1 **Synthesis of P(3HB-co-3HHx) copolymers with high molar fractions of 3-**
2 **hydroxyhexanoate monomer units synthesized by the wild-type strain *Cupriavidus***
3 ***eutrophus* B10646 and properties of these copolymers**
4
5 **Synthesis of P(3HB-co-3HHx) copolymers by the wild strain *Cupriavidus eutrophus* B10646**
6
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16
17 **Abstract**
18 **BACKGROUND:** P(3HB-co-3HHx) copolymers are very promising biomaterials. The main
19 challenge in the production of these polymers is to simultaneously achieve high cell biomass;
20 high P(3HB-co-3HHx) content; and high molar fraction of 3HHx in P(3HB-co-3HHx). The most
21 common approach to production of these copolymers is the use of recombinant bacterial strains.
22 The purpose of this study was to optimize the process of production of P(3HB-co-3HHx)
23 copolymers containing high molar fractions of 3HHx by using the wild-type strain *Cupriavidus*
24 *eutrophus* B10646.
25
26 **RESULTS:** Kinetic properties of *C. eutrophus* B10646 were studied during the cultivation of
27 the cells on substrates necessary for P(3HB-co-3HHx) synthesis: glucose, nitrogen, sodium
28 hexanoate, and sodium acrylate. The physiological ranges of their effects were determined
29 experimentally, and *C. eutrophus* B10646 was grown in culture media with different dosages of
30 these substrates. P(3HB-co-3HHx) copolymers with different molar fractions of 3HHx, including
31 high ones (12 to 68%), were synthesized, and their physicochemical and mechanical properties
32 were investigated.
33
34 **CONCLUSION:** For the first time, cultivation conditions of *Cupriavidus eutrophus* B10646
35 enabled production of high biomass yields (5-6 g/L) and high content of the polymer (60-75%)
36 that contained high 3HHx molar fraction. By varying the 3HB/3HHx ratio, one can change
37 physicochemical and mechanical properties of P(3HB-co-3HHx) copolymers.
38
39
40 *Keywords:* wild-type strain *Cupriavidus eutrophus* B10646, growth kinetics, poly(3-
41 hydroxybutyrate-co-3-hydroxyhexanoate), physicochemical and mechanical properties
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46 **1. Introduction**

47 Natural polyesters polyhydroxyalkanoates (PHAs) are synthesized by prokaryotic
48 microorganisms in a complex multistage biosynthetic process, with each stage catalyzed by
49 specific enzymes. This type of natural macromolecules includes structurally diverse polymers
50 composed of monomers with different chemical structure (saturated and unsaturated, linear and
51 branched, aliphatic, aromatic, etc.) and with different numbers of carbon atoms.[1] PHA synthase –
52 one of the key enzymes of PHA synthesis – catalyzes formation of ester bonds during
53 polymerization of monomers.[2,3] Based on the notion of substrate specificity of synthases, all
54 known types of PHAs have been divided into three groups: short-chain-length ($PHAs_{SCL}$),
55 medium-chain-length ($PHAs_{MCL}$), and long-chain-length ($PHAs_{LCL}$) PHAs. $PHAs_{SCL}$ are
56 composed of monomers consisting of three to five carbon atoms (C_3-C_5), $PHAs_{MCL}$ – C_6 to C_{14},
57 and $PHAs_{LCL}$ – more than C_{17} and C_{18}.[4]

58 PHA copolymers are of the greatest interest to researchers since the basic
59 physicochemical properties of this type of PHAs vary widely depending on the composition and
60 fractions of monomers comprising them.[5] The most variable properties of PHA copolymers are
61 their degree of crystallinity and temperature characteristics – parameters that determine the
62 conditions of polymer processing and properties of the resulting products. Copolymers
63 composed of short-chain-length (SCL) and medium-chain-length (MCL) monomers make up a
64 special group of PHAs, as these copolymers, such as poly(3-hydroxybutyrate-co-3-
65 hydroxyhexanoate), P(3HB-co-3HHx), or poly(3-hydroxybutyrate-co-hydroxyoctanoate),
66 P(3HB-co-3HO), have elastomeric properties and a decreased degree of crystallinity, in contrast
67 to the homogenous poly(3-hydroxybutyrate), P(3HB).[6]

68 Both wild-type and genetically modified microorganisms are capable of synthesizing
69 PHAs composed of SCL and MCL monomer units. Wild-type producers of SCL-MCL PHAs
70 described in the literature represent different taxa: *Pseudomonas putida*,[7] *Rhodococcus ruber*,[8]
71 *Aeromonas caviae* 440,[9] *Ectothiorhodospira shaposhnikovii*,[10] etc. This is by no means an

72 exhaustive list. The authors of those studies described synthesis of SCL-MCL PHAs on various
73 substrates (sugars, organic and fatty acids), which served as the main carbon source. The nutrient
74 media also contained additional carbon sources – the so-called precursor substrates for the
75 synthesis of medium-chain-length monomers (hexanoic acid, octanoic acid, etc.), most of which
76 inhibit the growth of PHA producing microorganisms. The total PHA yields were considerably
77 lower than potentially attainable ones, and the molar fractions of medium-chain-length
78 monomers were low. For instance, the fractions of 3HHx in the PHA reported so far varied from
79 several mol.% to a few dozen mol.%, but did not exceed 15-20 mol.%.

80 Recombinant PHA producing strains, harboring genes of PHA synthesis enzymes from
81 different bacteria, have been engineered to achieve more productive synthesis of SCL-MCL
82 PHAs. Recombinant PHA producers are engineered by using both PHA-synthesizing
83 microorganisms (*Pseudomonas, Aeromonas, Ralstonia*) and ones incapable of PHA synthesis (*E.*
84 *coli*). If PHA-synthesizing bacteria are used, the aim is overexpression of the genes that control
85 PHA synthesis. Non-PHA producers are also used to study the mechanism of PHA synthesis and
86 detect additional participants in biosynthesis. For instance, *E. coli* is a promising bacterium for
87 engineering recombinant strains because its metabolism has been studied thoroughly; moreover,
88 *E. coli* has high growth rate, can use a wide range of substrates, and has no intracellular
89 depolymerases, i.e. it may synthesize polymers in high yields.[11] However, even the use of
90 recombinant PHA producers to synthesize SCL-MCL PHA copolymers has not resulted in the
91 simultaneous production of large amounts of biomass and high total yields of polymers
92 containing high molar fractions of medium-chain-length monomers. Thus, until recently, the
93 highest molar fractions of medium-chain-length monomer units in the SCL-MCL PHAs studied
94 were no greater than 20 mol.%.[5] When high molar fractions (75-90 mol.%) of MCL monomers
95 such as 3HHx were achieved in the culture of a recombinant strain *Aeromonas hydrophila* AKJ1,
96 biomass production and total PHA yields were extremely low (1.67-3.0 g/L and 0.27-4.28% of
97 CDW).[12]

98 The highest molar fractions of medium-chain-length monomers, 3HHx, have been
99 recently produced in the culture of recombinant strain *Cupriavidus necator* Re2160/pCB113
100 with the cloned PHA synthase gene of *Rhodococcus aetherivorans* and an enol-CoA hydratase
101 gene from *Ps. aeruginosa*[13] on plant oils[14] and on crude palm kernel oil and soybean oil, with
102 3HHx molar fraction reaching 55 to 70 mol.% and polymer yields 45-48% of CDW.[15]

103 Chemolithoorganotrophic bacteria of the genus *Cupriavidus* (formerly known as
104 *Ralstonia*) are regarded as very promising PHA producers, as these bacteria are capable of
105 synthesizing PHAs in very high yields (80-90% of CDW) from various substrates.[4,16,17] It was
106 previously believed that the PHA synthase of *Ralstonia* strains, which is a Class I synthase, is
107 substrate specific, and, thus, wild-type bacteria of this genus are capable of accumulating only
108 short-chain-length PHAs.[1] The first data showing the ability of the wild-type strain *R. eutropha*
109 B5786 to synthesize P(3HB-co-3HHx) copolymers with 3HHx reaching 10-16 mol.% when
110 grown in autotrophic culture on CO_2 and hexanoate as an additional substrate were reported by
111 Volova et al.[18,19] Several years later, Green et al.[20] also showed that the wild-type *R. eutropha*
112 H16 was capable of synthesizing SCL-MCL PHAs containing 3HHx. In the copolymers
113 synthesized by the cells grown on octanoic acid as a carbon source, in the medium supplemented
114 with different concentrations of sodium acrylate, the molar fraction of 3HHx reached 5.7 mol.%.
115 The study comparing two strains – *R. eutropha* B5786 and *R. eutropha* H16 – showed that the
116 H16 strain was capable of synthesizing P(3HB-co-3HHx) as well.[21] The molar fraction of 3HHx
117 in P(3HB-co-3HHx) reached 50 mol.% in experiments with *R. eutropha* strains grown
118 heterotrophically on fructose, in the medium supplemented with sodium hexanoate and sodium
119 acrylate (the latter blocking reactions of the fatty acid oxidation cycle and preventing the carbon
120 chain of hexanoic acid from shortening).[22]

121 The PHA synthase gene of *R. eutropha* B5786 was cloned and characterized, and
122 molecular structure of the enzyme was compared with PHA synthases of several strains
123 accumulating SCL-MCL PHAs.[23] Homology of the PHA synthase of *R. eutropha* B5786 to the

124 PHA synthase of *R. eutropha* H16 was 99%. Homology of the *R. eutropha* B5786 synthase to
125 the synthases of some strains producing SCL-MCL PHAs was between 26% and 41%. Thus, no
126 direct relationship was found between molecular organization of PHA synthases and their
127 functions, namely, their ability to synthesize PHAs with certain structures.

