.
347 The maximum values of the specific growth rate of bacteria (by total and active biomass) on
348 glycerol purified occurred in the initial period of the first stage of the process on a complete
349 nutrient medium with a limited supply of nitrogen (50% of the physiological requirement of
350 bacteria), and amounted to 0.15 h^{-1} and 0.14 h^{-1}, respectively (Fig. 3). This period corresponds to
351 the most active consumption of glycerol by the culture, at an average rate of 4.0±0.2 g/g h. The
352 rate of polymer synthesis at this stage was 0.18 h^{-1}, and had a downward trend in time. After 30-
353 32 hours, the total cell biomass concentration was 42.1±1.7 g/L, the polymer content in the cells
354 reached 47.8±2.3%. During the second stage the supply of nitrogen to the culture was stopped;
355 the controlled supply of glycerol and mineral elements precluded the deficiency of these
356 substrates in the culture. At the second stage, there was a gradual decrease in the specific growth
357 rates of bacteria and polymer synthesis as the consumption of glycerol dropped. At the end of the
358 fermentation period (60 h), the total cell biomass yield was 69.3±3.5 g/L, the yield of the

359 polymer was 72.4±3.6%. The consumption of glycerol for the whole period was 3.5±0.2 kg,
360 which corresponds to the economic coefficient $Y_{P(3HB)}$ 0.29 ± 0.01 g/g.

361 On the second type of purified glycerol (Glycerol II) (Fig. 3), the maximum values of the
362 specific growth rate of bacteria (in terms of total and active biomass) at the first stage of the
363 process were 0.15 h^{-1} and 0.13 h^{-1}, which is comparable to the results for glycerol purified during
364 this period. The consumption of glycerol in this period was from 3.8 to 4.0 g/g h — while the
365 polymer synthesis rate was 0.18 ± 0.02 h^{-1} with a downward trend. After 30 hours, the total cell
366 biomass concentration was 45.6±2.2 g/L, the polymer content in the cells reached 56.1±2.7%. At
367 the end of the fermentation period, the total cell biomass yield was 69.4±3.5 g/L, the yield of the
368 polymer was 73.3±3.6%. The consumption of glycerol for the whole period was 3.4±0.2 kg,
369 which corresponds to the economic coefficient $Y_{P(3HB)}$) 0.29±0.01.

370 Glycerol III the maximum values of bacteria specific growth rate (in terms of total and
371 active biomass) at the first stage of the process were 0.14 h^{-1} and 0.13 h^{-1} respectively when
372 glycerol was consumed by the culture at a level of 4.2±0.2 g/g h. The average rate of polymer
373 synthesis at this stage was 0.17±0.02 h^{-1}. After 30 hours, the total cell biomass concentration was
374 46.2±1.9 g/L, the polymer content in the cells reached 52.2±2.1%. At the end of the fermentation
375 period, the total cell biomass yield was 69.3±2.9 g/L, the yield of the polymer was 78.1±3.2%.
376 The consumption of raw glycerol for the whole period amounted to 3.8±0.2 kg, which
377 corresponds to an economic coefficient for the polymer - 0.26±0.01. Taking into account that the
378 concentration of raw glycerol is 82.07%, $Y_{P(3HB)}$ in terms of absolute glycerol was 0.29±0.02 g/g.

379 Comparison of the results with publications shows that the use of crude glycerin, as a
380 rule, is accompanied by inhibition of bacterial growth and polymer synthesis. In the cultures of
381 *Cupriavidus necator* JMP 134 and *Paracoccus denitrificans* DSMZ 4134 on purified glycerol
382 with yeast extract additions, the biomass and polymer yield were 70 g/L and 70%, respectively;
383 on crude glycerol these indicators were lower: 50 g/L and 48%, respectively [28]. The authors
384 showed a stronger negative effect of the impurities of NaCl than of K_2SO_4. A similar inhibitory

385 effect of the impurities of crude glycerol on the yield of biomass and PHA was detected in the
386 culture of *C.necator* DSM 545 [7]. The mutant strain *Cupriavidus necator* DSM 545 was also
387 inhibited by the NaCl impurities (2-6 g/L) of crude glycerol [16]. A similar negative effect of
388 crude glycerol on the synthesis of PHA was obtained by using glucose, in addition to glycerol, in
389 cultures of the natural strains *Cupriavidus necator* DSM 545 and *Burkholderia sacchari* DSM
390 17165 [36]. In the work [4], the authors compared the synthesis of PHA on purified and crude
391 glycerol (waste of biodiesel production) and showed that the yields of biomass and polymer were
392 1.5 times lower on crude glycerol. Thus, it was shown that the synthesis of PHA on crude
393 glycerol is less productive than that on purified glycerol. Nevertheless, comparison of the results
394 of PHA production on crude and purified glycerol obtained in the process of biodiesel production
395 from plant raw materials showed that it is preferable for the effective synthesis of the polymer to
396 use purified glycerol, despite the costs associated with its purification. When using purified
397 glycerol, the consumption of carbon substrate for polymer synthesis is 8%, and not 40-45%, as is
398 the case with both sugars and other substrates [32, 33].

