Research Article

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Synthesis of P(3HB-co-3HHx) copolymers containing high molar fraction of 3-hydroxyhexanoate monomer by *Cupriavidus eutrophus* B10646

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Abstract

BACKGROUND: P(3HB-co-3HHx) copolymers are very promising biomaterials. The main challenge in the production of these polymers is to simultaneously achieve high cell biomass; high P(3HB-co-3HHx) content; and high molar fraction of 3HHx in P(3HB-co-3HHx). The most common approach to production of these copolymers is the use of recombinant bacterial strains. The purpose of this study was to optimize the process of production of P(3HB-co-3HHx) copolymers containing high molar fractions of 3HHx by using the wild-type strain *Cupriavidus eutrophus* B10646.

RESULTS: Kinetic properties of *C. eutrophus* B10646 were studied during cultivation of the cells on substrates necessary for P(3HB-co-3HHx) synthesis: glucose, nitrogen, sodium hexanoate, and sodium acrylate. The physiological ranges of their effects were determined experimentally, and *C. eutrophus* B10646 was grown in culture media with different dosages of these substrates. P(3HB-co-3HHx) copolymers with different molar fractions of 3HHx, including high ones (12 to 68%), were synthesized, and their physicochemical and mechanical properties were investigated.

CONCLUSION: For the first time, cultivation conditions of *Cupriavidus eutrophus* B10646 enabled production of high biomass yields (5–6 g L^{-1}) and high content of the polymer (60–75%) that contained high 3HHx molar fraction. By varying the 3HB/3HHx ratio, one can change physicochemical and mechanical properties of P(3HB-co-3HHx) copolymers.

Supporting information may be found in the online version of this article.

Keywords: wild-type strain *Cupriavidus eutrophus* B10646; growth kinetics; poly(3-hydroxybutyrate-co-3-hydroxyhexanoate); physicochemical and mechanical properties

INTRODUCTION

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

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

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P(3HB-co-3HO), have elastomeric properties and a decreased degree of crystallinity, in contrast to the homogenous poly(3-hydroxybutyrate), P(3HB).[6]

Both wild-type and genetically modified microorganisms are capable of synthesizing PHAs composed of SCL and MCL monomer units. Wild-type producers of SCL-MCL PHAs described in the literature represent different taxa: *Pseudomonas putida*,[7] *Rhodococcus sp.* NICMB 40126,[8] *Aeromonas caviae* 440,[9] *Ectothiorhodospira shaposhnikovii*,[10] etc. This is by no means an exhaustive list. The authors of those studies described synthesis of SCL-MCL PHAs on various substrates (sugars, organic and fatty acids), which served as the main carbon source. The nutrient media also contained additional carbon sources – the so-called precursor substrates for the synthesis of medium-chain-length monomers (hexanoic acid, octanoic acid, etc.), most of which inhibit the growth of PHA producing microorganisms. The total PHA yields were considerably lower than potentially attainable ones, and the molar fractions of medium-chain-length monomers were low. For instance, the fractions of 3HHx in the PHA reported so far varied from 1–5 mol% to 15–20 mol%.

Recombinant PHA producing strains, harboring genes of PHA synthesis enzymes from different bacteria, have been engineered to achieve more productive synthesis of SCL-MCL PHAs. Recombinant PHA producers are engineered by using both PHA-synthesizing microorganisms (*Pseudomonas, Aeromonas, Ralstonia*) and ones incapable of PHA synthesis (*E. coli*). If PHA-synthesizing bacteria are used, the aim is overexpression of the genes that control PHA synthesis. Non-PHA producers are also used to study the mechanism of PHA synthesis and detect additional participants in biosynthesis. For instance, *E. coli* is a promising bacterium for engineering recombinant strains because its metabolism has been studied thoroughly; moreover, *E. coli* has high growth rate, can use a wide range of substrates, and has no intracellular depolymerases, i.e. it may synthesize polymers in high yields.[11] However, even the use of recombinant PHA producers to synthesize SCL-MCL PHA copolymers has not resulted in the simultaneous production of large amounts of biomass and high total yields of polymers containing high molar fractions of medium-chain-length monomers. There are very few, if any, published studies that report a successful combination of all of these parameters. For instance, Jian *et al.* used a recombinant strain *Aeromonas hydrophila* AKJ1 to synthesize P(3HB-co-3HHx) copolymers and achieved high molar fractions (94.5–94.6 mol%) of 3HHx, but biomass production and total PHA yields were as low as 3.00–4.07 g L^{-1} and 25.99–54.49% DCW, respectively.[12] In a study by Wong *et al.*, recombinant strain *Cupriavidus necator* Re2160/pCB113 with the cloned PHA synthase gene of *R. aetherivorans* and an enol-CoA hydratase gene from *Ps. aeruginosa* produced higher biomass concentrations and polymer yields (6.27–6.73 g L^{-1} and 82–83% DCW, respectively), but the molar fraction of 3HHx was only 56–59 mol%.[13]

Chemolithoorganotrophic bacteria of the genus *Cupriavidus* (formerly known as *Ralstonia*) are regarded as very promising PHA producers, as these bacteria are capable of synthesizing PHAs in very high yields (80–90% of DCW) from various substrates.[4,14,15] It was previously believed that the PHA synthase of *Ralstonia* strains, which is a Class I synthase, is substrate specific, and, thus, wild-type bacteria of this genus are capable of accumulating only short-chain-length PHAs.[1] The first data showing the ability of the wild-type strain *R. eutropha* B5786 to synthesize P(3HB-co-3HHx) copolymers with 3HHx reaching 10–16 mol% when grown in autotrophic culture on CO_2 and hexanoate as an additional substrate were reported by Volova *et al.*[16,17] Several years later, Green *et al.*[18] also showed that the wild-type *R. eutropha* H16 was capable of synthesizing SCL-MCL PHAs containing 3HHx. In the copolymers synthesized by the cells grown on octanoic acid as a carbon source, in the medium supplemented with different concentrations of sodium acrylate, the molar fraction of 3HHx reached 5.7 mol%. The study comparing two strains – *R. eutropha* B5786 and *R. eutropha* H16 – showed that the H16 strain was also capable of synthesizing P(3HB-co-3HHx).[19] The molar fraction of 3HHx in P(3HB-co-3HHx) reached 50 mol% in experiments with *R. eutropha* B5786 strain grown heterotrophically on fructose, in the medium supplemented with sodium hexanoate and sodium acrylate (the latter blocking reactions of the fatty acid oxidation cycle and preventing the carbon chain of hexanoic acid from shortening).[20]