128 *Cupriavidus eutrophus* B10646, a recently isolated wild-type strain showing enhanced
129 tolerance to precursor C-substrates necessary for PHA_{MCL} synthesis, is capable of synthesizing
130 SCL-MCL PHAs. The first studies of this strain showed that when grown in autotrophic culture
131 on CO_2 as the main carbon source, with sodium hexanoate as an additional substrate,
132 *Cupriavidus eutrophus* B10646 cells synthesized PHA copolymers containing 20-25 mol.%
133 3HHx.[24] PHA terpolymers, P(3HB-co-3HV-co-3HHx) synthesized by *Cupriavidus eutrophus*
134 B10646 cells in heterotrophic culture on sugars and sodium valerate and sodium hexanoate as
135 additional substrates contained 13.6 mol.% 3HHx.[25]

136 The purpose of this study was to investigate accumulation of P(3HB-co-3HHx)
137 copolymers with high molar fractions of 3HHx synthesized by the wild-type strain *Cupriavidus*
138 *eutrophus* B10646 and characterize these copolymers.

139 **2. Experimental**

140 *2.1. Bacterial strain*

141 The strain used in this study was *C. eutrophus* B-10646, registered in the Russian
142 National Collection of Industrial Microorganisms. The strain has a broad organotrophic potential
143 and can use as carbon sources different substances; it is tolerant to concentrations of a number of
144 organic C-substrates (sodium valerate and hexanoate, γ-butyrolactone) reaching 3-5 g/L in the
145 culture medium and is able to use them to synthesize PHA copolymers containing short- and
146 medium-chain-length monomer units. As a nitrogen source, the strain utilizes nitrates,
147 ammonium salts, urea, and amino acids.[26]

148 *2.2. Media*

149 Schlegel's mineral medium was used as a basic solution for growing cells:[27]

150 $Na_2HPO_4 \cdot H_2O$ – 9.1; KH_2PO_4 – 1.5; $MgSO_4 \cdot H_2O$ – 0.2; $Fe_3C_6H_5O_7 \cdot 7H_2O$ – 0.025; $CO(NH_2)_2$ -
151 1.0 (g/L). The main carbon substrate was glucose, which was sterilized by membrane filtration
152 using Opticap XL300 Millipore Express SHC filters (U.S.), in order to prevent the pH from
153 falling. Nitrogen was provided in the form of urea, and, thus, no pH adjustment was needed. The
154 pH level of the culture medium was stabilized at 7.0±0.1. A solution of iron citrate (5 g/L),
155 which was used as a source of iron, was added to reach a concentration of 5 ml/L. Hoagland's
156 trace element solution was used: 3 ml of standard solution per 1 L of the medium. The standard
157 solution contains H_3BO_3 – 0.288; $CoCl_2 \cdot 6H_2O$ – 0.030; $CuSO_4 \cdot 5H_2O$ – 0.08; $MnCl_2 \cdot 4H_2O$ –
158 0.008; $ZnSO_4 \cdot 7H_2O$ – 0.176; $NaMoO_4 \cdot 2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g/L). Substrate feeding
159 strategies were varied depending on the technique of bacterial cultivation employed: culture in
160 flasks in a shaker or culture in a fermentation system.

161 *2.3. Growth conditions*

162 Cells were grown in the batch and continuous cultures. Inoculum was produced using an
163 Innova® 44 constant temperature incubator shaker (New Brunswick Scientific, U.S.). Inoculum
164 was prepared by resuspending the stock culture maintained on agar medium. Bacteria were
165 grown in 0.5-L to 2.0-L glass flasks half-filled with mineral medium, with the initial
166 concentration of glucose 10 g/L. In the phase of bacterial culture in the fermentor, the lowest
167 initial cell concentration was 1 g per L.

168 Growth kinetics of bacterial cells was studied in continuous mode in a BioFlo-115
169 automated laboratory fermentor, with an 8-L fermentation vessel and the working volume of the
170 culture from 3 L to 5 L, under strictly aseptic conditions. The mass flow of the fermentor is
171 controlled by the air flow rate and agitation speed; the latter can be varied from 300 to 1000 rpm,
172 and, thus, the oxygen transfer rate, KLa, varies from 120 to 480 1/h. The fermentor is equipped
173 with a control station with a liquid crystal display, which records the data of cultivation process,
174 pH probes, O_2 probes, a system for automatic substrate feeding, and a thermal stabilization
175 system. The air was continuously pumped through the culture medium, using microbiological

176 filters and an EL-200 air pump, of productivity 9 m^3/h and pressure 19.6 kPa. The air was
177 pumped into the culture medium automatically, at 1.5-3 L/min. Oxygen saturation level was
178 maintained at 25-30%; as cell concentration increased, oxygen concentration began to decrease,
179 and agitation speed increased (cascaded control).

180 The synthesis of P(3HB-co-3HHx) copolymers was studied in the batch culture, by using
181 the previously developed procedure.[25] Cultivation was performed in an Innova® 44 constant
182 temperature incubator shaker (New Brunswick Scientific, U.S.). Cells were grown in 2.0 L glass
183 flasks half-filled with mineral medium. Synthesis of PHA copolymers was achieved as follows:
184 the culture medium was supplemented with precursor substrate (different concentrations of
185 sodium hexanoate) (Sigma, U.S.) at 24 h, and cultivation lasted 48-72 h. In a number of
186 experiments, the culture medium was also supplemented with sodium acrylate (Sigma)
187 (simultaneously with sodium hexanoate supplementation), which blocked reactions of the fatty
188 acid β-oxidation cycle and enhanced the synthesis of 3HHx monomer units.

189 *2.3. Monitoring process parameters*

190 During the course of cultivation, samples of culture medium were taken for analysis
191 every 4 h (in the continuous culture in the fermentor) or every 8 h (in the batch culture in flasks);
192 cell concentration in the culture medium was determined based on the weight of the cell samples
193 dried at 105°C for 24 h (DCW) per 1 L. Cell concentration in the culture medium was monitored
194 every hour by converting the optical absorbance at 440 nm of culture broth to dry cell weight by
195 using a standard curve prepared previously.

196 Glucose concentration was determined using the “Glucose – FKD” kit, which contained
197 chromogenic enzyme substrate and a calibrator (a glucose solution of a known concentration).
198 Optical density of the study sample and calibration sample were compared photometrically with
199 the optical density of the blank, with optical path length 10 mm at wavelength 490 nm. Sodium
200 hexanoate concentrations in the culture medium were controlled using chromatographic analysis
201 of the culture medium samples, which was done after preliminary extraction with chloroform

202 from acidified samples. Nitrogen concentration in the culture medium was analyzed at different
203 time points, using a photometric method, with Nessler's reagent. To measure concentrations of
204 major and trace elements (S, K, Mg, P, Na, Ca, Fe, Cu, Mn, Mo, Zn, Co, Cr, Se), samples of the
205 culture medium were taken periodically and measured using inductively coupled plasma atomic
206 emission spectroscopy in an ICAP – 6000 Thermosystem (Thermo Electron Corporation, U.S.).

207 P(3HB-co-3HHx) biosynthesis was evaluated based on cell concentration, polymer yield,
208 the amount of the main growth substrate used, and process duration and productivity.
209 Conventional methods were used to determine kinetic and production parameters of the culture.
210 The cell biomass yield (X, g/L), and the specific growth rate (μ, h^{-1}) were calculated.

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

212 $$\mu=(dx)/dt$$

213 where x is biomass, g/L; t– duration of cultivation, h.

214 Kinetic constants of the culture were determined by using the conventional Lineweaver-
215 Burk semi-graphical method: by linearizing inverse values of the relationship between the
216 specific growth rate of bacterial cells and substrate concentration: *Ks* – by the Michaelis-Menten
217 equation and *Ki* – by the Monod-Ierusalimsky equation.

218 *2.4. Analysis of P(3HB-co-3HHx) structure and physicochemical properties*

219 Intracellular polymer content at different time points was determined by analyzing
220 samples of dry cell biomass. Intracellular PHA content and composition of extracted polymer
221 samples were analyzed by a GC-MS (6890/5975C, Agilent Technologies, U.S.). Both
222 lyophilized cells and extracted polymer were subjected to methanolysis in the presence of
223 sulfuric acid, and polymer was extracted and methyl esterified at 100°C for 4 h. Benzoic acid
224 was used as an internal standard to determine total intracellular PHA.[28] Monomer units of
225 P(3HB-co-3HHx) were identified in the extracted and purified polymer samples based on their
226 retention times and mass spectra. ^{1}H NMR spectra of the P(3HB-co-3HHx) copolymer were
227 recorded at room temperature in $CDCl_3$ on a Bruker AVANCE III 600 spectrometer (Germany)

228 operating at 600.13 MHz.