399 *3.5. Pilot production of PHA on glycerol*

400 The process of PHA synthesis by *C. eutrophus* B-10646 on Glycerol II was scaled and
401 studied under pilot production (PP) conditions. Pilot production includes: units for media and
402 inoculum preparation; unit for fermentation; unit for polymer extraction and purification. The PP
403 fermentation unit includes a steam generator (Biotron, South Korea) for sterilizing fermenters
404 and communications, a compressor (Remeza, Belarus) for air supply, a 30-liter seed culture
405 fermenter, a 150-liter production fermenter, an ultrafiltration unit (Vladisart, Russia) to
406 concentrate the culture, and a unit for cool dehumidification of the condensed bacterial
407 suspension (LP10R ILSHIN C, South Korea) (Fig. 4).

408 The seed culture *C. eutrophus* B-10646 was obtained from a museum culture stored on an
409 agarized medium by growing in 2.0-liter flasks on a complete nutrient medium in a shaker-
410 incubator. The resulting culture (24 L) was concentrated by centrifugation in compliance with

411 the sterility rules. A 5.0-liter inoculum with a cell concentration of 14-17 g/L was used to seed a
412 30-liter fermenter containing 6 L of phosphate buffer; the supply of sterile air was provided, and
413 the process of building up the seed material began. The initial concentration of cells in the
414 inoculum was about 7.5 g/L. The process of building up the seed material was carried out on a
415 complete nutrient medium. For this purpose, dosing pumps were used to feed continuously by
416 separate streams the solutions of glycerol, carbamide, magnesium sulfate, and ferric citrate with
417 trace elements into the fermenter. The residual concentration of glycerol in the culture was
418 maintained at 5-20 g/L; carbamide — 0.1-0.2 g/L; magnesium sulphate — 0.05-0.1 g/L. The
419 process continued for 15-20 hours. As a result of introducing the feeding solutions, the volume
420 of the culture increased to 15; the concentration of cells in the culture was about 20-25 g/L; the
421 polymer content was not higher than 10-15 g/L. A higher intracellular concentration of the
422 polymer would adversely affect the rate of cell growth when transferring the culture to the
423 production fermenter. At this stage, the average rates of cell growth and polymer synthesis were
424 about 0.11 h^{-1}.
425 The inoculum with a volume of 13-15 liters with a cell concentration of 20-25 g/L
426 obtained in a seed fermenter, in a sterile seeding line was pumped into a production fermenter
427 containing 50 liters of a sterile buffer solution of potassium and sodium phosphates; thus, the
428 initial concentration of the culture of 65-70 liters was 7.0 g/L. The process was carried out in two
429 stages with continuous supply to the culture of sterile air and feeding solutions. At the first stage,
430 glycerol and all components of the medium were supplied in excess; the supply of nitrogen was
431 50% of the physiological needs of the culture, that is, the growth of cells was limited. After 25-
432 30 hours the concentration of total biomass reached 75-80 g/L; the polymer content in cells was
433 45-50%. Further, the supply of nitrogen to the culture was stopped and the process was
434 continued for 25-30 hours on a nitrogen-free medium with a gradual decrease in the supply of
435 glycerol and air to the culture. At the end of fermentation, the culture volume was 95-100 L, the
436 concentration of biomass 110±5.5 g/L, the polymer content – 78±3.1%. Thus, in the production