The PHA synthase gene of *R. eutropha* B5786 was cloned and characterized, and molecular structure of the enzyme was compared with PHA synthases of several strains accumulating SCL-MCL PHAs.[21] Homology of the PHA synthase of *R. eutropha* B5786 to the PHA synthase of *R. eutropha* H16 was 99%. Homology of the *R. eutropha* B5786 synthase to the synthases of some strains producing SCL-MCL PHAs was between 26% and 41%. Thus, no direct relationship was found between molecular organization of PHA synthases and their functions, namely, their ability to synthesize PHAs with certain structures.

Cupriavidus eutrophus B10646, a recently isolated wild-type strain showing enhanced tolerance to precursor C-substrates necessary for PHA_{MCL} synthesis, is capable of synthesizing SCL-MCL PHAs. The first studies of this strain showed that when grown in autotrophic culture on CO_2 as the main carbon source, with sodium hexanoate as an additional substrate, *Cupriavidus eutrophus* B10646 cells synthesized PHA copolymers containing 20–25 mol% 3HHx.[22] PHA terpolymers, P(3HB-co-3HV-co-3HHx) synthesized by *Cupriavidus eutrophus* B10646 cells in heterotrophic culture on sugars and sodium valerate and sodium hexanoate as additional substrates contained 13.6 mol% 3HHx.[23]

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

EXPERIMENTAL

Bacterial strain

The strain used in this study was *C. eutrophus* B10646, registered in the Russian National Collection of Industrial Microorganisms. The strain has a broad organotrophic potential and can use different carbon sources; it is tolerant to concentrations of a number of organic C-substrates (sodium valerate and hexanoate, γ-butyrolactone) reaching 3–5 g L^{-1} in the culture medium and is able to use them to synthesize PHA copolymers containing short- and medium-chain-length monomer units. As a nitrogen source, the strain utilizes nitrates, ammonium salts, urea, and amino acids.[24]

Media

Schlegel's mineral medium was used as a basic solution for growing cells:[25] $Na_2HPO_4.H_2O$ – 9.1; KH_2PO_4 – 1.5; $MgSO_4 \cdot H_2O$ – 0.2; $Fe_3C_6H_5O_7 \cdot 7H_2O$ – 0.025; $CO(NH_2)_2$ – 1.0 (g L^{-1}). The main carbon substrate was glucose (China), which was sterilized by membrane filtration using Opticap XL300 Millipore Express SHC filters (US),

in order to prevent the pH from falling. Nitrogen was provided in the form of urea, and, thus, no pH adjustment was needed. The pH level of the culture medium was stabilized at 7.0 ± 0.1. A solution of iron citrate (5 g L^{-1}), which was used as a source of iron, was added to reach a concentration of 5 mL L^{-1}. Hoagland's trace element solution was used: 3 mL of standard solution per 1 L of the medium. The standard solution contains H_3BO_3 – 0.288; $CoCl_2 \cdot 6H_2O$ – 0.030; $CuSO_4 \cdot 5H_2O$ – 0.08; $MnCl_2 \cdot 4H_2O$ – 0.008; $ZnSO_4 \cdot 7H_2O$ – 0.176; $NaMoO_4 \cdot 2H_2O$ – 0.050; $NiCl_2$ – 0.008 (g L^{-1}). Substrate feeding strategies were varied depending on the technique of bacterial cultivation employed: culture in flasks in a shaker or culture in a fermentation system.

Growth conditions

Cells were grown in batch and continuous cultures. Inoculum was produced using an Innova® 44 constant temperature incubator shaker (New Brunswick Scientific, USA). Inoculum was prepared by resuspending the stock culture maintained on agar medium. Bacteria were grown in 0.5 L to 2.0 L glass flasks half-filled with mineral medium, with the initial concentration of glucose 10 g L^{-1} at 30 °C and 200 rpm. Cells were cultivated to reach a concentration of 1 g L^{-1} (with polymer content not higher than 10%).

Growth kinetics of bacterial cells was studied in continuous mode in a BioFlo-115 automated laboratory fermentor, with an 8 L fermentation vessel and the working volume of the culture from 3 L to 5 L, under strictly aseptic conditions. The mass flow of the fermentor is controlled by the air flow rate and agitation speed; the latter can be varied from 300 to 1000 rpm, and, thus, air transfer rate varies from 2 to 8 L min^{-1}. The fermentor is equipped with a control station with a liquid crystal display, which records the cultivation process data, pH probes, O_2 probes, a system for automatic substrate feeding, and a thermal stabilization system. Dissolved oxygen saturation level was maintained at 25–30%; as cell concentration increased, dissolved oxygen began to decrease and agitation speed increased (cascaded control).

