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Biosynthesis of Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) from Different 4-Hydroxybutyrate Precursors by New Wild-Type Strain *Cupriavidus necator* IBP/SFU-1

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Abstract: The study addresses the growth of the new wild-type strain *Cupriavidus necator* IBP/SFU-1 and the synthesis of poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) (P(3HB-*co*-4HB) on media containing fructose and three different precursors of 4HB (ε-caprolactone 1,4-butanediol and 1,6-hexanediol). It was found that ε-caprolactone is the best precursor for the synthesis of P(3HB-*co*-4HB) copolymers. By varying the concentration and number of doses of ε-caprolactone added into the bacterial culture, it was possible to find conditions that ensured the synthesis of P(3HB-*co*-4HB) copolymers with different contents of 4HB (from 3–5 to 22.4 mol.%). The physicochemical properties of the copolymers were investigated depending on the proportions of 4HB monomers. The effect of 4HB monomers was manifested in a certain decrease in the weight-average molecular weight (M_w) (272–353 kDa), number-average molecular weight (M_n) (47–67 kDa) of the samples, and an increase in polydispersity (5.09–6.71) compared with P(3HB). The crystallinity degree decreased with an increasing fraction of the 4HB units (from 72 to 59%, as the 4HB content increased from 0 to 22.4 mol.%). In addition, the increase in 4HB content affected the temperature parameters (melting point, glass transition temperature, crystallization temperature, and thermal degradation temperature).

Keywords: *Cupriavidus necator* IBP/SFU-1; polyhydroxyalkanoates; 4-hydroxybutyrate; ε-caprolactone; 1,4-butanediol; 1,6-hexanediol; properties

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1. Introduction

Polyhydroxyalkanoates (PHA) are polyesters of hydroxyalkanoic acids synthesized by microorganisms belonging to various taxa. These polymers are synthesized in the cell as a reserve source of carbon and energy in response to stress or nutrient depletion, such as nitrogen or phosphorus, and oxygen with an excess of a carbon source [1–3]. The uniqueness of polyhydroxyalkanoates is due to their properties, primarily biocompatibility and biodegradation. PHAs are biodegradable, having similar properties to synthetic plastics that make them a potential sustainable replacement for petroleum-based plastics [4]. P(3HB) is the most studied member of the PHA family; however, the high degree of crystallinity and melting point, and the brittleness caused by secondary crystallization processes, significantly limit its scope of application [5,6]. The preparation of copolymers containing monomers other than 3-hydroxybutyrate, such as 4-hydroxybutyrate, 3-hydroxyvalerate,

and 3-hydroxyhexanoate makes it possible to overcome the undesirable properties of the P(3HB) homopolymer [7,8].

Poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), P(3HB-*co*-4HB) is considered as one of the most popular polyhydroxyalkanoates that has a significant application potential, especially in the biomedical and pharmaceutical fields [9–11]. This is, firstly, due to the fact that PHAs have absolute biocompatibility, since butyric acid is a natural metabolite of living organisms of various levels of organization. Secondly, it is known that P(3HB-*co*-4HB) copolymers are known to degrade in vivo at the highest rates compared to other types of polymers of this family, which excludes the formation of rough fibrous capsules that occur during the implantation of P(3HB) products [12]. In addition, the different contents of 4HB monomers in the P(3HB-*co*-4HB) copolymers makes it possible to obtain materials with a wide variability in terms of physicochemical and mechanical properties [13–15]. These copolymers have reduced values in the degree of crystallinity [16], and products made from them are characterized by the highest elasticity (the elongation at break exceeds the values in orders of magnitude higher than the values of other PHAs, for example, the tensile strength of P(3HB-*co*-4HB) containing 64–100 mol.% 4HB increased from 17 to 104 MPa as the proportion of 4HB increased [17]). The temperature characteristics and other mechanical properties of P(3HB-*co*-4HB) also directly depend on the content of 4HB in the copolymer and can vary over a wide range [17–19]. Furthermore, it is known that 4HB-containing copolymers can undergo hydrolysis not only with the participation of P(3HB) depolymerases but also with lipases [18] which, in fact, accelerates the cleavage of such polymers by both simple and enzymatic hydrolysis [20]. This has caused heightened interest and a large number of publications on the study of P(3HB-*co*-4HB) copolymer synthesis.

It is known that some microorganisms can synthesize 4HB monomers to form a P(3HB-*co*-4HB) copolymer. The incorporation of monomers of this type into PHA was first shown in a culture of *Alcaligenes eutrophus* bacteria grown on 4-hydroxybutyric acid as the only carbon source [21]. Later, the ability to synthesize P(3HB-*co*-4HB) copolymers was shown for *Alcaligenes latus* [22], *Comamonas acidovorans* [18], *Comamonas testosteronii* [23], *Hydrogenophaga pseudoflava* [24], *Burkholderia sacchari* [25,26], *Aneurinibacillus* sp. [27]. Recombinant *E. coli* strains capable of accumulating P(3HB-*co*-4HB) with the content of 4HB monomers of 2.8–18.4 mol.% were constructed [28–31], as well as cyanobacteria *Synechococcus* sp. PCC 7002 [32] and halophilic microorganisms *Halomonas bluephagenesis* [33]. Two approaches based on the carbon sources were used to synthesize P(3HB-*co*-4HB) copolymers [14]. In the first case, a mixture of two C-substrates was used where one of them acted as the main substrate (sugars, fatty acids, oils, glycerol, etc.), and the second was a structurally related precursor for the synthesis of 4HB monomers [34–37]. The second approach involved the use of structurally related substrates as the sole carbon source [15,18,38,39] or their combination [40–42]. It has also been reported that recombinant *E. coli* harboring the genes for the PHA biosynthesis from *C. necator* (*phaA*, *phaB* and *phaC*) and the succinate degradation genes from *Clostridium kluyveri* (*sucD*, *4hbD* and *orfZ*) was able to synthesize P(3HB-*co*-4HB) when it was grown on glucose as the sole C-substrate [28].