229 Molecular weight and molecular-weight distribution of the copolymer were examined
230 using a gel permeation chromatograph (Agilent Technologies 1260 Infinity, U.S.) with a
231 refractive index detector, using an Agilent PLgel Mixed-C column. Chloroform was the eluent,
232 at a flow rate of 1.0 ml/min at 40°C. Typical sample volumes were 50 µl at a polymer
233 concentration of 2 mg/ml. Narrow polydispersity polystyrene standards (Agilent, U.S.) were
234 used to generate a universal calibration curve, from which molecular weights (weight average,
235 M_w, and number average, M_n) and polydispersity ($Đ = M_w/M_n$) were determined. The
236 measurement accuracy was 2%.

237 Thermal analysis of P(3HB-co-3HHx) specimens was performed using a DSC-1
238 differential scanning calorimeter (METTLER TOLEDO, Switzerland). Powdered samples (4.0
239 ±0.2 mg each) were placed into the aluminum crucible and compressed prior to measurement.
240 Every sample was measured at least 3 times. Samples were preheated to 60°C and cooled to
241 25°C. The specimens were heated to temperatures from 25°C to 300°C, at $5°C\times min^{-1}$
242 (measurement precision 1.5°C); melting point (T_m) and thermal decomposition temperature (T_d)
243 were determined from exothermal peaks in thermograms. The thermograms were analyzed using
244 the STARe v11.0 software.

245 In order to determine the crystallinity of the P(3HB-co-3HHx), 3 film samples 2 cm in
246 diameter and 0.15 mm thick were prepared from a 2% polymer solution in chloroform. The
247 samples had a circular shape because during measurement the sample spins in a direction
248 perpendicular to the surface. X-Ray structure analysis and determination of crystallinity of
249 copolymers were performed employing a D8 ADVANCE X-Ray powder diffractometer
250 equipped with a VANTEC fast linear detector, using CuKa radiation (Bruker, AXS, Germany).
251 The scan step was 0.016°, measurement time in each step 114 s, and scanning range from 5° to
252 60° (from 48° to 60° there only was a uniformly decreasing background); the registered
253 parameter was intensity of X-rays scattered by the sample; 55°/0.016°=3438 times. The degree

254 of crystallinity was calculated as a ratio of the total area of crystalline peaks to the total area of
255 the radiograph (the crystalline + amorphous components). Measurement accuracy: point
256 measurement accuracy ± 0.4 PPS, with the lowest intensity 1.5 PPS and the highest intensity 32
257 PPS; the error in determination of the degree of crystallinity, which was calculated based on
258 multiple measurements, was 2% or less.

259 *2.5. Analysis of P(3HB-co-3HHx) physical-mechanical properties*

260 The microstructure of the surface of P(3HB-co-3HHx) films prepared by coating glass
261 with the chloroform solution of the polymer followed by solvent evaporation was analyzed using
262 scanning electron microscopy (TM 3000, Hitachi, Japan). Surface properties were studied with a
263 Drop Shape Analyzer – DSA-25E (KRÜSS GmbH – Germany) for measuring contact angles of
264 water and diiodomethane drops by the Owens, Wendt, Rabel and Kaelble method: surface free
265 energy (SFE) and its dispersion and polar components were measured, and the data were
266 processed by the DSA-4 software.

267 Physical and mechanical properties of the films prepared from P(3HB-co-3HHx) with
268 different compositions were investigated using an Instron 5565 electromechanical tensile testing
269 machine (U.K.). Dumbbell-shaped samples 50 mm long, 6.1 mm wide, and 25–30 µm thick were
270 prepared for studying physical and mechanical properties of the films. The thickness of films
271 was measured prior to testing, using a LEGIONER EDM-25-0.001 electronic digital micrometer.

272 Samples were maintained under normal conditions for at least two weeks to reach
273 equilibrium crystallization. At least five samples were tested for each type of films.
274 Measurements were conducted at room temperature; the clamping length of the samples was 30
275 mm. The speed of the crosshead was 3 mm/min at room temperature. Young's modulus (E,
276 MPa), tensile strength (σ, MPa) and elongation at break (ε, %) were automatically calculated by
277 the Instron software (Bluehill 2, Elancourt, France). To obtain Young's modulus, the software
278 calculated the slope of each stress-strain curve in its elastic deformation region. Measurement
279 error did not exceed 10%.

280 *2.6. Statistics*

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

285 **3. Results**

286 The structure of this research was determined by the fact that bacterial culture
287 synthesizing PHA copolymers is a very complex system controlled by various factors. First, for
288 PHA synthesis to occur, a sufficient amount of carbon substrate should be present in the culture
289 medium; at the same time, substrate concentration should be maintained within the physiological
290 range for each specific strain, to avoid both substrate deficiency and its inhibitory effect. Second,
291 to promote PHA accumulation, one of the substrates of constructive metabolism (for the study
292 strain, this is nitrogen) must limit cell growth. Third, the culture medium must contain precursor
293 substrate (sodium hexanoate) concentrations that would be sufficient to enable 3HHx formation
294 but would not profoundly inhibit the cell culture. Fourth, in order to facilitate incorporation of 3-
295 hexanoate into the P(3HB-co-3HHx) copolymers, reactions of fatty acid β-oxidation should be
296 blocked by, e.g., acrylic acid, to prevent the carbon chain of sodium hexanoate from shortening;
297 acrylic acid, however, may inhibit cell growth. Hence, to achieve productive synthesis of P(3HB-
298 co-3HHx) by the wild-type strain *C. eutrophus* B10646, it was necessary to study the
299 relationship between specific growth rate of the cells and concentrations of glucose, nitrogen,
300 sodium hexanoate, and sodium acrylate and estimate the influence of these substrates on the
301 yield of P(3HB-co-3HHx) copolymers and their 3HHx fraction and on the total production of
302 bacterial biomass.

303 *3.1. The effects of the substrates necessary for the synthesis of P(3HB-co-3HHx)*
304 *polymers on kinetic properties of C. eutrophus B10646 culture*

305 This study was performed with continuous culture, in which parameters of the medium
306 can be maintained at preset levels for long time periods and which is used to determine the
307 accurate physiological ranges of effects of glucose, nitrogen, sodium hexanoate, and sodium
308 acrylate on the study strain, μ/S relationships, and substrate constants (K_s and K_i). We varied the
309 concentration of one substrate in the culture medium, while the other parameters of the medium
310 were maintained at optimal levels. Results are shown in Figure 1.

311 The study of the effect of glucose concentration on the growth rate of the glucose-
312 utilizing strain, *C. eutrophus* 10646, found the physiological limits of the effect of this substrate.
313 Results of experiments showed that for this strain, glucose concentration in the culture medium
314 should be maintained within a range of 5 to 35 g/L (Fig. 1a). Glucose concentrations below 5 g/L
315 and above 35 g/L adversely affected cell growth rate. Glucose concentrations below and above
316 these limits caused a decrease in the cell yield; at glucose concentration of 50 g/L, specific
317 growth rate of the cells dropped to 0.050 h^{-1}, and at 80-100 g/L, cell growth was completely
318 inhibited. Results of experiments were processed according to Varfolomeev and Kalyuzhny[29] to
319 obtain substrate constants of the study strain relative to the substrates (glucose and nitrogen): K_1
320 $= K_{gl} = 2.9$ g/L and $K_2 = K_{nitr} = 0.06$ g/L. Saturation constant (Ks) and inhibition constant (Ki) of
321 the main growth substrate (glucose) for *C. eutrophus* B10646 cells were found to be 12.7 g/L
322 and 38.5 g/L, respectively. Thus, in the case of the batch culture, with varying cell concentration
323 and substrate concentration, glucose should be fed to the culture medium periodically or
324 continuously, and its concentration should be constantly monitored.

325 Figure 1b shows the relationship between specific growth rate of *C. eutrophus* B10646
326 cells and nitrogen concentration. If the nutrient medium contains ammonium chloride, Cl^- ions
327 are accumulated in the pericellular medium, and their concentration in the cell culture
328 asymptotically reaches their concentration in the nutrient medium and acidifies it; thus, pH needs
329 to be adjusted. Therefore, in our experiments, we used urea as nitrogen source in the culture
330 medium: the urea molecule is completely assimilated by bacterial cells, the ionic composition of

331 the medium remains unchanged, and, thus, no adverse effects occur, unlike in the case of using
332 other nitrogen forms.
333 Experiments were carried out with varied nitrogen concentrations in the nutrient medium
334 to find the relationship between specific growth rate of *C. eutrophus* B10646 cells and nitrogen
335 supply and to determine nitrogen requirements of the cells. The nitrogen supply tested in these
336 experiments was varied between 0.1 and 0.22 g/g of the cell biomass synthesized. The highest
337 intracellular nitrogen concentration at the highest specific growth rate (about 0.24-0.27 h^{-1})
338 reached 0.12±0.005 g/g. Nitrogen supply below this value limits cell growth. In the case of
339 excessive nitrogen supply, its residual concentration in the culture medium increases. At the
340 same time, residual concentration of the element must not drop below the critical level, i.e. the
341 concentration gradient between the medium and the cell must be large enough for the rate of the
342 diffusion of molecules toward the cell surface to be greater than the rate of their consumption by
343 the cells. On the other hand, bacterial cultivation with considerable residual concentrations of
344 elements is technologically uneconomical, as large amounts of mineral substrate will be wasted.
345 Therefore, it is important to know the lowest concentration of the element that does not affect the
346 process of bacterial cultivation. In continuous culture, specific growth rate of the cells was not
347 affected by residual nitrogen concentration varied within a wide range of 0.05 to 0.3 g/L (Fig.
348 1b). It is generally possible to reduce residual nitrogen concentration to trace amounts, but then
349 this parameter must be continuously monitored. Another important issue is that during
350 cultivation of bacterial cells on urea, ammonia produced in hydrolysis of urea by bacterial urease
351 may accumulate in the pericellular medium. It has been found that in the case of using urea,
352 about half of the nitrogen in the pericellular medium is ammonia nitrogen. If residual nitrogen
353 concentration exceeds 0.3 g/L, large amounts of ammonia inhibit cell growth. Hence, during
354 bacterial cultivation, nitrogen should be constantly controlled. The saturation constant (*Ks*) and
355 inhibition constant (*Ki*) for nitrogen found in this study were 0.005 g/L and 0.28 g/L,
356 respectively.