The synthesis of P(3HB-co-3HHx) copolymers was studied in the batch culture, by using the previously developed procedure.[23] Cultivation was performed in an Innova® 44 constant temperature incubator shaker (New Brunswick Scientific, USA). Cells were grown in 2.0 L glass flasks half-filled with mineral medium at 30 °C and 200 rpm. Synthesis of PHA copolymers was achieved as follows: the culture medium was supplemented with precursor substrate (different concentrations of sodium hexanoate) (Acros Organics, USA) at 24 h, and cultivation lasted 48–72 h. In a number of experiments, the culture medium was also supplemented with sodium acrylate (Sigma, USA) (simultaneously with sodium hexanoate supplementation), which blocked reactions of the fatty acid β-oxidation cycle and enhanced the synthesis of 3HH× monomer units.

Monitoring process parameters

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

Glucose concentration was determined using the 'Glucose – FKD' ('Fermatsevtika i klinicheskaya diagnostika', Moscow, Russia), which contained chromogenic enzyme substrate and a calibrator (a 10 mmol L^{-1} glucose solution). Optical density of the study sample and calibration sample were compared photometrically with the optical density of the blank, with optical path length 10 mm at wavelength 490 nm. Glucose concentration in the samples was calculated using the following formula:

$$C = \left(E_0/E_k\right) \times 10$$

where C is glucose concentration, mmol L^{-1}; E_0 is optical density of the sample tested, units of optical density; E_k is optical density of the calibration sample, units of optical density; 10 is the glucose concentration in the calibrator, mmol L^{-1}.

Sodium hexanoate concentrations in the culture medium were controlled using a gas chromatography analysis (GC-MS 6890/5975C, Agilent Technologies, USA) of the culture medium samples, which was done after preliminary extraction with chloroform from acidified samples. Nitrogen concentration in the culture medium was analyzed at different time points, using a photometric method, with Nessler's reagent.[26]

P(3HB-co-3HHx) biosynthesis was evaluated based on cell concentration, polymer yield, the amount of the main growth substrate used, and process duration and productivity. The cell biomass yield (X, g L^{-1}), and the specific growth rate (μ, h^{-1}) were calculated.

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

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

where x is biomass, g L^{-1}, t is duration of cultivation, h.

Kinetic constants of the culture (saturation constant, *Ks* and inhibition constant, *Ki*) were determined by using the conventional Lineweaver–Burk semi-graphical method: by linearizing inverse values of the relationship between the specific growth rate of bacterial cells and substrate concentration.[27]

Analysis of P(3HB-co-3HHx) structure and physicochemical properties

Intracellular polymer content at different time points was determined by analyzing samples of dry cell biomass. Intracellular PHA content and composition of extracted polymer samples were analyzed by a GC-MS (6890/5975C, Agilent Technologies, USA). Both lyophilized cells and extracted polymer were subjected to methanolysis in the presence of sulfuric acid, and polymer was extracted and methyl esterified at 100 °C for 4 h. Benzoic acid was used as an internal standard to determine total intracellular PHA.[28] Monomer units of P(3HB-co-3HHx) were identified in the extracted and purified polymer samples based on their retention times and mass spectra.

Molecular weight and molecular-weight distribution of the copolymer were examined using a gel permeation chromatograph (1260 Infinity, Agilent Technologies, USA) with a refractive index detector, using an Agilent PLgel Mixed-C column. Chloroform was the eluent, at a flow rate of 1.0 mL min^{-1} at 40 °C. Typical sample volumes were 50 μL at a polymer concentration of 2 mg mL^{-1}. Narrow polydispersity polystyrene standards (Agilent, USA) were used to generate a universal calibration curve, from which molecular weights (weight average, M_w, and number average, M_n) and polydispersity ($Đ = M_w/M_n$) were determined. The measurement accuracy was 2%.

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

X-ray structure analysis and determination of crystallinity of copolymers were performed employing a D8 Advance X-ray powder diffractometer equipped with a Vantec fast linear detector, using CuKa radiation (Bruker, AXS, Germany). In order to determine the crystallinity of the P(3HB-co-3HHx), three film samples 2 cm in diameter and 0.15 mm thick were prepared from a 2% polymer solution in chloroform. The samples had a circular shape because during measurement the sample spins in a direction perpendicular to the surface. The scan step was 0.016°, measurement time in each step 114 s, and scanning range from 5° to 60° (from 48° to 60° there only was a uniformly decreasing background); the registered parameter was intensity of X-rays scattered by the sample; 55°/0.016° = 3438 times. The degree of crystallinity was calculated as a ratio of the total area of crystalline peaks to the total area of the radiograph (the crystalline + amorphous components). Measurement accuracy: point measurement accuracy ±0.4 PPS, with the lowest intensity 1.5 PPS and the highest intensity 32 PPS; the error in determination of the degree of crystallinity, which was calculated based on multiple measurements, was 2% or less.

Analysis of P(3HB-co-3HHx) physical–mechanical properties

Surface properties of P(3HB-co-3HHx) films prepared by coating glass with the chloroform solution of the polymer were studied with a Drop Shape Analyzer – DSA-25E (Krüss GmbH – Germany) for measuring contact angles of water and diiodomethane drops by the Owens–Wendt–Rabel–Kaelble method:[29–31] surface free energy (SFE) and its dispersion and polar components were measured, and the data were processed by the DSA-4 software.

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

Samples were maintained at room temperature in the laminar flow box for at least two weeks to reach equilibrium crystallization. At least five samples were tested for each type of film. Measurements were conducted at room temperature; the clamping length of the samples was 30 mm. The speed of the crosshead was 3 mm min^{-1} at room temperature. Young's modulus (E, MPa), tensile strength (σ, MPa) and elongation to break (ε, %) were automatically calculated by the Instron software (Bluehill 2, Elancourt, France). To obtain Young's modulus, the software calculated the slope of each stress–strain curve in its elastic deformation region. Measurement error did not exceed 10%.