An analysis of publications indicates that among the known P(3HB-*co*-4HB) producers, the various PHA-producing strains belonging to the *Cupriavidus* taxon are the most actively studied, since these microorganisms have a strong intracellular system for the synthesis of these valuable macromolecules, including various C-substrates, and also have the ability to synthesize PHA of varied chemical composition with a different set and ratio of monomers. A representative review [43] summarizes a huge array of data on the patterns of synthesis and properties of P(3HB-*co*-4HB) copolymers obtained in cultures of various producer strains. As a precursor for the synthesis of 4HB-containing copolymers, γ-butyrolactone was mainly used [13,38,44,45] and sporadically salts of 4-hydroxybutyric acid [14,46] mostly by wild-type strains of representatives of the genus *Cupriavidus*, and other species, for example, *Burkholderia sacchari*, *Halomonas bluephagenesis*, *Bacillus cereus*, etc., with polymer yields in a wide range, including with a 4HB fraction from 1.6 to 99.0 mol.% [43]. In addition to wild-type PHA producers, to increase the content of 4HB monomers in copolymers,

genetically modified microorganisms were obtained, including the construction of strains synthesizing 100% P(4HB) [47,48]. Our team [45] also showed good results when using γ-butyrolactone in the culture of the wild-type strain of *Cupriavidus necator* B-10646 with 4HB content of 10.4–75.0 mol.%. Currently, there are data on the metabolic pathways for the inclusion of such precursors, although the exact mechanisms of this process have not yet been established. It is believed that structurally related carbon sources are transformed to 4-hydroxybutyrate, which is further transformed to hydroxybutyryl-CoA, which can already be included in the PHA chain with the participation of PHA synthase [43].

In addition to the above, to synthesize P(3HB-*co*-4HB) copolymers, alkanediols with different chain lengths (1,4-butanediol, 1,6-hexanediol, 1,8-octanediol, 1,10-decanediol, 1,12-dodecanediol) have also been used as precursors [15,37,38]. Regarding the metabolic pathways of this group of precursors, it is known that alkanediols are oxidized at the first stage to the corresponding ω-hydroxy fatty acid, after which they are transformed to the coenzyme A thioester and undergo β-oxidation to 4HB-CoA, which can be directly polymerized by PHA synthase [49]. Substrates 1,4-butanediol and 1,6-hexanediol are more accessible and cheaper than 4-hydroxybutyric acid or γ-butyrolactone. It makes it possible to consider them as promising substrates for the synthesis of 4HB-containing copolymers. Data on producer strains, production parameters, composition, and properties of P(3HB-co-4HB) copolymers obtained using those co-substrates are discussed less widely. Despite the large number of papers devoted to studying those precursors of 4HB-containing polymers, data on temperature properties and degree of crystallinity of such copolymers are presented fragmentarily. We have recently shown ε-caprolactone as another substrate for the synthesis of 4HB monomers in the culture of the well-described strain *C. necator* B-10646 [50–52]. It has been shown that this precursor can be very promising for the synthesis of P(3HB-*co*-4HB) copolymers, but this issue has not been considered in detail.

Based on the literature data, achieving a high content of 4HB is mostly quite problematic and is accompanied by a decrease in productivity indicators due to the toxic effects of the co-substrates used: only a few studies show a bacterial biomass yield of more than 5.0 g/L with a total copolymer content of more than 60%. For the efficient biosynthesis of P(3HB-*co*-4HB) copolymers, it is extremely important not only to achieve a high content of 4HB monomers but also to maintain high levels of both the biomass concentration and the polymer content minimizing the inhibition effects of the precursors. This makes it extremely urgent to search for new producers with a strong metabolic system of PHA synthesis, which, for example, representatives of the genus *Cupriavidus* have, and precursor substrates that are less toxic and provide greater inclusion of target monomers.

Based on the complex task of the need to search for new strains and precursors, this work is devoted to the study of the conditions, patterns of synthesis, and properties of P(3HB-*co*-4HB) copolymers with different ratios of monomers from the new wild-type strain *Cupriavidus necator* IBP/SFU-1 while using caprolactone as a precursor, which has not been previously studied in detail, in comparison with the previously described 1,4-butanediol and 1,6-hexanediol.

2. Materials and Methods

2.1. Microorganisms

Recently isolated from the soil (Krasnoyarsk, Russia), a highly productive wild-type strain of *Cupriavidus necator* IBP/SFU-1 was used in all experiments of this study. Isolation, identification and its characterization were carried out as described in [53].

2.2. Culture Medium and Cultivation Conditions

Schlegel's mineral medium was employed for the cultivation of stain and contained the following components (g/L): Na_2HPO_4 (9.1), KH_2PO_4 (1.5), $MgSO_4$ (0.2), $C_6H_5O_7Fe$ (0.025) along with a trace element solution (3 mL/L medium), which had the following composition (g/L) H_3BO_3 (0.288), $CoCl_2$ (0.030), $CuSO_4$ (0.08), $MnCl_2$ (0.008), $ZnSO_4$ (0.176), $NaMoO_4$ (0.050), $NiCl_2$ (0.008) [54]. NH_4Cl was used in a limiting concentration