357 As sodium hexanoate is a necessary precursor substrate for synthesis of P(3HB-co-3HHx)
358 copolymers, we carried out experiments with different amounts of sodium hexanoate added to
359 the culture medium. The relationship between specific growth rate of *C. eutrophus* B10646 cells
360 and sodium hexanoate concentration in the culture medium is shown in Figure 1c. As this strain
361 was selected for sodium hexanoate tolerance, the physiological range of the sodium hexanoate
362 effect on this strain is rather wide. Cell growth is inhibited by sodium hexanoate concentrations
363 over 4 g/L; the *Ki* for this strain is 3.96 g/L.

364 Another study substrate was sodium acrylate (Fig. 1d). When fatty acids are added as a
365 supplementary substrate, *β*-oxidation is the most likely source of monomers for heteropolymer
366 synthesis. Short-chain-length FAs – butyric acid, valeric acid – are reduced to D-*β*-OH-
367 derivatives during *β*-oxidation, skipping the main pathway of monomer synthesis. These two
368 monomers exhibit high affinity for PHA synthase, and, thus, they are not involved in the cycle of
369 FA *β*-oxidation any more, but are immediately directed to the synthesis of the polymer. When
370 the synthesis of P(3HB-co-3HHx) is induced by addition of sodium hexanoate, just a small part
371 of it is incorporated into the polymer as 3HHx, while its major part is converted to 3HB after
372 losing the C_2-fragment in reactions of *β*-oxidation. The reason may be that the affinity of
373 synthase for this monomer is two orders of magnitude lower than for 3HB and 3HV, so the
374 larger part of sodium hexanoate goes through at least one cycle of *β*-oxidation. Hence,
375 incorporation of 3HHx into the PHA can be enhanced by creating the conditions favoring
376 intracellular accumulation of this monomer. 3-ketoacyl-CoA thiolase inhibition is the most likely
377 way to suppress the *β*-oxidation reaction, which is responsible for the shortening of the carbon
378 chains of monomers, 3HHx in particular. Acrylic acid is one of 3-ketoacyl-CoA thiolase
379 inhibitors. Acrylic acid strongly inhibits *C. eutrophus* B10646 cell growth (Fig. 1d); only at
380 concentrations below 0.3 g/L, this substrate has no inhibitory effect on the strain. At sodium
381 acrylate concentration of 0.5 g/L, specific growth rate of *C. eutrophus* B10646 cells drops by
382 nearly half, i.e. the *Ki* for acrylate is 0.23 g/L.

383 Based on the relationships between substrate concentrations necessary for P(3HB-co-
384 3HHx) synthesis and specific growth rate of *C. eutrophus* B10646 cells and the physiological
385 ranges of effects of these substrates on the strain found in the experiments described above, we
386 created the conditions necessary for the synthesis of P(3HB-co-3HHx) in two-stage batch culture
387 (Fig. 2). In Stage 1, which lasted 32 h, cells were grown in the medium containing an excessive
388 amount of glucose, under nitrogen deficiency. Instead of 1 g/ L, 0.5 g/L of urea was added to the
389 nutrient medium. After 24 h of cultivation, when cell concentration reached 3.0 g/L and
390 intracellular polymer content 28-30% of CDW, sodium hexanoate was added in one portion, at a
391 non-inhibitory concentration (1 g/L) (Fig. 2a). In 8 h after sodium hexanoate was added to the
392 culture medium, the second stage of cultivation started: bacterial cells were grown in nitrogen-
393 free medium, with controlled glucose concentration maintained at levels that did not limit cell
394 growth. Bacterial biomass and intracellular polymer concentration increased: at the end of the
395 experiment (72 h), X (g/L) was 7.5 g/L and copolymer content reached 85% of CDW. The 3HHx
396 molar fraction of the copolymer reached its maximum (11 mol.%) in 16 h after sodium
397 hexanoate was added to the culture medium. Then, however, the 3HHx fraction decreased while
398 the molar fraction of 3-hydroxybutyrate increased. The end concentration of 3HHx in the
399 copolymer was 5.5 mol.%.

400 Results of the experiment done in a similar fashion but with sodium hexanoate and
401 sodium acrylate (0.1 g/L) simultaneously added to the culture medium are shown in Figure 2b.
402 The trends in changes of the study parameters were similar to those in the previous experiment,
403 but the simultaneous joint effect of two factors (sodium hexanoate and sodium acrylate) inhibited
404 cell growth, although when added separately, the same concentrations of these substrates had not
405 caused any inhibition of cell growth (Fig. 1c, d). After 72 h of cultivation, cell concentration and
406 intracellular polymer content were 5.8 g/L and 72% of CDW, respectively, or 14-23% lower than
407 in the experiment with sodium hexanoate alone added to the culture medium. The maximum
408 fraction of 3HHx was also reached in 16 h after sodium hexanoate and sodium acrylate were

409 added to the culture medium, but it was larger (35 mol.%). Then, as cell concentration and
410 intracellular polymer content grew some what, the 3HHx fraction decreased while the 3HB
411 fraction increased. The end concentration of 3HHx in the copolymer was 25 mol.%.

412 Thus, results of experimental studies showed that 1) the highest intracellular copolymer
413 content and the largest 3HHx fraction of the copolymer are reached at different time points and
414 2) the combined inhibitory effect of sodium hexanoate and sodium acrylate on *C. eutrophus*
415 B10646 cells is stronger than the separate effects of these substrates. Further research is needed
416 to maximize the major parameters of P(3HB-co-3HHx) synthesis: bacterial biomass yield, total
417 copolymer content, and the 3HHx fraction of the copolymer.

418 *3.2. A study of the conditions necessary for effective synthesis of P(3HB-co-3HHx) in the*
419 *culture of C. eutrophus B10646*

420 In order to find the conditions for increasing cell concentration, P(3HB-co3HHx) content,
421 and the 3HHx molar fraction, in a series of experiments, we varied the amounts of sodium
422 hexanoate and sodium acrylate added to the culture medium and used different supplementation
423 schedules while maintaining glucose and nitrogen concentrations of the medium within the
424 physiological ranges of their effects on *C. eutrophus* B10646.

425 Results of studying the effect of sodium hexanoate concentration in the culture medium
426 on cell concentration, copolymer content, and the 3HHx fraction are shown in Figure 3a. Based
427 on the results suggesting a decrease in the molar fraction of 3HHx at longer time intervals after
428 the addition of sodium hexanoate to the culture medium, the cultivation lasted no more than 40
429 to 48 h. As sodium hexanoate concentration in the culture medium was increased, the 3HHx
430 fraction of the copolymer increased, too. The highest molar fraction of 3HHx (12 mol.%) was
431 achieved at sodium hexanoate concentration of 2 g/L. The total biomass production and
432 copolymer yield remained almost unchanged: 4.7-5.0 g/L and 63-70 % of CDW, respectively.

433 The other factor influencing the formation of 3HHx monomer units is acrylic acid, which
434 blocks the reactions of fatty acid β-oxidation cycle. Results of studying the effect of sodium

435 acrylate concentration on the parameters of the *C. eutrophus* B10646 culture are shown in Figure
436 3b. Sodium acrylate concentration in the culture medium was varied between 0.1 g/L and 1.0
437 g/L, while sodium hexanoate concentration was maintained at a steady level of 1.0 g/L.

438 From Figure 3b we can see that as sodium acrylate concentration was increased from 0.1
439 to 0.4 g/L, the 3HHx molar fraction of the copolymer increased from 5 mol.% to 56 mol.%, the
440 cell concentration showed little change, but polymer content decreased. At sodium acrylate
441 concentration of 0.5 g/L, a decrease in cell concentration and an even more significant decrease
442 in copolymer content were observed, while the 3HHx molar fraction increased to 65.7 mol.%. As
443 the concentration of sodium acrylate in the nutrient medium was increased further (from 0.5 to
444 1.0 g/L), the cell concentration dropped to 3.3 g/L and polymer content to 25% of CDW,
445 whereas the 3HHx molar fraction remained rather high, reaching 58 mol.%.