Statistics

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

RESULTS

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

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

The study of the effect of glucose concentration on the growth rate of the glucose-utilizing strain, *C. eutrophus* B10646, determined the physiological limits of the effect of this substrate. Results of experiments showed that for this strain, glucose concentration in the culture medium should be maintained within a range of 5 to 35 g L^{-1} (Fig. 1(a)). Glucose concentrations below 5 g L^{-1} and above 35 g L^{-1} adversely affected cell growth rate. Glucose concentrations below and above these limits caused a decrease in the cell yield; at glucose concentration of 50 g L^{-1}, specific growth rate of the cells dropped to 0.050 h^{-1}, and at 80–100 g L^{-1}, cell growth was completely inhibited. Saturation constant (*Ks*) and inhibition constant (*Ki*) of the main growth substrate (glucose) for *C. eutrophus* B10646 cells were found to be 12.7 g L^{-1} and 38.5 g L^{-1}, respectively. Thus, in the case of the batch culture, with varying cell concentration and substrate concentration, glucose should be fed to the culture medium periodically or continuously, and its concentration should be constantly monitored.

Figure 1(b) shows the relationship between specific growth rate of *C. eutrophus* B10646 cells and nitrogen concentration. If the nutrient medium contains ammonium chloride, Cl^- ions are accumulated in the culture medium, and their concentration in the cell culture asymptotically reaches their concentration in the nutrient medium and acidifies it;[32] thus, pH needs to be adjusted. Therefore, in our experiments, we used urea as nitrogen source in the culture medium: the urea molecule is completely assimilated by bacterial cells, the ionic composition of the medium remains unchanged, and, thus, no adverse effects occur, unlike in the case of other nitrogen forms.

Experiments were carried out with varied nitrogen concentrations in the nutrient medium to find the relationship between specific growth rate of *C. eutrophus* B10646 cells and nitrogen supply and to determine nitrogen requirements of the cells. The nitrogen supply tested in these experiments was varied between 0.1 and 0.22 g g^{-1} of the cell biomass synthesized. The highest intracellular nitrogen concentration at the highest specific growth rate (about 0.24–0.27 h^{-1}) reached 0.12 ± 0.005 g g^{-1}. Nitrogen supply below this value limits cell growth. In the case of excessive nitrogen supply, its residual concentration in the culture medium increases. At the same time, residual concentration of the element must not drop below the critical level, i.e. the concentration gradient between the medium and the cell must be large enough for the rate of the diffusion of molecules toward the cell surface to be greater than the rate of their consumption by the cells. On the other hand, bacterial cultivation with considerable residual

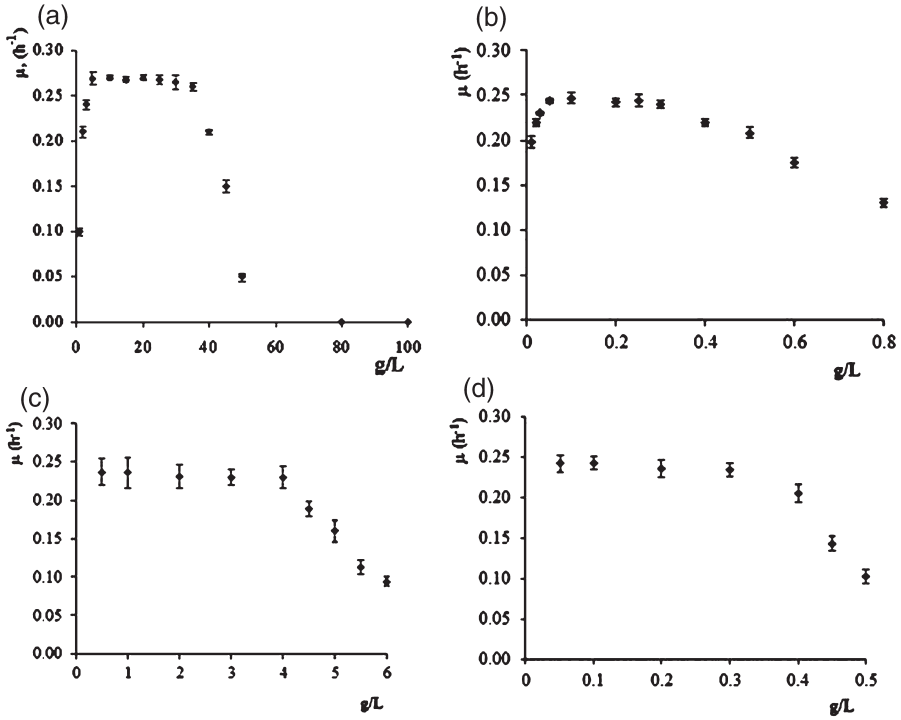

Figure 1. Specific growth rate of *C. eutrophus* B10646 cells in continuous culture as related to (a) glucose, (b) urea, (c) sodium hexanoate, and (d) sodium acrylate concentrations (g L^{-1}) in the culture medium.

concentrations of elements is technologically uneconomical, as large amounts of mineral substrate will be wasted. Therefore, it is important to know the lowest concentration of the element that does not affect the process of bacterial cultivation. In continuous culture, specific growth rate of the cells was not affected by residual nitrogen concentration varied within a wide range of 0.05 to 0.3 g L^{-1} (Fig. 1(b)). It is generally possible to reduce residual nitrogen concentration to trace amounts, but then this parameter must be continuously monitored. Another important issue is that during cultivation of bacterial cells on urea, ammonia produced in hydrolysis of urea by bacterial urease may accumulate in the culture medium. It has been found that in the case of using urea, about half of the nitrogen in the culture medium is ammonia nitrogen. If residual nitrogen concentration exceeds 0.3 g L^{-1}, large amounts of ammonia inhibit cell growth. Hence, during bacterial cultivation, nitrogen should be constantly controlled. The saturation constant (*Ks*) and inhibition constant (*Ki*) for nitrogen found in this study were 0.005 g L^{-1} and 0.28 g L^{-1}, respectively.