437 fermenter, in the adopted mode, .7.7 kg of homopolymer P(3HB) were synthesized in hours with
438 an average productivity of 1.33±0.1 g/L h and a polymer synthesis rate of 0.17 h^{-1}.
439 An important indicator of microbiological processes is the economic coefficient, that is,
440 the expenditure of the substrate for the formation of the desired product. The expenditure of
441 various substrates for PHA synthesis and the economic coefficient vary considerably, amounting,
442 for example, to 0.3-04. g/g of glucose; 1 g/g of palm oil [42] and 1.0 g/g of hydrogen [44]. The
443 expenditure of purified glycerol for polymer synthesis was 3.4-3.5 kg; accordingly, the economic
444 coefficient for this substrate was $Y_{P(3HB)}$ 0.29 g/g; on crude glycerol — somewhat lower, 0.26
445 g/g. This is consistent with the data of the work [28]. However, it should be noted that in the
446 works of different authors using different cultures this index varies from 0.05 to 0.37 g/g [5, 17,
447 36]. Concerning the scaling of PHA synthesis technologies using glycerol, in the available
448 literature there is a report on the scaling of P(3HB) synthesis process [49]. In a 400-liter
449 fermenter with a fill factor of 0.5 in a culture of 200 liters of the native strain *Burkholderia*
450 *cepacia ATCC 17759* on crude glycerin (85%), the yield of biomass and the yield of polymer,
451 after 120 hours, was 23.6 g/l and 31%, respectively. It was shown that with an increase in the
452 concentration of glycerol from 3 to 9%, the value Mw decreases from 300 to 170 kDa. These
453 indicators are much inferior to the results achieved in the work presented.

454 *3.6. Properties of PHA synthesized by C.eutrophus B-10646 on glycerol*

455 The conditions for cultivation of microorganisms, especially carbon substrate, affect the
456 composition and properties of PHAs. In a number of works, the properties of PHAs synthesized
457 on glycerol are analyzed. Special attention is paid to the molecular mass characteristics. This is
458 due to the fact that glycerol, a multi-hydroxy component of plant oil, has been reported to
459 function as a catalyzed chain transfer reaction (CT) agent in PHA polymerization, resulting in
460 the formation of low molecular weight PHA. In case of a CT reaction, the PHA chain number
461 increases in inverse proportion to the PHA molecular weight [41]. However, the results for the
462 values of Mw and Mn are not free of contradictions. A series of studies showed a decrease in the

463 Mw value of P(3HB) synthesized on glycerol, to 260-400 kDa [42] and lower [3, 4], but there
464 are also publications in which very high Mw values on glycerol are reported, up to 620 and 750
465 kDa [28], up to 790-960 kDa [7]. It should be noted that the values of molecular weight are an
466 important indicator of polymers, in particular, PHAs, in which the values Mw and Mn are a very
467 variable parameter — usually, the number average molecular weight (Mn) of bacterially
468 synthesized PHA is in the range of (10 -100) x 10^4. Because higher molecular weight gives PHA
469 higher mechanical strength [25].

470 The results of the study of the chemical composition and physical-chemical properties of
471 PHA samples synthesized on glycerol of different purification degrees are presented in Table 2.
472 Investigation of the properties of PHA samples synthesized by *C. eutrophus* B-10646 on three
473 types of glycerol did not reveal dramatic changes when using purified and crude glycerol. The
474 polymer synthesized by the glycerol-adapted productive culture *C. eutrophus* B-10646 on two
475 types of purified glycerol had similar values Mn 104 and 115 kDa; and Mw - 355 and 416 kDa
476 as well as polydispersity, respectively, 3.42 and 3.63. When using crude glycerol, the Mn and
477 Mw values were somewhat lower, 87 and 304 kDa. These values are generally lower than those
478 obtained earlier on other substrates. The ^{1}H-NMR spectra of three PHA samples synthesized on
479 three glycerol grades were similar (Fig. 5), and showed the expected resonances for P(3HB) as
480 demonstrated by the methyl group at 1.25 ppm, the methylene group between 2.45 and 2.65
481 ppm, and the methine group at 5.25 ppm. The ^{1}H-NMR-obtained spectra of PHA samples
482 synthesized on glycerol are similar to the published studies [3, 36, 49]. The decrease in
483 molecular weight was previously recorded for polymer samples synthesized by a non-glycerol-
484 adapted strain *C. eutrophus* B-10646 in shake flask culture (Mn and Mw, respectively, 59 and
485 210 kDa), while on sugars these values were 130-150 and 495-640 kDa, and on CO_2+H_2 - 250
486 and 830 kDa [48].

487 PHAs belong to semicrystalline polymers in which the ratio of amorphous and crystalline
488 phases varies depending on the chemical composition and the set and ratio of monomers.