446 Since sodium hexanoate and sodium acrylate simultaneously added to the culture medium
447 inhibited cell growth, we tried adding these substrates portion-wise. The batch culture of *C.*
448 *eutrophus* B10646 in the mode of PHA synthesis was supplemented with sodium hexanoate
449 portions (one to five) (one portion of 0.5 g/L) every 6-8 h. Sodium acrylate was added
450 simultaneously with sodium hexanoate, at concentrations that did not limit cell growth of the
451 study strain (0.1 g/L) (Fig. 3c). Intracellular polymer content varied from 31% to 75% of CDW
452 in different experiments, and the 3HHx molar fraction reached 17-68 mol.%. The largest molar
453 fraction of 3-hydroxyhexanoate (68 mol.%) was achieved when 2.5 and 0.5 g/L of sodium
454 hexanoate and sodium acrylate (total concentration) were added to the culture medium; the
455 biomass yield in that experiment was 3.5 g/L, and polymer content was rather low (31% of
456 CDW). The experiments showed that the adverse influence of sodium acrylate on polymer
457 synthesis and biomass yield is greater than that of sodium hexanoate. The highest 3HHx fraction
458 of the copolymer was achieved when sodium hexanoate and sodium acrylate were added to the
459 culture medium with decreased residual glucose concentration.

460 Thus, by varying the conditions of carbon supply and supplementation schedules of the
461 additional substrate (sodium hexanoate) and sodium acrylate, we managed to achieve increased
462 intracellular polymer content and biomass yield in *C. eutrophus* B10646 culture and synthesis of
463 P(3HB-co-3HHx) copolymers with different 3HHx molar fractions, including high ones.

464 *3.3. Physicochemical properties of P(3HB-co-3HHx) copolymers with different molar*
465 *fractions of 3HHx*

466 The composition and physicochemical properties of P(3HB-co-3HHx) copolymers with
467 different 3HHx molar fractions are given in Table 1. Figure 4 shows an example of ^{1}H NMR
468 spectra of polymer sample with 3HHx. Among the major parameters characterizing the
469 properties of high-molecular-weight compounds are their molecular weight characteristics,
470 which determine processing behavior of the polymers and parameters of their processing into
471 specialized products. PHA molecular weight is a very variable parameter, which is influenced by
472 a number of factors, including carbon source, cultivation duration, and polymer recovery
473 technique employed [3].

474 Analysis of the influence of the 3HHx fraction on molecular weight parameters of the
475 copolymers did not reveal any strong relationship between the weight-average molecular weight
476 of the polymer and the 3HHx fraction (M_w/3HHx) (Table 1). However, this parameter was lower
477 in all P(3HB-co-3HHx) copolymers than in the P(3HB) homopolymer. The highest M_w values
478 were found for the P(3HB-co-3HHx) copolymers with the high 3HHx molar fractions (55-68
479 mol.%). Polydispersity (the parameter of inhomogeneity, or the proportions of the numbers of
480 macromolecules with different molecular weights in a given polymer sample) of the P(3HB-co-
481 3HHx) samples was not related to the 3HHx fraction and varied between 3.1 and 5.42.

482 Temperature characteristics and the ability of polymers to crystallize are important
483 parameters, as they determine thermomechanical behavior of the polymers and, hence, their melt
484 processability. Table 1 shows that melting temperature and thermal decomposition temperature of
485 P(3HB-co-3HHx) copolymers are generally lower than those of P(3HB), but no clear

486 relationship was found between these parameters and 3HHx fractions. The low temperature of
487 crystallization of the homogenous P(3HB) is an obstacle to melt processing of this polymer. One
488 way to increase the PHA crystallization temperature is to synthesize copolymers with other
489 monomer units, such as 3HHx, incorporated into 3HB polymer chains. It is important to note that a
490 decrease in the melting point and thermal decomposition temperature of P(3HB-co-3HHx) does
491 not reduce the characteristic difference between these temperatures. The considerable (about
492 100°C) difference between T_m and T_d is an essential processing property of P(3HB-co-3HHx)
493 copolymers, making it processable into different products by using conventional polymer
494 processing techniques (solution casting, extrusion, pressure casting, etc.).

495 X-ray structure analysis revealed a significant influence of the 3HHx fraction on the
496 crystalline to amorphous ratio in different P(3HB-co-3HHx) samples (Table 1). As the 3HHx
497 molar fraction increased, the degree of crystallinity (C_x) of the copolymers consistently
498 decreased. The copolymers with molar fractions of 3HHx ranging between 10 mol.% and 43
499 mol.% consisted of nearly equal amorphous and crystalline phases, and their degree of
500 crystallinity was close to 50%. In the copolymer with a higher 3HHx fraction, the amorphous
501 phase was greater than the crystalline one, and the C_x dropped. The copolymer with 55 mol.%
502 3HHx had the C_x of 45% and the copolymer with 68 mol.% 3HHx had the C_x of 21%. Thus, by
503 varying the 3HHx molar fractions of P(3HB-co-3HHx) copolymers, we managed to prepare
504 thermally stable samples with significantly different degrees of crystallinity.

505 *3.4. Physical-mechanical properties of P(3HB-co-3HHx) copolymers with different molar*
506 *fractions of 3HHx*

507 Physical and mechanical properties of P(3HB-co-3HHx) copolymers were studied in
508 smooth 0.03-mm-thick films prepared from solutions of copolymers with different fractions of
509 monomer units. Differences in the composition and physicochemical properties of P(3HB-co-
510 3HHx) copolymers influenced the surface structure and properties of the films (Fig. 5). In
511 contrast to the close-grained surface of P(3HB) films, with a few small pores, the surface of the

512 copolymer films was covered by different numbers of 1- to 5-μm diameter pores. The sample
513 with 12 mol.% 3HHx was the smoothest. The sample with 24.6 mol.% 3HHx was the most
514 porous, with numerous pores about 5 μm in diameter covering its entire surface. The surfaces of
515 the films prepared from P(3HB-co-3HHx) copolymers with higher 3HHx molar fractions (55 and
516 65.7%) were covered by more numerous pores, and they had smaller diameters.

517 Hydrophilic/hydrophobic balance of the surface is a major parameter that indirectly
518 characterizes biological compatibility and influences cell adhesion and viability. Table 2 shows
519 that the water contact angle of P(3HB-co-3HHx) was higher than that of P(3HB), reaching 95.9-
520 109°. No correlation was found between the contact angle of diiodomethane and liquid surface
521 tension and the 3HHx molar fraction in polymer films. At the same time, the polar part of liquid
522 surface tension in P(3HB-co-3HHx) films decreased significantly.

523 The mechanical properties of a polymer product should correspond to its intended use.
524 Some devices need enhanced mechanical strength, determined by Young's modulus and tensile
525 strength; in other cases, the product should be elastic, i.e. show high elongation at break. In PHA
526 products, these parameters largely depend on the production technique employed and chemical
527 composition of the polymers used [5].

528 As can be seen from Table 1, mechanical properties of P(3HB-co-3HHx) films were
529 considerably influenced by the molar fraction of 3HHx in the copolymer. All copolymer films
530 had significantly higher values of elongation at break, as a parameter of polymer elasticity, than
531 films prepared from the highly crystalline P(3HB) homopolymer (2.5%). Elongation of P(3HB-
532 co-3HHx) films was enhanced as the 3HHx molar fraction of the copolymer was increased: it
533 was almost 40-50 orders of magnitude higher in P(3HB-co-3HHx) films with 3HHx over 60
534 mol.% than in the films with 12 mol.% 3HHx. Higher elasticity of P(3HB-co-3HHx) films,
535 characteristic of medium-chain-length copolymers, was accompanied by lower mechanical
536 strength of the films, expressed as Young's modulus and tensile strength. Young's modulus of
537 all P(3HB-co-3HHx) films was considerably lower than that of the homogenous P(3HB) (2071.2

538 MPa). As the 3HHx molar fraction of the copolymers was increased (from 12 mol.% to 68
539 mol.%), Young's modulus dropped from 1286.4 to 217.0 MPa and tensile strength – to 6.6 – 7.8
540 MPa. Tensile strength and Young's modulus express stiffness and brittleness of the films, while
541 elongation at break indicates polymer elasticity. Thus, the low values of tensile strength and
542 Young's modulus of P(3HB-co-3HHx) films suggest that the presence of the 3HHx fraction in
543 the copolymer increases flexibility of the films. Hence, the degree of crystallinity and
544 mechanical properties of P(3HB-co-3HHx) products can be significantly altered by varying the
545 fractions of 3HHx monomer units in the PHA.

546 **4. Discussion**

547 This study describes integrated investigations of growth kinetics of the wild-type strain,
548 *Cupriavidus eutrophus* B10646, which is able to synthesize PHA copolymers from various
549 substrates and shows enhanced tolerance to precursor substrates that are needed to synthesize
550 medium-chain-length monomer units, and synthesis of PHA copolymers by this strain.

551 Synthesis of medium-chain-length PHAs is a complex process, as it involves the use of
552 precursor substrates, which are usually toxic to PHA producing strains. Therefore, production of
553 high yields of bacterial biomass, with high intracellular content of polymers that contain MCL
554 monomer units is a challenging task. As already mentioned in Section 1 above, the available
555 literature describes synthesis and composition of PHAs produced by wild-type strains of various
556 genera (*Ralstonia, Pseudomonas, Rhodospirillum*, *Rhodococcus*, *Thiocapsa*, *Aeromonas*,
557 *Ectothiorhodospirai*), which generally contain rather low molar fractions of medium-chain-
558 length monomer units (from several mol.% to 10-20 mol.%). Various PHA producing bacteria
559 are used and special cultivation modes are developed to attain high yields of bacterial biomass
560 and polymers and to produce polymers with high molar fractions of MCL monomer units.