(0.4–0.6 g/L) as a nitrogen source. To prepare the inoculate, the collection culture was transferred from an agarized nutrient medium to a liquid containing 15 g/L of fructose (Panreac, Barcelona, Spain) as a carbon source. Sterilization of fructose was carried out using the membrane filtration method, the filter pore size was 0.21 μm (Opticap XL300 Millipore Express SHC filters, Merck, Darmstadt, Germany). The starting culture was grown for 24 h in 0.5 L flasks using a thermostatically controlled incubator shaker Innova 44 (New Brunswick Scientific, Edison, NJ, USA); after that, it was employed in further experiments. The cultivation processes were conducted in a periodic mode according to a technique previously developed and described for the synthesis of PHA [55]. To synthesize P(3HB-*co*-4HB) copolymers, precursors of 1,4-butanediol (Panreac, Barcelona, Spain), 1,6-hexanediol (Acros Organics, Geel, Belgium), and ε-caprolactone (Geyer GmbH & Co., Renningen, Germany) were added to the cell culture at different concentrations. To trace the residual fructose concentration in the medium, the resorcinol method [56] was applied. The polymer content in bacterial cells (% of the dry cell biomass) and the bacterial biomass concentration (X, g/L) were used as indicators of the synthesis of PHA by the producer using the various precursor substrates necessary for the synthesis of 4-hydroxybuturate (4HB) monomers. The polymer ($Y_{P/S}$, g PHA/g substrate) and 4HB monomer yields ($Y_{4HB/precursor}$) were calculated.

2.3. PHA Recovery from Cell Biomass

To extract the polymer, bacterial cells were separated from the nutrient medium in an Avanti J-HC centrifuge (centrifugation for 10 min at 6000 rpm, BeckmanCoulter, Indianapolis, IN, USA), washed and lyophilized (LP10R freezing, drying, ilShinBioBase, Dongducheon, Republic of Korea). To extract PHA from the raw cell biomass, fatty acids and lipids were first removed by ethanol treatment, then the polymer extraction process was carried out using dichloromethane, after which the solvent was evaporated (rotary evaporator P/210 In Buchi, Flaville, Switzerland) and the polymer was precipitated with hexane. To purify the obtained samples, the polymers were dissolved several times in chloroform, again precipitated and dried at 40 °C [57].

2.4. PHA Chemical Composition

The gas chromatography method (7890A chromatograph-mass spectrometer equipped with a 5975C mess detector, Agilent Technologies, Santa Clara, CA, USA) was used to analyze the intracellular content and composition of synthesized PHA samples. For this purpose, the procedure of methanolysis of cellular biomass, as well as isolated and purified polymer samples, was carried out, after which the obtained methyl esters of fatty acids were identified by retention times and mass spectra [58].

2.5. Physicochemical Properties of PHAs

In this study, standard methods described in detail in [59] were used to evaluate the physicochemical properties of synthesized PHA samples.

To determine the molecular weight characteristics of synthesized PHA samples, the size-exclusion chromatography method (Agilent Technologies 1260 Infinity, Waldbronn, Germany, DB-35MS column) was used. Parameters such as the average molecular weight (M_w), the average molecular weight (M_n) and polydispersity ($Đ = M_w/M_n$) were evaluated. Chloroform was used as an eluent, the flow rate of which was 1.0 mL/min. The concentration of the injected sample was 5.0 mg/mL, and its volume was 50.0 μL. Polystyrene standards (Agilent Technologies, Santa Clara, CA, USA) were used for the calibration procedure.

In order to determine the thermal properties of the obtained PHAs, the method of thermal analysis used involved a differential scanning calorimeter DSC-1 (Mettler Toledo, Schwerzenbac, Switzerland) and TGA (Mettler Toledo, Schwerzenbac, Switzerland). The obtained thermograms were analyzed using the STARe v11.0 software (Mettler Toledo, Schwerzenbac, Switzerland), on the basis of which, parameters such as glass transition

temperature (T_g), melting point (T_{melt}) and thermal degradation temperature (T_{degr}) were determined by endothermic peaks, and crystallization temperature (T_c) was determined by exothermic ones [53].

The crystallinity of the synthesized samples was determined using X-ray diffraction analysis (D8 ADVANCE X-Ray powder diffractometer a VANTEC fast linear detector (Bruker AXS, Karlsruhe, Germany).

2.6. Statistics

To determine the statistical reliability of the obtained data, the Student's *t*-test (significance level: $p \leq 0.05$) was used. All calculations, including the evaluation of standard deviations and arithmetic means, were carried out using Microsoft Excel software (Version 2109). All experiments in this study were performed three times.

3. Results

The synthesis of P(3HB-*co*-4HB) copolymers with a high mole fraction of 4HB monomers, while maintaining high bacterial biomass and total PHA yields, is a complex biotechnological task. As a rule, to produce copolymers, wild-type strains require the use of additional substrates (precursors)—carbon sources structurally related to the target monomers. Most of those precursors, even in low concentrations, are toxic, and have an inhibitory effect on bacterial growth, and reduce the accumulation of PHAs. For bacteria belonging to the *Cupriavidus* taxon, the ability to grow on 4HB precursors, such as 4-hydroxybutyric acid, γ-butyrolactone, 1,4-butanediol, 1,6-hexanediol, etc., alkanediols, as the sole carbon source, have been shown by many authors [18,36,37,60], but in most of those cases, high mole fractions of 4HB are accompanied by a decrease in the biomass concentration and polymer content. The use of sugars, fatty acids, and vegetable oils as the main carbon sources in combination with co-substrates makes it possible to increase the production parameters of the culture, but results in reducing the content of 4HB in the P(3HB-*co*-4HB) copolymers. Therefore, for a specific strain producer of these copolymers, it is extremely important to select co-substrates and modes of their use in order to obtain PHA of a certain composition and maintain a high level of production indicators of the bacterial culture.