489 Concerning the degree of crystallinity (Cx), it is generally accepted that P(3HB) is a highly
490 crystalline polymer in which the crystalline phase dominates over amorphous phase, and Cx is
491 65-80% [25]. In the samples of P(3HB) synthesized on glycerol, the value Cx was reduced (50-
492 55%), thus, the amorphous and crystalline phases were aligned. A similar effect was noted in
493 other works. The Cx value of P(3HB) synthesized on glycerol by *Cupriavidus sp.* USMAHM13
494 was 49% [34]; a series of polymer samples synthesized by *Cupriavidus necator* IPT 026 was
495 also reduced and varied in the range of 52-62% [6].

496 No deviations in the parameters of the temperature characteristics were found in the
497 investigated samples of P(3HB), Tm and Td values were in the previously identified value
498 limits, respectively, 172-176 and 295-296 ^{o}C.

499 Thus, when synthesized on glycerol, there are changes in physical-chemical properties: a
500 decrease in the degree of crystallinity and molecular weight of P(3HB), but the temperature
501 characteristics remain unchanged

502 **4. Conclusion**

503 The performed studies and the obtained results showed that the studied natural strain of
504 bacteria *C. eutrophus* B-10646 provides a productive synthesis of PHA on glycerol, comparable
505 to the process on sugars. It is important to note the proven possibility of implementing the
506 productive process of PHA synthesis not only on purified glycerol, but also on crude glycerol
507 containing impurities, without drastically reducing the productivity of the bacterial culture. In a
508 scaled-up pilot variant, a highly efficient process with high yields of total biomass (110 g/l) and
509 polymer (78%) was implemented using purified glycerol.

510 **Conflict of interest**

511 No conflict of interest to declare.

512 **Acknowledgment**

513 This study was financially supported by Project “Agropreparations of the new generation:
514 a strategy of construction and realization” (Agreement No 14.Y26.31.0023) in accordance with

515 Resolution No 220 of the Government of the Russian Federation of April 9, 2010, "On measures
516 designed to attract leading scientists to the Russian institutions of higher learning".

517 **References**

518 1. Aditi, S., D'Souza Shalet, N.M., Pranesh, R., Katyayini, T., 2015. Microbial production
519 of polyhydroxyalkanoates (PHA) from novel sources: A Review. Int. J. Res. BioSci. 4,
520 16-28.

521 2. Anjum, A., Zuber, M., Zia, K.M., Noreen, A., Anjum, M.N., Tabasum, S., 2016.
522 Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of
523 recent advancements. Int. J. Biol. Macromol. 89, 161–174.

524 3. Ashby, R.D., Solaiman, D.K.Y., Foglia, T.A., 2005. Synthesis of short-/medium-chain-
525 length poly(hydroxyalkanoate) blends by mixed culture fermentation of glycerol.
526 Biomacromolecules 6, 2106–2112.

527 4. Ashby, R.D., Solaiman, D.K.Y., Strahan, G.D., 2011. Efficient utilization of crude
528 glycerol as fermentation substrate in the synthesis of poly(3-hydroxybutyrate)
529 biopolymers. J. Am. Oil Chem. Soc . 88, 949–959.

530 5. Bormann, E.J., Roth, M., 1999. The production of polyhydroxybutyrate by
531 *Methylobacterium rhodesianum* and *Ralstonia eutropha* in media containing glycerol and
532 casein hydrolysates. Biotechnol. Lett. 21, 1059–1063.

533 6. Campos, M.I., Figueiredo, T.V.B., Sousa, L.S., Druzian, J.I., 2014. The influence of
534 crude glycerin and nitrogen concentrations on the production of PHA by *Cupriavidus*
535 *necator* using a response surface methodology and its characterizations. Ind. Crops Prod.
536 52, 338–346.

537 7. Cavalheiro, J.M.B.T., de Almeida, M.C.M.D., Grandfils, C., da Fonseca, M.M.R., 2009.
538 Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol.
539 Process Biochem. 44, 509–515.