As sodium hexanoate is a necessary precursor substrate for synthesis of P(3HB-co-3HHx) copolymers, experiments were carried out with different amounts of sodium hexanoate added to the culture medium. The relationship between specific growth rate of *C. eutrophus* B10646 cells and sodium hexanoate concentration in the culture medium is shown in Fig. 1(c). As this strain was selected for sodium hexanoate tolerance, the physiological range of the sodium hexanoate effect on this strain is rather wide. Cell growth is inhibited by sodium hexanoate concentrations more than 4 g L^{-1}; the *Ki* for this strain is 3.96 g L^{-1}.

Another substrate studied was sodium acrylate (Fig. 1(d)). When fatty acids are added as a supplementary substrate, β-oxidation is the most likely source of monomers for heteropolymer synthesis. Short-chain-length FAs – butyric acid, valeric acid – are reduced to D-β-OH-derivatives during β-oxidation, skipping the main pathway of monomer synthesis. These two monomers exhibit high affinity for PHA synthase, and, thus, they are not involved in the cycle of FA β-oxidation any more, but are immediately directed to the synthesis of the polymer.[33] When the synthesis of P(3HB-co-3HHx) is induced by addition of sodium hexanoate, just a small part of it is incorporated into the polymer as 3HHx, while the major part is converted to 3HB after losing the C_2-fragment in reactions of β-oxidation. The reason may be that the affinity of synthase for this monomer is two orders of magnitude lower than for 3HB and 3HV, so the larger part of sodium hexanoate goes through at least one cycle of β-oxidation. Hence, incorporation of 3HHx into the PHA can be enhanced by creating the conditions favoring intracellular accumulation of this monomer. 3-ketoacyl-CoA thiolase inhibition is the most likely way to suppress the β-oxidation reaction, which is responsible for the shortening of the carbon chains of monomers, 3HHx in particular. Acrylic acid is one of 3-ketoacyl-CoA thiolase inhibitors.[18] Acrylic acid strongly inhibits *C. eutrophus* B10646 cell growth (Fig. 1(d)); at concentrations below 0.3 g L^{-1}, this substrate has no noticeable inhibitory effect on the strain. At a sodium acrylate concentration of 0.5 g L^{-1}, specific growth rate of *C. eutrophus* B10646 cells drops by nearly half, i.e. the *Ki* for acrylate is 0.23 g L^{-1}.

Based on the relationships between substrate concentrations necessary for P(3HB-co-3HHx) synthesis and specific growth rate of *C. eutrophus* B10646 cells and the physiological ranges of effects of these substrates on the strain found in the experiments described above, we created the conditions necessary for the synthesis of P(3HB-co-3HHx) in a two-stage batch culture (Fig. 2). In Stage 1, which lasted 32 h, cells were grown in medium containing an excessive amount of glucose, under nitrogen deficiency. Instead of 1 g L^{-1}, 0.5 g L^{-1} of urea was added to the nutrient medium. After 24 h of cultivation, when cell concentration reached 3.0 g L^{-1} and intracellular polymer content 28–30% of DCW, sodium hexanoate

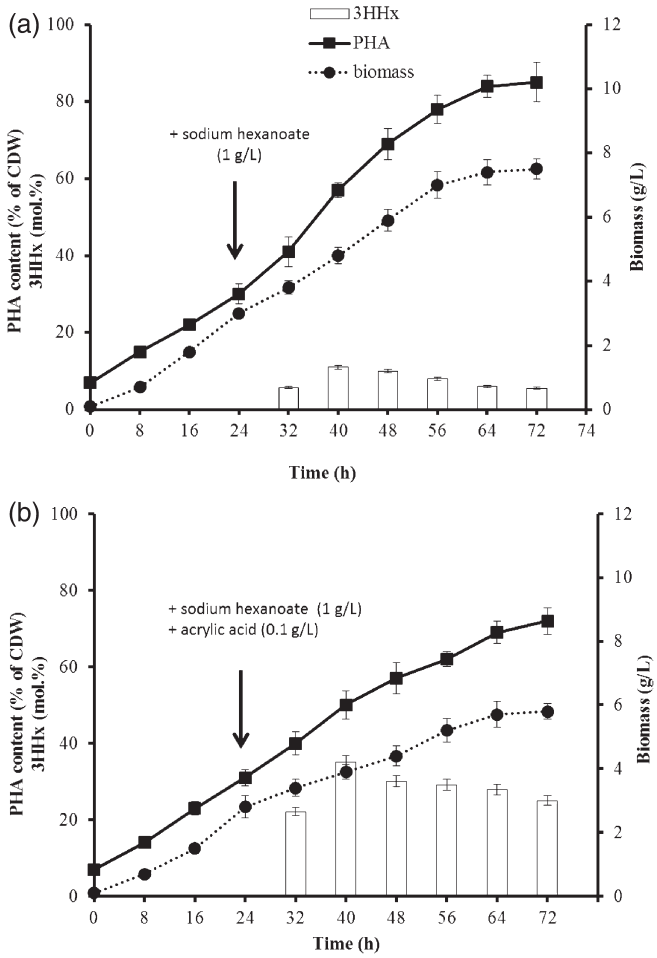

Figure 2. Parameters of continuous culture of *C. eutrophus* B10646 (1 – end cell concentration, *X* g L^{-1}; P(3HB-co-3HHx) yield, % of DCW; 3HHx fraction – mol%): (a) with a single addition of sodium hexanoate (1 g L^{-1}) to the culture medium; (b) with a single addition of sodium hexanoate (1 g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

was added in one portion, at a non-inhibitory concentration (1 g L^{-1}) (Fig. 2(a)). In 8 h after sodium hexanoate was added to the culture medium, the second stage of cultivation started: bacterial cells were grown in nitrogen-free medium, with controlled glucose concentration maintained at levels that did not limit cell growth. Bacterial biomass and intracellular polymer concentration increased: at the end of the experiment (72 h), *X* (g L^{-1}) was 7.5 g L^{-1} and copolymer content reached 85% of DCW. The 3HHx molar fraction of the copolymer reached its maximum (11 mol%) at 40 h after sodium hexanoate was added to the culture medium. Then, however, the 3HHx fraction decreased while the molar fraction of 3-hydroxybutyrate increased. The end concentration of 3HHx in the copolymer was 5.5 mol%.