In this paper, the conditions and regularities of the synthesis of the P(3HB-*co*-4HB) copolymers, as well as their composition and properties, in the culture of a new strain-producer *C. necator* IBP/SFU-1, when using ε-caprolactone, as well as 1,4-butanediol and 1,6-hexanediol, not previously studied in detail, as precursors of the synthesis of 4HB monomers. The strain under study was obtained recently; its growth and accumulation of PHA was characterized on a number of carbon substrates [53], but its ability to synthesize copolymer PHA is discussed here for the first time.

3.1. Effect of ε-Caprolactone as a 4HB Precursor on the Biomass Concentration, Content and Composition of the Polymer Obtained in the Culture of the New Strain Cupriavidus necator IBP/SFU-1

The results of the study of the P(3HB-*co*-4HB) copolymers synthesis using ε-caprolactone, which was introduced into the medium at the beginning of cultivation at concentrations of 1.0–5.0 g/L, are presented in Figure 1. We have previously shown the possibility of the synthesis of copolymers with 4HB when ε-caprolactone was used as a precursor of 4HB when wild-type strain *Cupriavidus necator* B-10646 was grown on glycerol, molasses and *Jerusalem artichoke* hydrolysate [50–52]. No information about the use of ε-caprolactone as a precursor substrate for the synthesis of the P(3HB-*co*-4HB) copolymer was found in the papers of other authors in the available literature.

The biomass concentration, PHA and 4HB content obtained in 24 h cultivation, as well as the dynamics of substrate consumption by the strain *Cupriavidus necator* IBP/SFU-1 are shown in Figure 1.

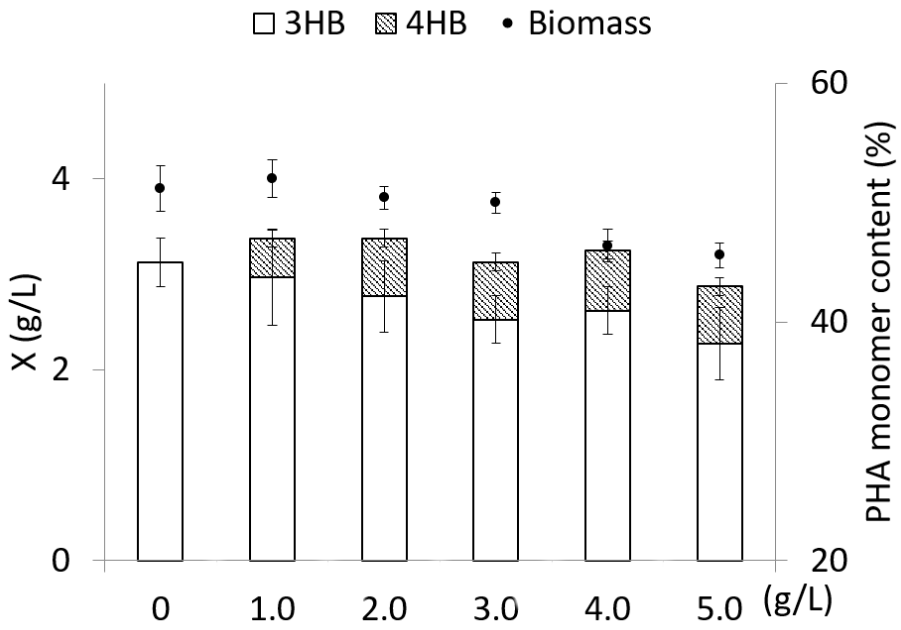

Figure 1. Biomass concentration of *Cupriavidus necator* IBP/SFU-1, content and composition of PHA in experiments with fructose as the main C-substrate and addition of ε-caprolactone at various concentrations supplemented into medium at 0 h of cultivation (incubated for 24 h).

The analysis of the residual concentration of ε-caprolactone in the medium showed that when ε-caprolactone was used at concentrations of 1.0, 2.0 and 3.0 g/L, at the end of cultivation (24 h), its residual concentration was 0.45, 0.75 and 1.50 g/L, respectively. ε-Caprolactone added into the medium at a concentration of 4.0–5.0 g/L was utilized less efficiently (the residual concentrations of it were 2.80 and 3.80 g/L, respectively).

A single addition of ε-caprolactone to the bacterial culture led to a gradual and weakly expressed decrease in the biomass concentration from 4.0–3.7 to 3.3–3.2 g/L when the concentration of ε-caprolactone was 1.0–3.0 and 4.0–5.0 g/L, respectively. The addition of ε-caprolactone did not have a negative effect on the synthesis of the polymer by the strain: 43–47% of PHA was obtained during 24 h of cultivation, which was not inferior to the control value obtained in the experiment without ε-caprolactone. In addition, in this study, the use of this substrate turned out to be more effective in comparison with the similar addition of 1,4-butanediol and 1,6-hexanediol. With an increase in the concentration of ε-caprolactone from 0.5 to 5.0 g/L, the content of 4HB also increased from 7.0 to 11.2 mol.%.

In order to increase the content of 4HB monomers in the composition of the copolymer P(3HB-*co*-4HB) and avoid the effect of inhibiting the growth of the culture and the accumulation of PHA observed when using high concentrations of precursor, the fractional supply of ε-caprolactone to the culture of *C. necator* IBP/SFU-1 was studied. The experiments included fractional four-fold additions of the co-substrate into the bacterial culture in single concentrations of 1.0–2.0 g/L (Figure 2). In the first experiment (Figure 2a), the precursor substrate was introduced into the medium at a concentration of 1 g/L for 24, 32, 48 h of cultivation (a total of 3.0 g/L of the precursor was introduced into the culture). In the second experiment (Figure 2b), ε-caprolactone was introduced according to the following scheme: 2.0 g/L for 24 and 48 h of culture growth (4.0 g/L of co-substrate was added in total). In the third case (Figure 2c), four additives of 1.0 g/L were added for 24, 32, 48 and 56 h of culture growth (4.0 g/L of ε-caprolactone was added in total).