540 8. Cavalheiro, J.M.B.T., Raposo, R.S.M., de Almeida M.C.M.D., Cesário, M.T., Sevrin, C.,
541 Grandfils, C., da Fonseca, M.M.R., 2012. Effect of cultivation parameters on the
542 production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and poly(3-
543 hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) by *Cupriavidus necator* using
544 waste glycerol. Bioresour. Technol. 111, 391–397.
545 9. Chanprateep, S., 2010. Current trends in biodegradable polyhydroxyalkanoates. J. Biosci.
546 Bioeng. 110, 621–632.
547 10. Chen, G.-Q., 2010. Plastics from bacteria: natural functions and applications,
548 Microbiology Monographs, Springer-Verlag, Berlin, Heidelberg.
549 11. Choi, J., Sang, Y.L., 1997. Process analysis and economic evaluation for poly(3-
550 hydroxybutyrate) production by fermentation. Bioprocess Eng.17, 335–342.
551 12. da Silva, G.P., Mack, M., Contiero, J, 2009.. Glycerol: a promising and abundant carbon
552 source for industrial microbiology. Biotechnol. Adv. 27, 30–39.
553 13. de Paula, F.C., de Paula, C.B.C., Gomez, J.G.C., Steinbüchel, A., Contiero, J., 2017
554 Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) production from biodiesel by-product and
555 propionic acid by mutant strains of *Pandoraea* sp. -*Biotechnol. Prog.*, 2017(33):1077–
556 1084.
557 14. Du, C., Sabirova, J., Soetaert, W., Lin, S.K.C., 2012. Polyhydroxyalkanoates production
558 from low-cost sustainable raw materials. Curr. Chem. Biol. 6, 14–25.
559 15. Fernández-Dacosta, C., Posada, J.A., Kleerebezem, R., Cuellar, M.C., Ramirez, A., 2015.
560 Microbial community-based polyhydroxyalkanoates (PHAs) production from
561 wastewater: Techno-economic analysis and ex-ante environmental assessment.
562 Bioresour. Technol. 185, 368–377.
563 16. Garcia, I.L., Lypez, J.A., Dorado, M.P., Kopsahelis, N., Alexandri, M., Papanikolaou, S.,
564 Villar, M.A., Koutinas, A.A., 2013. Evaluation of by-products from the biodiesel

565 industry as fermentation feedstock for poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
566 production by *Cupriavidus necator.* Bioresour. Technol. 130, 16–22.
567 17. Hsiao, L.-J., Lee, M.-C., Chuang, P.-J., Kuo, Y.-Y., Lin, J.-H., Wu, T.-M., Li, S.-Y.,
568 2018. The production of poly(3-hydroxybutyrate) by thermophilic *Caldimonas*
569 *manganoxidans* from glycerol. J. Polym. Res. 25, 85–92.
570 18. Jost, V., 2018. Packaging related properties of commercially available biopolymers – An
571 overview of the status quo. eXPRESS Polym. Lett. 12, 429–435.
572 19. Khahha, S., Goyal, A., Moholkar, V.S., 2012. Microbiol conversion of glycerol: present
573 status and future prospects. Crit. Rev. Biotechnol. 32, 235–262.
574 20. Koller, M., Sandholzer, D., Salerno, A., Braunegg, G., Narodoslawsky, M., 2013.
575 Biopolymer from industrial residues: Life cycle assessment of poly(hydroxyalkanoates)
576 from whey. Resour. Conserv. Recycl. 73, 64–71.
577 21. Koller, M., Maršálek, L., de Sousa Dias, M.M., Braunegg, G., 2017. Producing microbial
578 polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. New Biotechnol. 37,
579 24–38.
580 22. Kootstra, M., Elissen, H., Huurman, S., 2017. PHA's (polyhydroxyalkanoates): General
581 information on structure and raw materials for their production. A running document for
582 "Kleinschalige Bioraffinage WP9: PHA", Task 5: ACRRES, Wageningen UR, February
583 2017, Report 727.
584 23. Kourmentza, C., Plácido, J., Venetsaneas, N., Burniol-Figols, A., Varrone, C., Gavala,
585 H.N., Reis, M.A.M., 2017. Recent advances and challenges towards sustainable
586 polyhydroxyalkanoate (PHA) production. Bioengineering, 4, e55.
587 24. Kumar, P., Jun, H.-B., Kim, B.S., 2018. Co-production of polyhydroxyalkanoates and
588 carotenoids through bioconversion of glycerol by *Paracoccus* sp. strain. Int. J. Biol.
589 Macromol. 107, 2552–2558.
590 25. Laycock, B., Peter, H., Pratt, S., Werker, A., Lant, P., 2013. The chemomechanical