Results of an experiment done in a similar fashion but with sodium hexanoate and sodium acrylate (0.1 g L^{-1}) simultaneously added to the culture medium are shown in Fig. 2(b). The trends in changes of the study parameters were similar to those in the previous experiment, but the simultaneous joint effect of two factors (sodium hexanoate and sodium acrylate) inhibited cell growth, although when added separately, the same concentrations of these substrates had not caused any inhibition of cell growth (Fig. 1(c), (d)). After 72 h of cultivation, cell concentration and intracellular polymer content were 5.8 g L^{-1} and 72% of DCW, respectively, or 14–23% lower than in the experiment with sodium hexanoate alone added to the culture medium (Fig. 2(a)). The maximum fraction of 3HHx was also reached at 40 h after sodium hexanoate and sodium acrylate were added to the culture medium, but it was larger (35 mol%). Then, as cell concentration and intracellular polymer content grew somewhat, the 3HHx fraction decreased while the 3HB fraction increased. The end concentration of 3HHx in the copolymer was 25 mol%.

Thus, results of experimental studies showed that the highest intracellular copolymer content and the largest 3HHx fraction of the copolymer are reached at different times. Further research is needed to maximize the major parameters of P(3HB-co-3HHx) synthesis: bacterial biomass yield, total copolymer content, and the 3HHx fraction of the copolymer.

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

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

Results of studying the effect of sodium hexanoate concentration in the culture medium on cell concentration, copolymer content, and the 3HHx molar fraction are shown in Fig. 3(a). Based on the results suggesting a decrease in the molar fraction of 3HHx at longer time intervals after the addition of sodium hexanoate to the culture medium, the cultivation lasted no more than 40 to 48 h. As sodium hexanoate concentration in the culture medium was increased, the 3HHx fraction of the copolymer increased, too. The highest molar fraction of 3HHx (12 mol%) was achieved at sodium hexanoate concentration of 2 g L^{-1}. The total biomass production and copolymer yield remained almost unchanged: 4.7–5.0 g L^{-1} and 63–70% of DCW, respectively.

The other factor influencing the formation of 3HHx monomer units is acrylic acid, which blocks the reactions of the fatty acid β-oxidation cycle. Results of studying the effect of sodium acrylate concentration on the parameters of the *C. eutrophus* B10646 culture are shown in Fig. 3(b). Sodium acrylate concentration in the culture medium was varied between 0.1 g L^{-1} and 1.0 g L^{-1}, while sodium hexanoate concentration was maintained at a steady level of 1.0 g L^{-1}.

From Fig. 3(b) we can see that as sodium acrylate concentration was increased from 0.1 to 0.4 g L^{-1}, the 3HHx molar fraction of the copolymer increased from 5 mol% to 56 mol%, the cell concentration showed little change, but polymer content decreased. At sodium acrylate concentration of 0.5 g L^{-1}, a decrease in cell concentration and an even more significant decrease in copolymer content were observed, while the 3HHx molar fraction increased to 65.7 mol%. As the concentration of sodium acrylate in the nutrient medium was increased further (from 0.5 to 1.0 g L^{-1}), the cell concentration dropped to 3.3 g L^{-1} and polymer content to 25% of DCW, whereas the 3HHx molar fraction remained rather high, reaching 58 mol%.

Since sodium hexanoate and sodium acrylate simultaneously added to the culture medium inhibited cell growth, we tried adding these substrates portion-wise. The batch culture of *C. eutrophus* B10646 in the mode of PHA synthesis was supplemented with