The biomass concentration of *C. necator* IBP/SFU-1 registered for 72 h of culture growth, with an increase in the total concentration of ε-caprolactone in the medium, from 3.0 to 4.0 g/L, with single additives of 1 g/L co-substrate, was comparable and the difference was statistically nonsignificant ($p < 0.05$) (7.3 and 7.5 g/L). The biomass concentration in the second experiment, where 2.0 g/L was added for 24 and 48 h of culture

growth, was significantly ($p < 0.05$) lower 7.1 g/L. The fractional feed of the co-substrate also made it possible to achieve sufficiently high intracellular polymer content (71–74%) regardless of the total amount of the co-substrate additives.

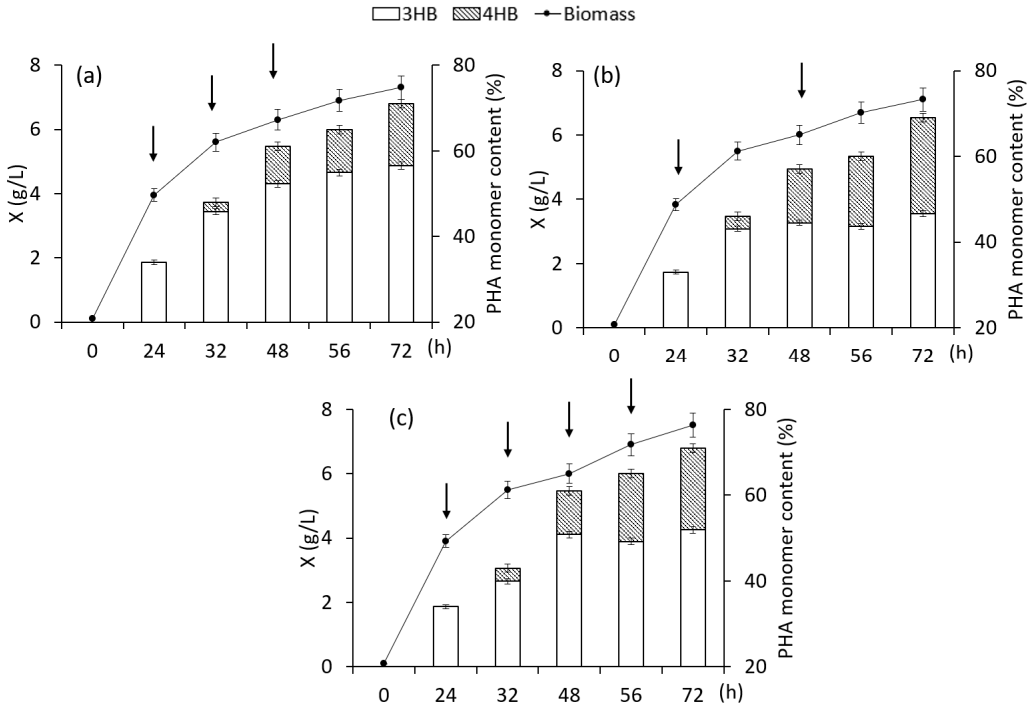

Figure 2. Biomass concentration of bacteria *Cupriavidus necator* IBP/SFU-1, content and composition of PHA in experiments with fructose as the main C-substrate and addition of ε-caprolactone at different concentrations: (**a**) 1.0 + 1.0 + 1.0; (**b**) 2.0 + 2.0; (**c**) 1.0 + 1.0 + 1.0 + 1.0 g/L (the time of supplementation is shown by arrows).

When ε-caprolactone was added fractionally, it was shown that after 32 h of bacterial growth (8 h after the first supply of ε-caprolactone), a low content of 4HB (2.23–3.10 mol.%) was recorded. The fractional application of the co-substrate made it possible to obtain the P(3HB-*co*-4HB) copolymer with sufficiently high mole fractions of 4HB from 14.4 to 22.4 mol.%. The maximum content of these monomers was revealed in an experiment with a four-fold supply of ε-caprolactone of 1 g/L.

3.2. Effect of 1,4-Butanediol as a Precursor of 4HB on the Biomass Concentration, Content and Composition of the Polymer Obtained in the Culture of the New Strain Cupriavidus necator IBP/SFU-1

The second precursor substrate for the synthesis of 4HB-containing copolymers from the new strain of *C. necator* IBP/SFU-1 was 1,4-butanediol, which was introduced into the medium at the beginning of cultivation at concentrations from 0.5 to 5.0 g/L (Figure 3).

C. necator IBP/SFU-1 was grown in a medium containing fructose as the main C-substrate, and 1,4-butanediol as the precursor substrate in concentrations from 0.5 to 5.0 g/L. The addition of 1,4-butanediol in concentrations up to 1.5 g/L did not have a clear inhibitory effect on the growth of *C. necator* IBP/SFU-1 culture. The bacterial biomass yield was not inferior to the control values obtained in the experiment without the addition of 1,4-butanediol (3.9 g/L), and amounted to 3.8–4.0 g/L. The intracellular PHA content was also comparable with the control value (45.0%) and amounted to 39.0–43.6%. An increase in the concentration of 1,4-butanediol to 2–5 g/L had a noticeable inhibitory effect on both productivity measures: the biomass concentration reached only 2.6–3.4 g/L with an intracellular polymer content of 28.7–32.3%. Measurement of the concentration of 1,4-butanediol at the end of cultivation showed a decrease in the concentration of the precursor by 0.3–0.6 g/L from the initial values. The low utilization degree of the precursor was also confirmed by the poor values of the 4HB

content in the polymer. When 1,4-butanediol was added in concentrations from 0.5 to 1.0 g/L, the content of 4HB monomers in the copolymer was almost the same and amounted to only 0.9 mol.%. An increase in the precursor concentration to 1.0 g/L contributed to an increase in the 4HB content (up to 2.5 mol.%) in the copolymer. The presence of the co-substrate in the medium at concentrations of 2.0–5.0 g/L did not lead to a significant increase in the content of 4HB in P(3HB-*co*-4HB) copolymer; its fraction did not exceed 4.0–4.5 mol.%.