591 properties of microbial polyhydroxyalkanoate. Prog. Polym. Sci. 38, 536–583.
592 26. Mattam, A.J., Clomburg, J.M., Gonzalez, R., Yazdani, S.S., 2013. Fermentation of
593 glycerol and production of valuable chemical and biofuel molecules. Biotechnol. Lett. 35,
594 831–842.
595 27. Mozejko-Ciesielska, J., Kiewisz, R., 2016. Bacterial polyhydroxyalkanoates: Still
596 fabulous? Microbiol. Res. 192, 271–282.
597 28. Mothes, G., Schnorpfeil, C., Ackermann, J.-U., 2007. Production of PHB from crude
598 glycerol. Eng. Life Sci. 7, 475–479.
599 29. Nakamura, M., Iso, H., Kitamura, A., Imano, H., Noda, H., Kiyama, M., Sato, S.,
600 Yamagishi, K., Nishimura, K., Nakai, M., Vesper, H.W., Teramoto, T., Miyamoto, Y.,
601 2016. Annals of clinical biochemistry. Int. J. Labor. Med. 53, 632–639.
602 30. Pablo, N., Pettinary, M.J., Calvagno, M., Mendwez, B., 2008. Poly(3-hydroxybutyrate)
603 synthesis from glycerol by a recombinant *Escherichia coli* arsA mutant in fed-batch
604 microaerobic cultures. Appl. Microbiol. Biotechnol. 77, 1337–1343.
605 31. Papanikolaou, S., Fakas, S., Fick, M., Chevalot, I., Galiotou-Panayotou, M., Komaitis,
606 M., Marc, I., Aggelis, G., 2007. Biotechnological valorization of raw glycerol discharged
607 after bio-diesel (fatty acid methyl esters) manufacturing process: production of 1,3-
608 propanediol, citric acid and single cell oil. Biomass Bioenergy 32, 60–71.
609 32. Posada, J.A., Naranjo, J.M., Lypez, J.A., Higuita, J.C., Cardona, C.A., 2011. Design and
610 analysis of poly-3-hydroxybutyrate production processes from crude glycerol. Process
611 Biochem. 46, 310–317.
612 33. Posada, J.A., Rincón, L.E., Cardona, C.A., 2012. Design and analysis of biorefineries
613 based on raw glycerol: Addressing the glycerol problem. Bioresour. Technol. 111, 282–
614 293.
615 34. Ramachandran, H., Amirul, A.A., 2014. Bioconversion of glycerine pitch into a novel
616 yellow-pigmented P(3HB-co-4HB) copolymer: Synergistic effect of ammonium acetate

617 and polymer characteristics. Appl. Biochem. Biotechnol. 172, 891–909.

618 35. Rebouillat, S., Pla, F., 2016. Recent strategies for the development of biosourced-
619 monomers, oligomers and polymers-based materials: A review with an innovationand a
620 bigger data focus. J. Biomater. Nanobiotechnol., 7, 167–213.

621 36. Rodríguez-Contreras, A., Koller, M., de Sousa Dias, M.M., Calafell-Monfort, M.,
622 Braunegg, G., Marqués-Calvo, M.S., 2015. Influence of glycerol on poly(3-
623 hydroxybutyrate) production by *Cupriavidus necator* and *Burkholderias acchari.*
624 Biochem. Eng. J. 94, 50–57.

625 37. Sabbagha F., Muhamada, I.I., 2017. Production of poly-hydroxyalkanoate as secondary
626 metabolite with main focus on sustainable energy. Renew. Sustain. Energy Rev. 72, 95–
627 104.

628 38. Saharan, B.S., Grewal, A., Kumar, P., 2014. Biotechnological production of
629 polyhydroxyalkanoates: A review on trends and latest developments. Chin. J. Biol. article
630 ID 802984, 18 pages.

631 39. Taidi, B., Anderson, A., Dawes, E.A., Byrom, D., 1994. Effect of carbon sources and
632 concentration on the molecular mass of poly(3-hydroxybutyrate) produced by
633 *Methylobacterium extorquens* and *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol.
634 40, 786–790.

635 40. Tan, G.-Y.A., Chen, C.-L., Li, L., Ge, L., Wang, L., Razaad, I.M.N., Li, Y., Zhao, L.,
636 Mo, Y., Wang, J.-Y., 2014. Start a research on biopolymer polyhydroxyalkanoate (PHA):
637 A review. Polymers 6, 706–754.

638 41. Tomozawa, S., Saito,Y., Nakamura, Y., Abe, H., Tsuge, T., 2010. Chain transfer reaction
639 catalyzed by various PHA synthases with poly(ethylene glycol) as an exogenous chain
640 transfer agent. Appl. Microbiol. Biotechnol. 87, 1427–1435.