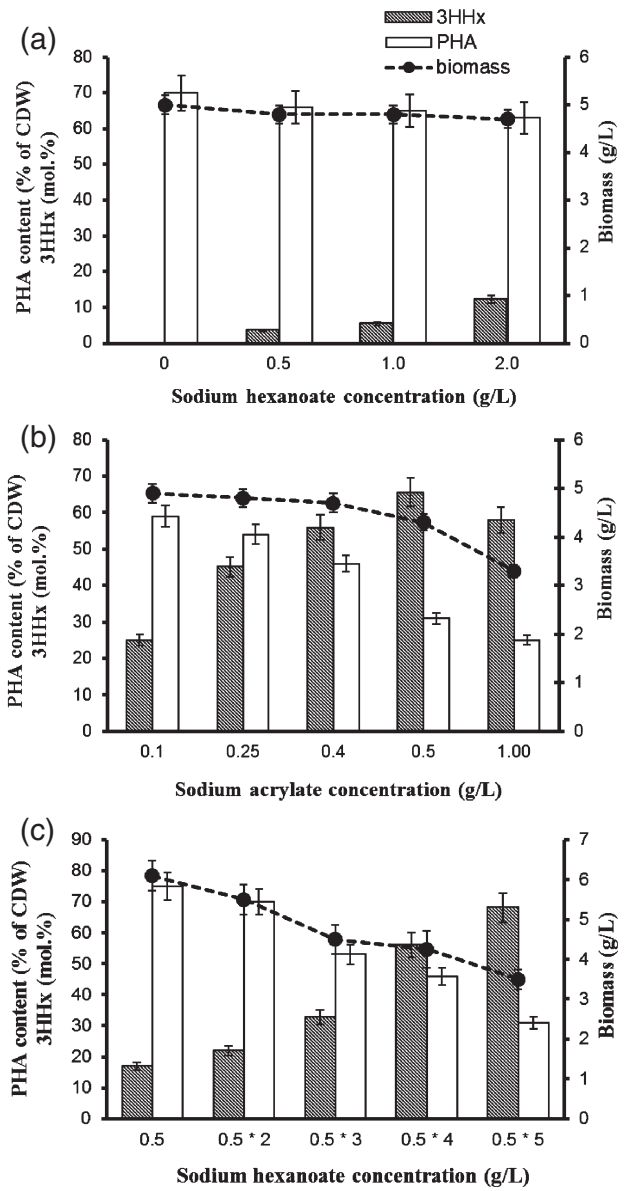

Figure 3. Parameters of the *C. eutrophus* B10646 culture (1 – end cell concentration, *X* g L^{-1}; P(3HB-co-3HHx) yield, % of DCW; 3HHx fraction – mol%) synthesizing P(3HB-co-3HHx) under different modes of sodium hexanoate and acrylate supplementation of the culture medium: (a) under different sodium 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 g L^{-1}; (c) dependent on the number of sodium hexanoate portions added to the culture medium (one portion of 0.5 g L^{-1}) with sodium acrylate added at the same time (one portion of 0.1 g L^{-1}). Sodium hexanoate and sodium acrylate were first added at 24 h of culture; subsequent additions were done every 6 h. Time of cultivation is 48 h. In the experiment with five additions of sodium hexanoate and sodium acrylate, cultivation lasted 54 h.

sodium hexanoate portions (one to five) (one portion of 0.5 g L^{-1}) every 6 h. Sodium acrylate was added simultaneously with sodium hexanoate, at concentrations that did not limit cell growth of the study strain (0.1 g L^{-1}) (Fig. 3(c)). Intracellular polymer content varied from 31% to 75% of DCW in different experiments, and the 3HHx molar fraction reached 17–68 mol%. The largest molar fraction of 3-hydroxyhexanoate (68 mol%) was achieved when 2.5 and 0.5 g L^{-1} of sodium hexanoate and sodium acrylate (total concentration), respectively, were added to the culture medium; the biomass yield in that experiment was 3.5 g L^{-1}, and polymer content was rather low (31% of DCW). The experiments showed that the adverse influence of sodium acrylate on polymer synthesis and biomass yield is greater than that of sodium hexanoate. The highest 3HHx fraction of the copolymer was achieved when sodium hexanoate and sodium acrylate were added to the culture medium with decreased residual glucose concentration.

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

Physicochemical and mechanical properties of P(3HB-co-3HHx) copolymers with different molar fractions of 3HHx.

Physicochemical and mechanical properties of P(3HB-co-3HHx) copolymers with different 3HHx molar fractions are given in Supplementary Tables 1 and 2. Analysis of the influence of the 3HHx molar fraction on molecular weight parameters of the copolymers did not reveal any strong relationship between the weight-average molecular weight of the polymer and the 3HHx fraction (M_w/3HHx) (Supplementary Table 1). Melting temperature and thermal decomposition temperature of P(3HB-co-3HHx) copolymers are generally lower than those of P(3HB), but no clear relationship was found between these parameters and 3HHx molar fractions. It is important to note that a decrease in the melting point and thermal decomposition temperature of P(3HB-co-3HHx) does not reduce the characteristic difference between these temperatures. The considerable (about 100 °C) difference between T_m and T_d is an essential processing property of P(3HB-co-3HHx) copolymers. X-ray structure analysis revealed significant influence of the 3HHx fraction on the crystalline to amorphous ratio in different P(3HB-co-3HHx) samples. As the 3HHx molar fraction increased, the degree of crystallinity (C_x) of the copolymers consistently decreased. By varying the 3HHx molar fractions of P(3HB-co-3HHx) copolymers, we managed to prepare thermally stable samples with significantly different degrees of crystallinity.

Physical and mechanical properties of P(3HB-co-3HHx) copolymers were studied in smooth 0.03-mm-thick films prepared from solutions of copolymers with different fractions of monomer units. The water contact angle of P(3HB-co-3HHx) was higher than that of P(3HB), reaching 95.9–109° (Supplemental Table 2). No correlation was found between the contact angle of diiodomethane and liquid surface tension and the 3HHx molar fraction in polymer films. At the same time, the polar part of liquid surface tension in P(3HB-co-3HHx) films decreased significantly.

The mechanical properties of a polymer product largely depend on the production technique employed and chemical composition of the polymers used.[5] Mechanical properties of P(3HB-co-3HHx) films were considerably influenced by the molar fraction of 3HHx in the copolymer. All copolymer films had significantly higher values of elongation to break, as a parameter of polymer elasticity, than films prepared from the highly crystalline P(3HB) homopolymer (2.5%) (Supplemental Table 1). Elongation of P(3HB-co-3HHx) films was enhanced as the 3HHx molar fraction of the copolymer was increased: it was almost 40–50 orders of magnitude higher in P(3HB-co-3HHx) films with 3HHx more than 60 mol% than in the films with 12 mol% 3HHx. Higher elasticity of P(3HB-co-3HHx)

films, characteristic of medium-chain-length copolymers, was accompanied by lower mechanical strength of the films, expressed as Young's modulus and tensile strength. Young's modulus of all P(3HB-co-3HHx) films was considerably lower than that of the homogenous P(3HB) (2071.2 MPa). As the 3HHx molar fraction of the copolymers was increased (from 12 mol% to 68 mol%), Young's modulus dropped from 1286.4 to 217.0 MPa and tensile strength – to 6.6–7.8 MPa. Hence, the degree of crystallinity and mechanical properties of P(3HB-co-3HHx) products can be significantly altered by varying the fractions of 3HHx monomer units in the PHA.