561 Another approach to synthesizing high fractions of MCL monomer units is engineering
562 recombinant producers of PHA copolymers. The highest molar fractions of medium-chain-length
563 monomers, 3HHx, have been recently produced in the culture of recombinant strain *Cupriavidus*

564 *necator* Re2160/pCB113 with the cloned PHA synthase gene of *R. aetherivorans* and an enol-
565 CoA hydratase gene from *Ps. Aeruginosa*.[13] Cultivation of this strain on plant oils resulted in
566 copolymer yield and 3HHx molar fraction reaching 74% of CDW and 20-40 mol.%,
567 respectively[14]. On crude palm kernel oil and soybean oil (2.5 g/L), the 3HHx molar fraction
568 reached 55 to 70 mol.% and polymer yields 45-48% of CDW, and the 3HHx fraction did not
569 decrease in the steady-state phase of the culture, as is usually the case, when the total polymer
570 yield is the highest.[15] Good results were obtained by Japanese researchers. [30] The authors studied
571 the influence of deletion of some genes (fadB', fadB1 and fadB2) on synthesis of PHA
572 copolymers by recombinant strain *R. eutropha* H16 and showed that fadB1 deletion was
573 effective for increasing the 3HHx molar fraction in the copolymer, with the total PHA
574 production being rather high (65.7% of CDW). The 3HHx fraction reached 23.6 mol.% in the
575 early phase of the culture, but then decreased to 15.7 mol.%.

576 In this study, high yields of P(3HB-co-3HHx) copolymers containing large molar
577 fractions of 3HHx (reaching 50-68 mol.%) were synthesized by the wild-type strain *C. eutrophus*
578 B10646. Kinetic properties of the culture were studied in experiments with four substrates
579 involved in P(3HB-co-3HHx) synthesis: glucose (major C-substrate); nitrogen (the factor
580 limiting cell growth and inducing accumulation of storage PHAs); sodium hexanoate (the
581 precursor substrate for synthesis of 3HHx monomer units); and sodium acrylate (the factor
582 blocking reactions of fatty acid β-oxidation cycle and enhancing incorporation of 3HHx into
583 P(3HB-co-3HHx)).

584 Based on results of these experimental studies, bacterial cells were cultivated under
585 different conditions of sodium hexanoate and sodium acrylate supply. We studied the effects of
586 the sodium hexanoate and sodium acrylate supplementation schedules on bacterial biomass
587 production, copolymer content, and the 3HHx molar fraction of the copolymer. We found that
588 intracellular polymer content and the 3HHx molar fraction of the copolymer reached their peaks
589 at different time points, and that the combined inhibitory effect of sodium hexanoate and sodium

590 acrylate on *C. eutrophus* B10646 cell growth was stronger than the separate effects of these
591 substrates. Synthesis of P(3HB-co-3HHx) copolymers with 45-68 mol.% 3HHx was achieved by
592 using scheduled portion-wise supply of sodium hexanoate and sodium acrylate; the total P(3HB-
593 co-3HHx) content varied from 25 to 54% of CDW, depending on the number of the
594 supplementations and the duration of the cultivation following the supplementations. Thus, the
595 parameters of P(3HB-co-3HHx) synthesis by the wild-type strain *C. eutrophus* B10646 are
596 comparable with the best results reported in the literature,[15] which were obtained by using the
597 recombinant strain *Cupriavidus necator* Re2160/pCB113 with the cloned PHA synthase gene of
598 *R. aetherivorans* and an enol-CoA hydratase gene from *Ps. Aeruginosa*.[13]

599 The study of the properties of purified P(3HB-co-3HHx) samples with different monomer
600 fractions showed that the molar fraction of 3HHx influenced physicochemical properties of the
601 polymers. The molecular weight and temperature parameters of P(3HB-co-3HHx) decreased as
602 the molar fraction of 3HHx increased, and this result is consistent with the data reported by other
603 authors.[14,15] The molar fraction of 3HHx had the strongest influence on the degree of crystallinity
604 of copolymer samples, decreasing the C_x to 20%, or more than threefold, when the 3HHx molar
605 fraction of the copolymer increased from 10 to 68 mol.%.

606 The composition of P(3HB-co-3HHx) and the 3HHx molar fraction have a considerable
607 effect on mechanical properties of P(3HB-co-3HHx) films. As the 3HHx molar fraction grows,
608 the elasticity of P(3HB-co-3HHx) films is increased considerably, as indicated by elongation at
609 break, which increases from several percent to several hundred percent, while mechanical
610 strength of the films decreases to a similar extent, as indicated by tensile strength and Young's
611 modulus. The trends of elasticity increase and mechanical strength decrease with the increase in
612 the molar fraction of 3HHx in the P(3HB-co-3HHx) copolymers are consistent with the data
613 reported by other authors,[5,9,14,15] but the values of elongation at break of P(3HB-co-3HHx) films
614 are different. In a number of studies,[31,32] elongation at break of films with 3HHx molar fractions
615 between several mol.% and 20-25 mol.% was found to be 100 to 500 %, which is in good

616 agreement with the results obtained in this study. Other authors,[9,15] however, report higher
617 values: up to 800% and even 1075%. It is difficult to interpret these differences, as most of the
618 authors did not describe the conditions of producing P(3HB-co-3HHx) films and their physical
619 size. The physical and mechanical properties of the P(3HB-co-3HHx) films reported in this study
620 only characterize the thin films (0.03 mm) prepared by the solvent evaporation technique.

621 Thus, by varying the 3HB and 3HHx composition of P(3HB-co-3HHx) copolymers
622 synthesized by the wild-type strain *C. eutrophus* B10646 grown under different cultivation
623 conditions, one can alter physicochemical and mechanical properties of the P(3HB-co-3HHx)
624 products and prepare P(3HB-co-3HHx) copolymers with high 3HHx molar fractions and with
625 improved properties.

626

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629

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Table 1

3HB and 3HHx fractions of P(3HB-co-3HHx) copolymers and their physicochemical and mechanical properties

PHA composition (mol.%)		M_w (kDa)	$Đ$	C_x (%)	T_m (°C)	T_d (°C)	E (MPa)	σ (MPa)	ε (%)
3HB	3HHx								
100.0	0	920±15	2.52±0.01	76	180	295	2071.2±23.8	16.7±0.7	2.5±0.1
88.0	12.0	440±19	3.10±0.05	56	170	275	1286.4±90.8	18.3±1.8	3.6±0.1
75.4	24.6	620±25	5.42±0.04	53	167	270	1207.5±21.4	21.6±0.8	4.1±0.1
57.0	43.0	310±9	3.88±0.04	54	176	282	938.0±14.1	19.0±0.3	5.0±0.1
45.0	55.0	700±18	4.10±0.07	45	171	281	311.4±21.8	6.6±0.3	13.6±0.3
34.3	65.7	680±24	3.90±0.04	35	173	276	209.5±17.0	7.8±0.7	140.6±5.5
32.0	68.0	720±6	4.84±0.01	21	172	282	217.0±11.3	7.4±0.7	177.0±4.8

T_m – melting point; T_d – thermal degradation temperature; C_x – crystallinity; M_w – weight average molecular weight; D – polidispersity; E - Young's modulus, σ - tensile strength, ε - elongation at break.

Table 2
Surface properties of the films prepared from solutions of P(3HB-co-3HHx) copolymers with different molar fractions of 3HHx

PHA composition. mol.%		Water contact angle (θ)	Contact angle of diiodomethane (θ)	Liquid surface tension (mN/m)	Liquid surface tension – polar part (mN/m)
3HB	3HHx				
100	0	92.8±0.7	49.4±0.6	35.9±0.4	1.30±0.08
88	12.0	95.9±0.9	38.3±1.3	40.7±0.6	0.34±0.07
75.4	24.6	103.0±0.7	44.5±1.0	37.2±0.6	0.04±0.02
57	43.0	106.2±0.8	48.4±1.1	35.2±0.6	0.03±0.02
45	55.0	104.7±0.9	49.5±0.2	34.6±0.1	0.04±0.03
34.3	65.7	109.0±0.5	46.9±0.9	36.1±0.5	0.14±0.03
32	68.0	99.0±1.2	53.1±0.6	33.1±0.4	0.54±0.15

Figure Legends

Fig. 1. Specific growth rate of *C. eutrophus* B10646 cells in continuous culture as related to a) glucose, b) urea, c) sodium hexanoate, d) sodium acrylate concentrations (g/L) in the culture medium

Fig. 2. Parameters of continuous culture of *C. eutrophus* B10646 (1 – end cell concentration, X g/L; P(3HB-co-3HHx) yield, % of CDW; 3HHx fraction – mol.%): a – with a single addition of sodium hexanoate (1 g/L) to the culture medium; b – with a single addition of sodium hexanoate (1 g/L) together with sodium acrylate (0.1 g/L). Arrows show additions of sodium hexanoate and sodium acrylate to the culture medium. Time of cultivation is 72 h.