641 42. Tsuge, T., Ko, T., Tago, M., Abe, H., 2013. Effect of glycerol and its analogs on
642 polyhydroxyalkanoate biosynthesis by recombinant *Ralstonia eutropha*: A quantitative

643 structure-activity relationship study of chain transfer agents. Polym. Degrad. Stab. 98,
644 1586–1590.
645 43. Volova, T.G., Kalacheva, G.S., 1990. Polyhydroxybutyrate – thermoplastic
646 biodegradable plymer (obtaining, properties, application). Preprint 131B, Krasnoyarsk.
647 47 p (in Russian).
648 44. Volova, T.G., Shishatskaya, E.I., Sinskey, A.J., 2013. Degradable Polymers: Production,
649 Properties, Applications. Nova Science Pub. Inc., New York.
650 45. Volova, T., Kiselev, E., Shishatskaya, E., Zhila, N., Boyandin, A., Syrvacheva, D.,
651 Vinogradova, O., Kalacheva, G., Vasiliev, A., Peterson, I., 2013. Cell growth and PHA
652 accumulation from CO_2 and H_2 of a hydrogen-oxidizing bacterium, *Cupriavidus*
653 *eutrophus* B-10646. Bioresour. Technol. 146, 215–222.
654 46. Volova, T.G., Zhila, N.O., Shishatskaya, E.I., Mironov, P.V., Vasil'ev, A.D., Sukovatyi,
655 A.G., Sinskey, A.J., 2013. The Physicochemical properties of polyhydroxyalkanoates
656 with different chemical structures. Polymer Science, Ser. A. 55, 427-437.
657 47. Volova, T.G., Kiselev, E.G., Vinogradova, O.N., Nikolaeva, E.D., Chistyakov, A.A.,
658 Sukovatyi, A.G., Shishatskaya, E.I., 2014. A glucose-utilizing strain, *Cupriavidus*
659 *eutrophus* B-10646: growth kinetics, characterization and synthesis of multicomponent PHAs.
660 PloS One. 9, e87551.
661 48. Zhila, N.O., Kalacheva, G.S., Volova, T.G., 2015. Fatty acid composition and
662 polyhydroxyalkanoates production by *Cupriavidus eutrophus* B-10646 cells grown on
663 different carbon sources. Process Biochem. 50, 69–78.
664 49. Zhu, C., Nomura, C.T., Perrotta, J.A., Stipanovic, A.J., Nakas, J.P., 2010. Production and
665 characterization of poly-3-hydroxybutyrate from biodiesel-glycerol by *Burkholderia*
666 *cepacia* ATCC 17759. Biotechnol. Prog. 26, 424–430.

667
668
669

Table 1 Summary of polyhydroxyalkanoates synthesis of different microorganisms using glycerol

Strain	Conditions	Substrate	Composition of PHA	biomass, g/L	PHA, %	Reference
Methylobacterium rhodesianum MB 126, wild *Ralstonia eutropha* DSM 11348,wild	fermentor 2.5 L	glycerol+casein hydrolysates glycerol+casein peptone, casamino acids	P(3HB)	22 45	50 65	[5]
Cupriavidus necator JMP 134, wild *Paracoccus denitrificans* DSMZ 4134, wild	fermentor 2.0 L	pure glycerol crude glycerol+ yeast extract	P(3HB)	50 25	45 20	[28]
Cupriavidus necator DSM 545, mutant	fermentor 2 L	pure glycerol crude glycerol+yeast extract	P(3HB)	82.5 68.8	62 38	[7]
Cupriavidus necator DSM 545, mutant	fermentor 2 L	waster glycerol+ γ-butyrolactone + propionic acid	P(3HB/4HB) (21.5 mol.%) P(3HB/4HB/3HV) (43.6/9.8 mol.%)	50-70	40	[8]
Cupriavidus necator DSM 545, mutant	shake flasks	crude glycerol + FAN	P(3HB/3HV) (2-8 mol.%)	5.1-15	46-50	[16]
Ralstonia eutropha KNK-DCD1, recombinant	shake flasks	glycerol + oleic acid	P(3HB/3HHx) (2 mol.%)	2,2	72-84	[42]
E. coli CT106, recombinant	fermentor 2 L	glycerol +yeast extract	P(3HB)	38	51	[30]
Cupriavidus sp. USMAHM13, wild	shake flasks fermentor 3.6 L	glycerine pitch+1,4-butanediol	P(3HB/4HB) (3-40 mol.%)	14	32	[34]
Cupriavidus necator IPT 026, wild	shake flasks	crude glycerol	P(3HB)	4,34	65	[6]
Cupriavidus necator DSM 545, wild *Burkholderia sacchari* DSM 17165, wild	fermentor 7.5 L	crude glycerol+glucose	P(3HB)	69 44	65 10	[36]