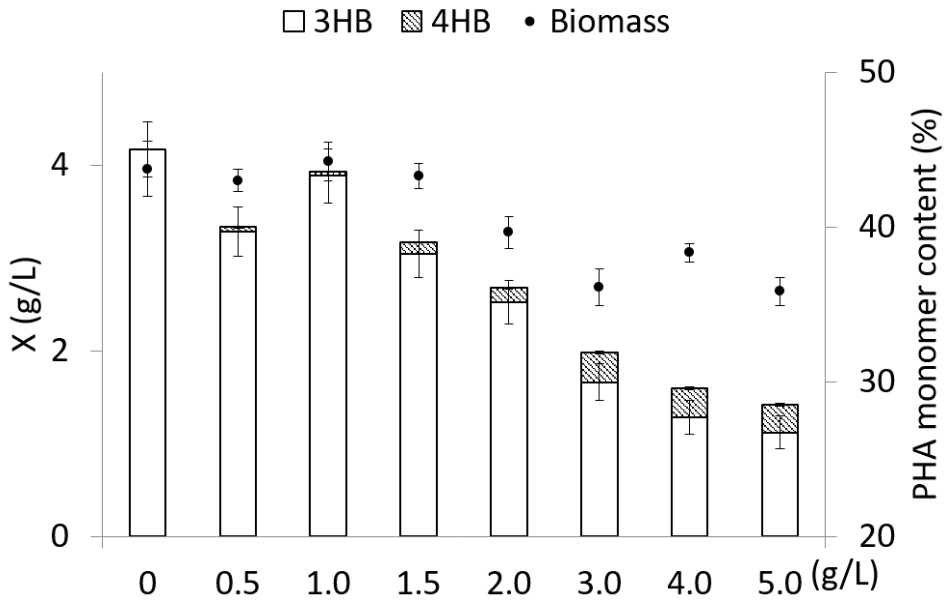

Figure 3. Biomass concentration of *Cupriavidus necator* IBP/SFU-1, content and composition of PHA in experiments with fructose as the main C-substrate and addition of 1,4-butanediol at various concentrations supplemented into medium at 0 h of cultivation (incubated for 24 h).

The mode of fractional dosing of 1,4-butanediol (similar to ε-caprolactone) did not lead to an increase in the 4HB content in the copolymer.

According to the literature data [14,15,34,36–38,46,61], 1,4-butanediol is often considered as a promising precursor substrate for the synthesis of copolymers containing 4HB monomers. It is reported that bacteria belonging to the *Cupriavidus* taxon are able to efficiently metabolize 1,4-butanediol as a single substrate [15,37,46] or as a co-substrate [34,36,37]. Thus, in Huang et al. [37], it is reported that 1,4-butanediol can be used by the *Cupriavidus* sp. USMAA1020 as the sole carbon source: up to 5.4 g/L of bacterial biomass with a total copolymer yield of up to 37% was obtained during 48 h of cultivation; the mole fraction of 4HB was estimated as 39 mol.%. The use of oleic acid as the main C-substrate allowed the authors to increase the productivity of the strain, and up to 8.8 g/L of bacterial biomass was obtained for 48 h with a copolymer content of up to 69% containing a fraction of 4HB monomers up to 14 mol.%. With respect to the use of 1,4-butanediol as a sole substrate, it was shown that the biomass concentration of *C. necator* A-04 grown in a medium containing 20 g/L 1,4-butanediol as the sole carbon source reached up to 4.35 g/L; polymer and 4HB content was 43% and 12 mol.%, respectively. It was also noted that the fraction of the 4HB monomer in P(3HB-*co*-4HB) copolymer rose in proportion to the increase in the content of 1,4-butanediol in the nutrient medium [15]; however, a similar effect was not found in the present study.

In general, the results obtained in the present study in terms of culture productivity are comparable to those described in the literature or exceed them, especially considering the duration of the process of cultivation (24 h). For example, in the paper [36], the use of sugars (glucose and fructose) as the main C-substrate in the culture of *Cupriavidus* sp. USMAAHM13 made it possible to achieve a concentration of bacterial biomass after 72 h of up to 4.5 and 6.0 g/L, respectively. The polymer content and the content of 4HB monomers were 25–32% PHA and 12–22 mol.%, respectively.

Regarding strains belonging to genera other than *Cupriavidus*, lower yields of bacterial biomass and PHA accumulation were obtained, but a higher content of 4HB monomers. So, Lee et al. reported that this substrate was extensively investigated as a precursor to the synthesis of P(3HB-*co*-4HB) copolymers in the culture of *Comamonas acidovorans* [14]. When 1,4-butanediol was used as the sole carbon source, the copolymer content was quite low: by 48 h of cultivation process, 2.3 g/L of bacterial biomass containing 25% of PHA was obtained; the content of 4HB monomers was estimated as 84 mol.%. Various combinations of glucose and 1,4-butanediol did not lead to a higher concentration of bacterial biomass (up to 2.5 g/L was obtained), although the total copolymer content increased to 53%; nevertheless, the authors showed that an increase in the concentration of 1,4-butanediol contributed to an increase in content of 4HB monomers, from 28 to 84 mol.%.

3.3. Effect of 1,6-Hexanediol as a 4HB Precursor on the Biomass Concentration, Content and Composition of the Polymer Obtained in the Culture of the New Strain Cupriavidus necator IBP/SFU-1

The compound 1,6-hexanediol has been investigated as a third precursor for the synthesis of the P(3HB-*co*-4HB) copolymer. The biomass concentration of *C. necator* IBP/SFU-1, the content and composition of PHA are shown in Figure 4.