Fig. 3. Parameters of the *C. eutrophus* B10646 culture (1 – end cell concentration, X g/L; P(3HB-co-3HHx) yield, % of CDW; 3HHx fraction – mol.%) synthesizing P(3HB-co-3HHx) under different modes of sodium hexanoate and acrylate supplementation of the culture medium: a – under different sodium hexanoate concentrations of the medium, without sodium acrylate addition; b – under different sodium acrylate concentrations of the medium, with sodium hexanoate maintained at a steady level of 1-2 g/L; c – as dependent on the number of sodium hexanoate portions added to the culture medium (one portion of 0.5 g/L) with sodium acrylate added at the same time (one portion of 0.1 g/L). Time of cultivation is 48 h.

Fig. 4. ^{1}H NMR-spectrum of the P(3HB-co-3HHx) copolymer.

Fig. 5. SEM images of the surfaces of the films prepared from P(3HB-co-3HHx) with different molar fractions of 3HHx.

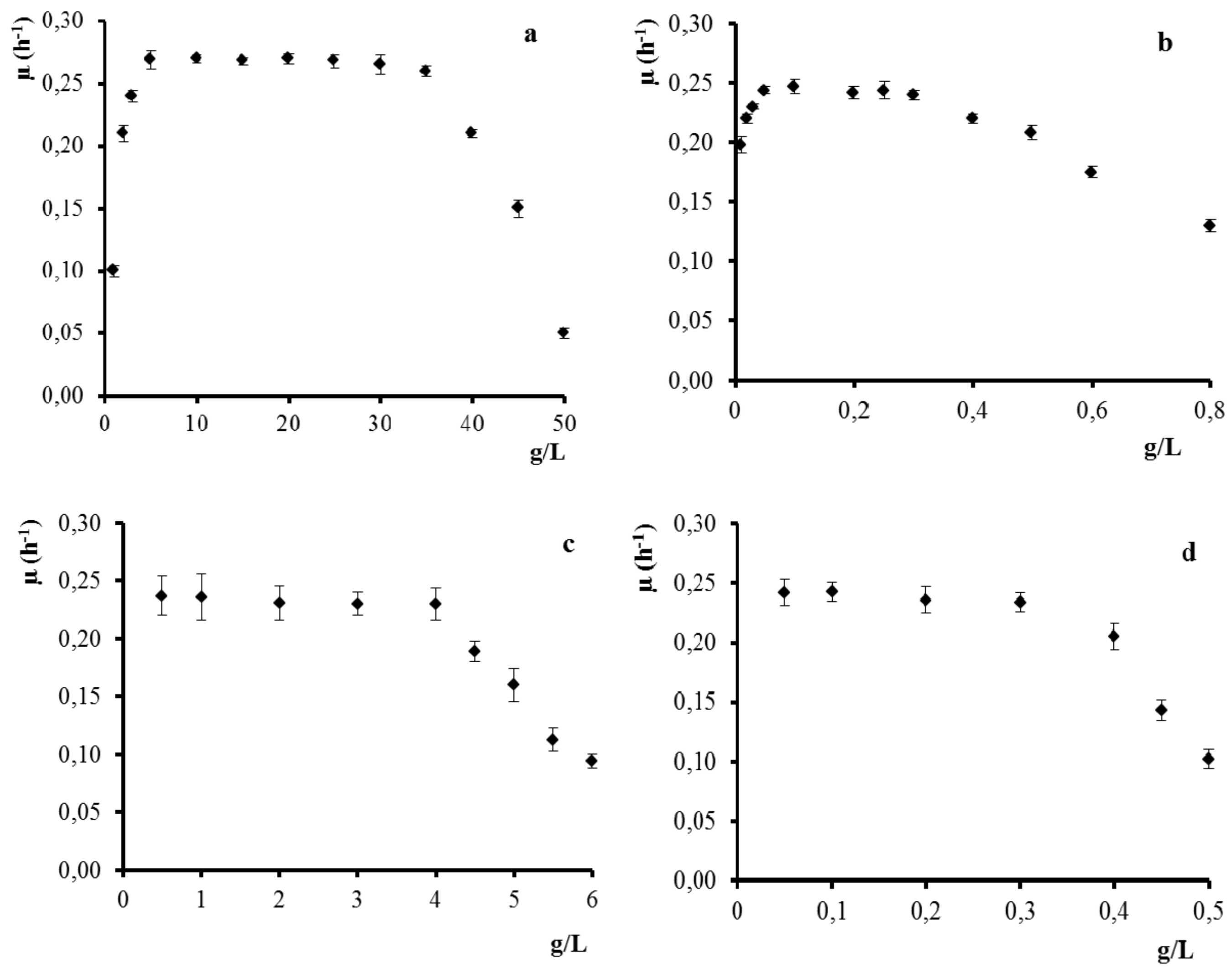
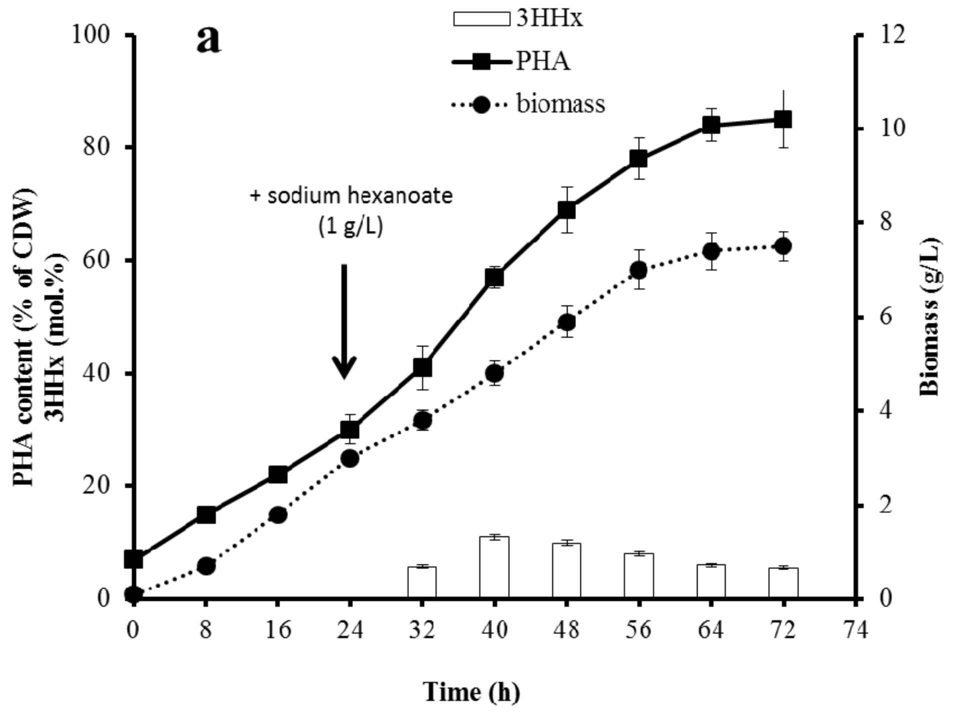


Fig. 1.



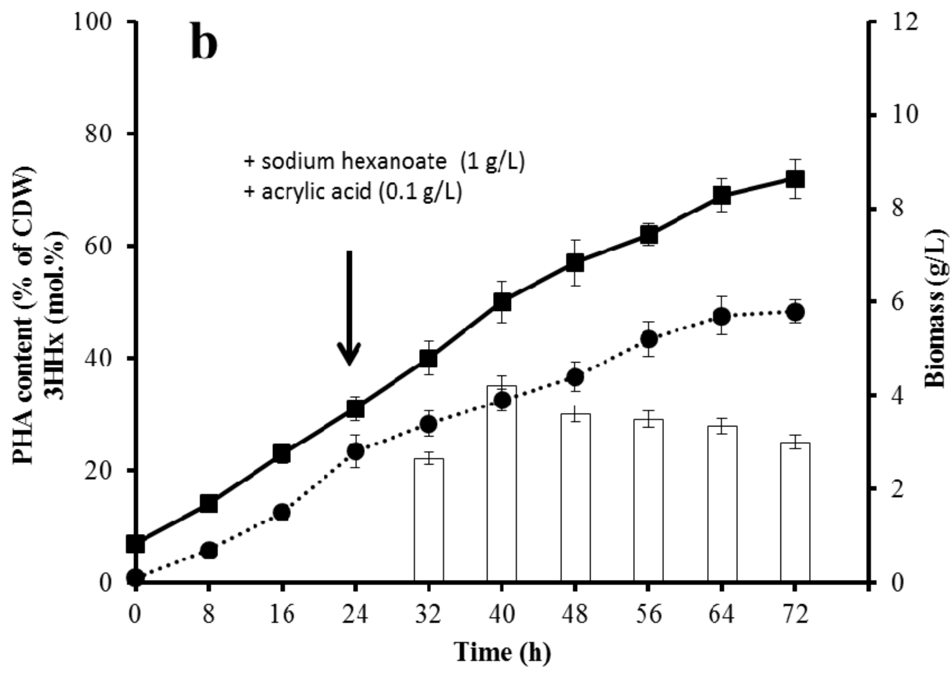


Fig. 2.