Pseudomonas oleovorans NRRLB-14682, wild *Pseudomonas corrugate* 388, wild	shake flasks	pure glycerol	P(3HB) *mcl*-PHA)	1,9 3,4	27 20	[3]
Pseudomonas oleovorans NRRL B-14682, wild	fermentor 12 L	pure glycerol (99%) crude glycerol (47 and 77%)	P(3HB)	4.0 2.3-3.0	50 30-40	[4]
Burkholderia cepacia ATCC 17759, wild	fermentor 400 L	crude glycerol (85%)	P(3HB)	23,6	31	[49]
Paracoccus sp. LL1, wild	fermentor 2-5 L	Crude glycerol	P(3HB)	24,2	39.3	[24]
Pandoraea sp. prp25, mutant	shake flask	crude glycerol+propionate or valeric acid	P(3HB/3HV) (30-34 mol%	4-6	59.1-62.3	[13]
	fermentor 10 L		P(3HB/3HV) (12-17 mol%)	8-10	23-47	
Caldimonas manganoxidans, wild	shake flask	crude glycerol	P(3HB)	8,4	71	[17]

Table 2 Composition and properties of PHA synthesized by *C. eutrophus* B-10646 using glycerol

Substrate	PHA composition, mol.%			M_n, kDa	M_w, kDa	Đ	C_x, %	T_g, °C	T_c, °C	T_{melt}, °C	T_{degr}, °C
	3HB	3HV	4HB								
Glucose*	100	0	0	365	920	2.52	76	n.d.	92	178	295
Glycerol-I	100	0	0	104	355	3.42	50	2.9	96	174	296
Glycerol-II	100	0	0	115	416	3.63	55	n.d.	103	176	296
Glycerol-III	100	0	0	87	304	3.49	52	2.7	99	172	295
Glycerol-II + potassium valerate (1 g/L)	72.0	28.0	0	97	253	2.61	41	-0.4	79	153 164	261
Glycerol-II + potassium propionate (1 g/L)	76.9	23.1	0	113	265	2.35	46	-0.8	67 69	171	296
Glycerol-II + ε-caprolactone (1 g/L)	95.0	0	5.0	111	299	2.69	52	1.0	93	157 168	296
Glycerol-II + potassium valerate (1 g/L+1 g/L)	64.3	35.7	0	119	287	2.41	44	-0.9	65 63	168 153	261
Glycerol-II + potassium propionate (1 g/L+1 g/L)	64.3	28.5	0	102	273	2.68	45	1.1	89 87	154 166	291
Glycerol-II + ε-caprolactone (1 g/L+1 g/L)	90.2	0	9.8	110	290	2.64	46	-0.4	77 74	151 162	263

*data from Volova et al. [46]
n.d.- not detected

1 **Figure Legends**

2 Fig.1 Production characteristics of the non-adapted and adapted museum strains *R.*
3 *eutropha* B 5786, *R. eutropha* B 8562 and *C. eutrophus* B-10646 grown on Glycerol II: yield of
4 cell biomass (g/L) and polymer (% of dry biomass weight).

5 Fig.2 Synthesis of PHA copolymers by the *C. eutrophus* B-10646 cultured on Glycerol II.
6 Arrows show additions of sodium valerate, sodium propionate or ε-caprolactone.

7 Fig.3 The fed-batch culture parameters of *C. eutrophus* B-10646 in a 30-l fermenter on
8 various sources of glycerol: cell biomass concentrations (total and catalytic) and polymer content
9 in cells and dynamics of the specific growth rate of total (μ) and catalytic (μ_c) cell biomass and
10 specific rate of polymer synthesis ($\mu_{polymer}$)

11 Fig.4 Photo and block diagram of the fermentation line of PHA pilot production: 1- steam
12 generator; 2 - compressor; 3 - incubator shaker; 4 - centrifuge; 5 - fermentor NLF 30; 6 - exhaust
13 air cooler; 7 - containers for feeding substrates; 8 - peristaltic dosing pumps; 9 - pilot scale
14 fermentor P150; 10 - ultrafiltration plant; 11 - freeze drying.

15 Fig.5 ^{1}H-NMR of P(3HB) produced by *C. eutrophus* B-10646 grown on Glycerol I,
16 Glycerol II and Glycerol III.

17