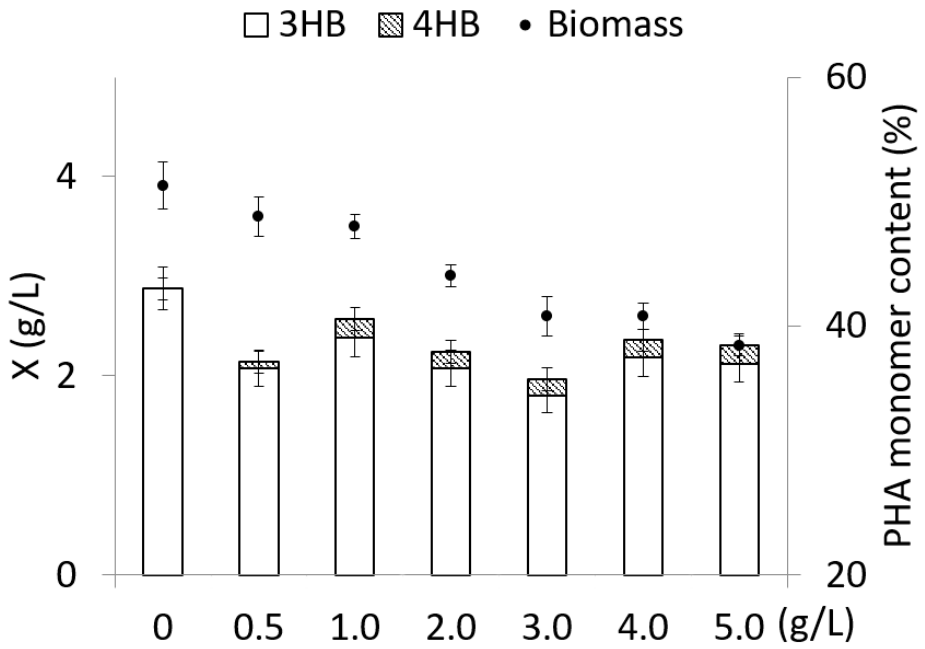

Figure 4. Biomass concentration of *Cupriavidus necator* IBP/SFU-1, content and composition of PHA in experiments with fructose as the main C-substrate and addition of 1,6-hexanediol at various concentrations supplemented into medium at 0 h of cultivation (incubated for 24 h).

A more notable inhibitory effect on the growth of the studied strain was shown when 1,6-hexanediol was used. The use of 1,6-hexanediol as a co-substrate led to a gradual decrease in the concentration of the bacterial biomass from 3.9 g/L to 2.3 g/L as the concentration of the precursor increased from 0 to 5.0 g/L. A similar effect of 1,6-hexanediol on polymer synthesis was recorded: the intracellular PHA content decreased from 45.0 to 28.6%.

The addition of 1,6-hexanediol to the medium led to the synthesis of P(3HB-*co*-4HB) copolymers with different contents of 4HB monomers. When the co-substrate was used at a concentration of 0.5 and 1.0 g/L, the 4HB content was 1.38 and 3.67 mol.%, respectively. The subsequent increase in the concentration of 1,6-hexanediol in the medium did not contribute to an increase in the fraction of 4HB in the copolymer. The mole fraction of 4HB in the polymer in the presence of 1,6-hexanediol at concentrations of 2.0–5.0 g/L was comparable and amounted to about 3.5–4.0 mol.%. The analysis of the residual concentration of 1,6-hexanediol showed that the utilization of this substrate was not active:

its concentration was lower than the initial value by 0.2–0.8 g/L, which also affected the inclusion of 4HB in the P(3HB-*co*-4HB) copolymer.

The mode of fractional dosing of 1,6-hexanediol (similar to ε-caprolactone) did not lead to an increase in the 4HB content in the copolymer. In addition, the fractional introduction of this precursor led to a decrease in biomass concentration and polymer content.

The use of 1,6-hexanediol as a precursor for the synthesis of 4HB monomers has been less widely studied in comparison with 1,4-butanediol. As a rule, most studies reported a sufficiently high content of 4HB monomers, up to 60 mol.%; however, the productivity indicators in those studies remained low, barely reaching 5.0 g/L of the biomass concentration and 60% of the copolymer content. Thus, Iqbal and Amirul [61] reported that 1.14–1.22 g/L of P(3HB-*co*-4HB) copolymer was obtained after 24 h, where the content of 4HB reached 60.0–62.0 mol.% when *Cupriavidus* sp. USMAA2-4 used 1,6-hexanediol as the sole carbon source. The authors also reported that when oleic acid was used as the main carbon source and 1,6-hexanediol as a co-substrate, the total copolymer content increased to 2.09 g/L, but the 4HB monomer content decreased to 20 mol.%. In the culture of the same strain, when 1,6-hexanediol was used as a single substrate, only 2.7 g/L of bacterial biomass containing 28% copolymer with an extremely high inclusion of 4HB monomer (99 mol.%) was obtained [38]. In another paper [37], *Cupriavidus* sp. USMAA1020 was grown on oleic acid and 1,6-hexanediol. The biomass concentration reached 6.8 g/L, and an intracellular copolymer content of up to 69% was obtained after 48 h; however, the 4HB content was low, up to 12 mol.%. The authors also reported that in the case of using 1,6-hexanediol as the sole carbon source, with an increase in its content in the medium from 0.1 to 0.3 wt%, the concentration of biomass and polymer content increased, which was accompanied by a decrease in the fraction of 4HB from 43.0 to 36.0 mol.%; a further increase in the concentration of 1,6-hexanediol led to a decrease in productivity indicators, but the content of 4HB monomers increased to 48.0 mol.%.