DISCUSSION

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

Synthesis of medium-chain-length PHAs is a complex process, as it involves the use of precursor substrates, which are usually toxic to PHA producing strains. Therefore, production of high yields of bacterial biomass with high intracellular content of polymers that contain MCL monomer units is a challenging task. As already mentioned above, the available literature describes synthesis and composition of PHAs produced by wild-type strains of various genera (*Ralstonia, Pseudomonas, Rhodospirillum*, *Rhodococcus*, *Thiocapsa*, *Aeromonas*, *Ectothiorhodospirai*), which generally contain rather low molar fractions of medium-chain-length monomer units (from 2–6 mol% to 10–20 mol%). Various PHA-producing bacteria are used and special cultivation modes are developed to attain high yields of bacterial biomass and polymers and to produce polymers with high molar fractions of MCL monomer units.

Another approach to synthesizing high fractions of MCL monomer units is engineering recombinant producers of PHA copolymers. The highest molar fractions of medium-chain-length monomers, 3HHx, have been recently produced in the culture of recombinant strain *Cupriavidus necator* Re2160/pCB113 with the cloned PHA synthase gene of *R. aetherivorans* and an enol-CoA hydratase gene from *Ps. aeruginosa*. Cultivation of this strain on plant oils resulted in copolymer yield and 3HHx molar fraction reaching 74% of DCW and 20–40 mol%, respectively.[34] On crude palm kernel oil and soybean oil (2.5 g L^{-1}), the 3HHx molar fraction reached 55 to 70 mol% and polymer yields 45–48% of DCW, and the 3HHx fraction did not decrease in the steady-state phase of the culture, as is usually the case, when the total polymer yield is the highest.[13] Insomphun *et al.* studied the influence of deletion of some genes (fadB′, fadB1 and fadB2) on synthesis of PHA copolymers by recombinant strain *R. eutropha* H16 and showed that fadB1 deletion was effective for increasing the 3HHx molar fraction in the copolymer, with the total PHA production being rather high (65.7% of DCW).[35] The 3HHx fraction reached 23.6 mol.% in the early phase of the culture, but then decreased to 15.7 mol%.

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

Based on results of these experimental studies, bacterial cells were cultivated under different conditions of sodium hexanoate and sodium acrylate supply. We studied the effects of the sodium hexanoate and sodium acrylate supplementation schedules on bacterial biomass production, copolymer content, and the 3HHx molar fraction of the copolymer. We found that intracellular polymer content and the 3HHx molar fraction of the copolymer reached their peaks at different times, and that the combined inhibitory effect of sodium hexanoate and sodium acrylate on *C. eutrophus* B10646 cell growth was stronger than the separate effects of these substrates. Synthesis of P(3HB-co-3HHx) copolymers with 45–68 mol% 3HHx was achieved by using scheduled portion-wise supply of sodium hexanoate and sodium acrylate; the total P(3HB-co-3HHx) content varied from 25 to 54% of DCW, depending on the number of supplementations and the duration of the cultivation following supplementations. Thus, the parameters of P(3HB-co-3HHx) synthesis by the wild-type strain *C. eutrophus* B10646 are comparable with the best results reported in the literature,[13] which were obtained by using the recombinant strain *Cupriavidus necator* Re2160/pCB113 with the cloned PHA synthase gene of *R. aetherivorans* and an enol-CoA hydratase gene from *Ps. aeruginosa*.

The study of the properties of purified P(3HB-co-3HHx) samples with different monomer fractions showed that the molar fraction of 3HHx influenced physicochemical properties of the polymers. The molecular weight and temperature parameters of P(3HB-co-3HHx) decreased as the molar fraction of 3HHx increased, and this result is consistent with the data reported by other authors.[13,34] The molar fraction of 3HHx had the strongest influence on the degree of crystallinity of copolymer samples, decreasing the C_x to 20%, or more than threefold, when the 3HHx molar fraction of the copolymer increased from 10 to 68 mol%.

The composition of P(3HB-co-3HHx) and the 3HHx molar fraction have a considerable effect on mechanical properties of P(3HB-co-3HHx) films. As the 3HHx molar fraction grows, the elasticity of P(3HB-co-3HHx) films is increased considerably, as indicated by elongation to break, which increases from several per cent to several hundred per cent, while mechanical strength of the films decreases to a similar extent, as indicated by tensile strength and Young's modulus. The trends of elasticity increase and mechanical strength decrease with the increase in the molar

fraction of 3HHx in the P(3HB-co-3HHx) copolymers, consistent with the data reported by other authors,[5,9,13,34] but the values of elongation to break of P(3HB-co-3HHx) films are different. The available data on elongation to break are very contradictory. Alata *et al.* reported elongation to break of the copolymers with 12 mol% 3HHx to be equal to 579.5%.[36] However, in the study by Zhao and Chen, copolymers with the same molar fraction of 3HHx had much lower elongation to break – 107.7%.[37] Moreover, Asrar *et al.* reported elongation to break of merely 25–60% for samples that contained 8–13 mol% 3HHx.[38] Chen *et al.* studied copolymers with similar molar fractions of 3HHx (9.5 and 10%), but their elongation to break differed almost tenfold: 43% and 400%, respectively.[39] In a study by Wong *et al.* no clear relationship was found between molar fractions of 3HHx and elongation to break. Films with 32 mol% 3HHx had elongation to break of 856%, while elongation to break of a sample with 56 mol% 3HHx was only 368%.[13] It is difficult to interpret these differences, as most of the authors did not describe the conditions of producing P(3HB-co-3HHx) films (whether they were produced from the solution or melt), the thickness of the specimens tested, and their shape and size. The physical and mechanical properties of the P(3HB-co-3HHx) films reported in this study only characterize the thin films (0.03 mm) prepared by the solvent evaporation technique.

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

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Supporting Information

Supporting information may be found in the online version of this article.

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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 (^oC)	T_d (^oC)	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 to 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