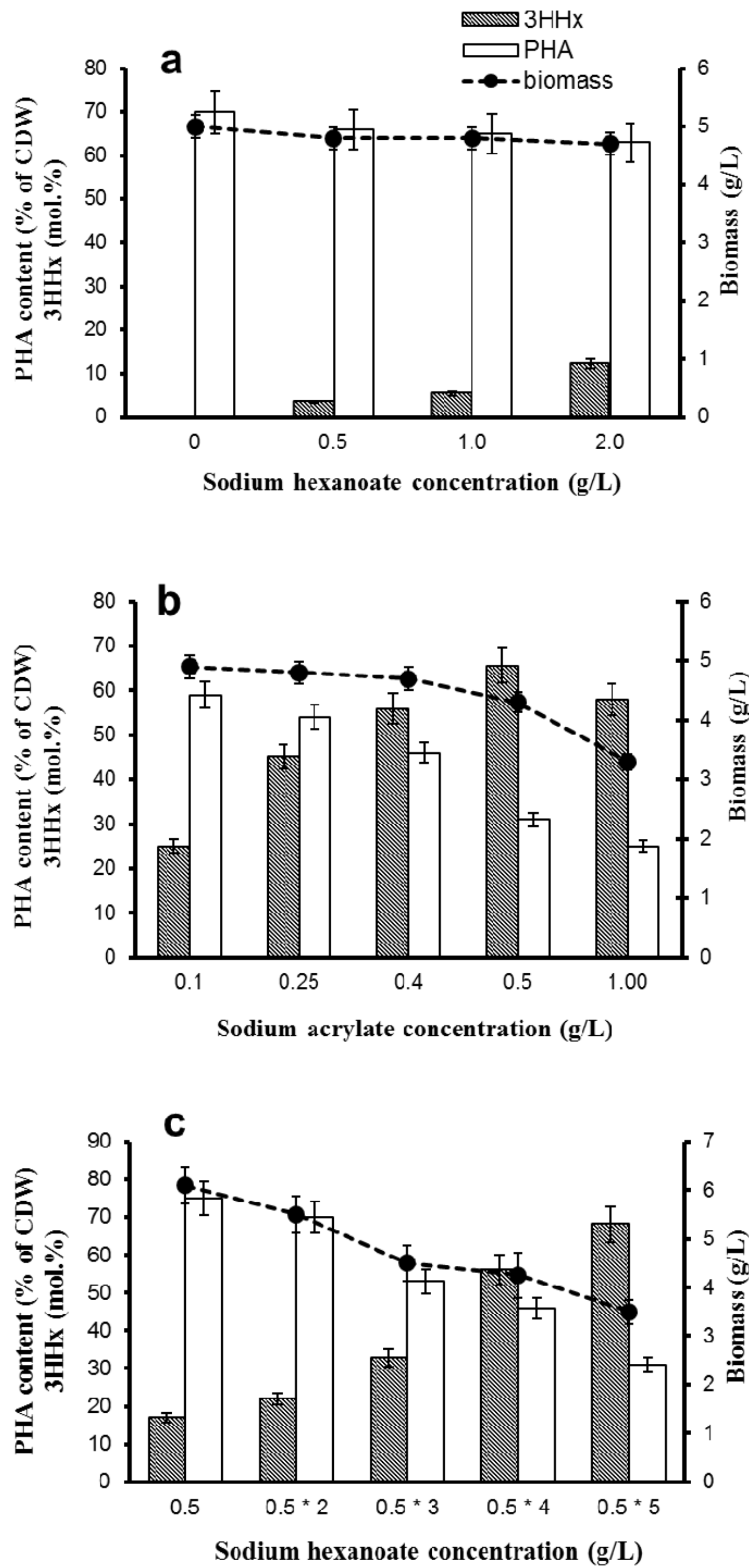


Fig.3

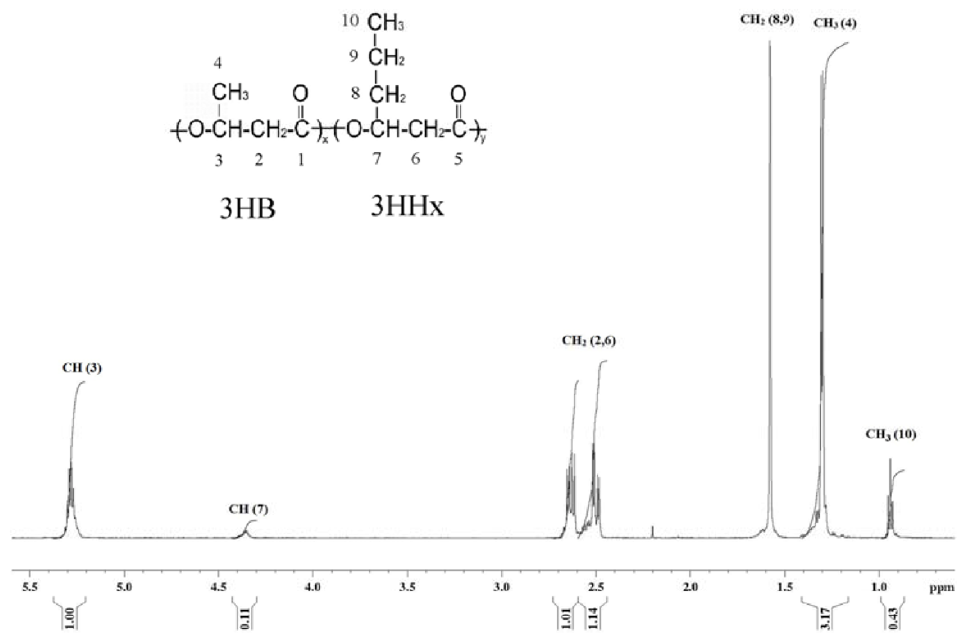
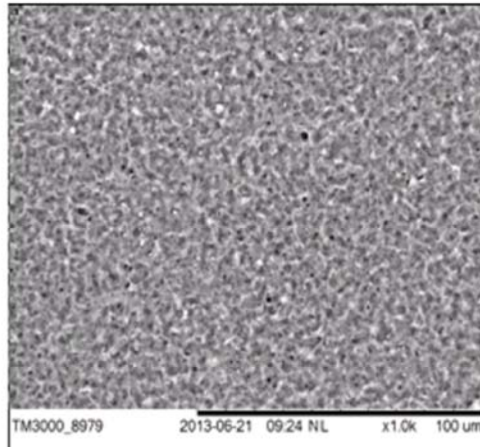
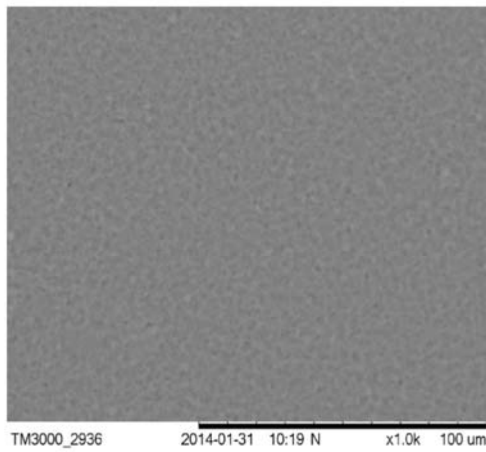


Fig.4

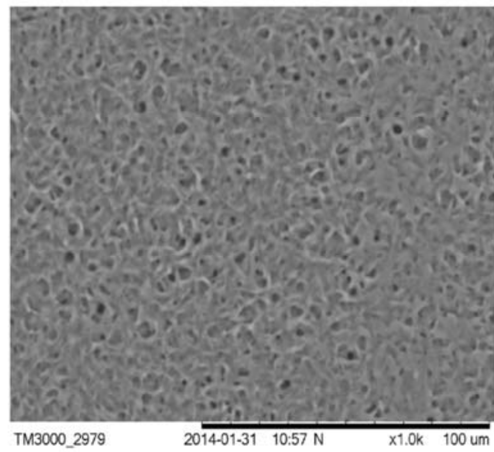
Fig. 5.



P(3HB) (100)



P(3HB-co-3HHx) (88/12)



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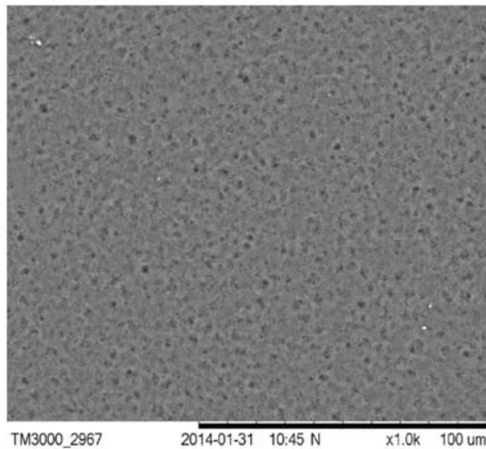

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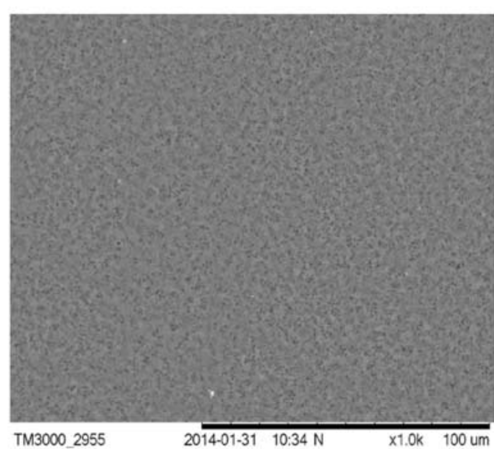

P(3HB-co-3HHx) (34.3/65.7)

Fig. 5.