The present study has shown that ε-caprolactone is an effective precursor substrate for the synthesis of 4HB monomers. Fractional feeding of this co-substrate enabled the cell biomass concentration (7.1–7.5 g/L) and the total copolymer yield (71–74% PHA intracellularly) to be preserved at sufficiently high levels, while the content of 4HB monomers reached over 20 mol.%.

$Y_{P/S}$ of 0.09–0.15 g/g were obtained from fructose as the main carbon source and ε-caprolactone, 1,4-butanediol and 1,6-hexanediol as precursor substrates (Table 1). These values correspond to 19–31% of maximum theoretical yield values of 0.48 from glucose and fructose [62].

Table 1. Comparison of the polymer yields with the maximum theoretical ones for *Cupriavidus necator* B-10646 grown on fructose as main carbon substrate and co-substrates.

Co-Substrate	Polymer Yields ($Y_{P/S}$, g PHA/g Substrate)	% of Maximum Theoretical $Y_{P/S}$
ε-caprolactone	0.13–0.15	27–31%
1,4-butanediol	0.09–0.14	19–29%
1,6-hexanediol	0.11–0.13	23–27%

When using ε-caprolactone as a precursor substrate, $Y_{4HB/\varepsilon\text{-caprolactone}}$ was 0.04–0.07 g/g. When using 1,4-butanediol and 1,6-hexanediol as precursor compounds, this indicator was close: $Y_{4HB/1,4\text{-butanediol}}$ = 0.01–0.04 g/g for 1,4-butanediol and $Y_{4HB/1,6\text{-hexanediol}}$ = 0.02–0.05 for 1,6-hexanediol, which was inferior to the results achieved using ε-caprolactone. The data obtained in this work on the use of previously unstudied ε-caprolactone exceeded the corresponding values ($Y_{P/S}$ = 0.09–0.10 g/g) achieved in the *C. necator* DSM 545 culture using glycerol and γ-butyrolactone as carbon sources [44], but were significantly inferior to those obtained in the *R. eutropha* KCTC 2662 culture grown on soybean oil and γ-butyrolactone ($Y_{P/S}$ = 0.42 g/g), which was due to a higher conversion of oils into PHA compared to sugars. Concerning the yield of 4HB, recalculated per 1 g of the precursor

used, the obtained results were not inferior to those obtained in the *B. sacchari* culture using glucose and γ-butyrolactone ($Y_{4HB/\gamma\text{-butyrolactone}}$ = 0.08 g/g) [63]. Thus, the 4HB content in the P(3HB-*co*-4HB) copolymers was from 3–5 up to 22.4 mol.%, while the copolymer content reached about 70%.

3.4. Physicochemical Properties of P(3HB-co-4HB) Copolymers

Molecular weight is one of the most important parameters characterizing the properties of polymers since it determines the technological properties of the material and the possibility of its processing. It is known that the molecular weight value is a very variable parameter depending on the physiological and biochemical characteristics of the producer strain, the conditions of carbon nutrition, and the extraction mode.

The results of the study of the molecular weight characteristics of P(3HB-*co*-4HB) copolymers are presented in Table 2. The values of M_w and M_n varied from 195 to 352 kDa and from 47 to 67 kDa, respectively, and did not exhibit a clear correlation to the content of 4HB in the copolymer while being lower than those of P(3HB). The polydispersity of the copolymers was 1.8–2.4 times higher than that of P(3HB) and amounted to 5.09–6.71. A decrease in M_w, M_n and an increase in the polydispersity of the P(3HB-*co*-4HB) copolymers compared to the P(3HB) homopolymer was also shown by other authors [26,36,39,61].

Table 2. Physicochemical properties of P(3HB-*co*-4HB) with different ratios of monomers synthesized by *Cupriavidus necator* IBP/SFU-1 using ε-caprolactone.

N	Composition of Monomers, mol.%		M_n, kDa	M_w, kDa	Đ	C_x, %	T_{melt}, °C	T_{degr}, °C	T_g, °C	T_c °C
	3HB	4HB								
1	88.7	11.3	47	292	6.21	68	171.1	124.3 271.3	–3.3	56.2 51.8
2	86.0	14.0	67	352	5.25	67	171.1	137.2 274.6	–3.4	60.4 49.8
3	84.0	16.0	58	295	5.09	64	127.5 167.1	154.1 276.7	–4.4	55.7
4	81.4	18.6	49	329	6.71	62	171.1	269.3	–4.8	65.0 49.5
5	79.4	22.4	51	272	5.33	59	170.9	134.6 271.4	–5.7	54.3
6	100	0	158	436	2.76	72	173	292	4.7	84.7

M_n—number-average molecular weight; M_w—weight-average molecular weight; Đ—polydispersity; C_x—crystallinity degree; T_{melt}—melting point; T_{degr}—thermal degradation temperature; T_g—glass transition temperature; T_c—crystallization temperature.

However, in other studies, the decrease in M_w and M_n was not accompanied by an increase in Đ for copolymers with 4HB synthesized by *Cupriavidus* sp. and *Delftia acidovorans* [15,38,64–66]. Apparently, the content of 4HB in the copolymer cannot be the main factor affecting the molecular weight of the P(3HB-*co*-4HB) copolymers, and changes in the molecular weight of the P(3HB-*co*-4HB) copolymers can be caused by various factors, one of which is the type and concentration of the carbon substrate. Thus, Saito et al. [18] and Huong et al. [37] found that *R. eutropha* grown on various 4HB precursors synthesized P(3HB-*co*-4HB) copolymers with a similar content of 4HB but their molecular weights were different. Norhafini et al., 2019 [67] showed that P(3HB-*co*-4HB) copolymers with the same 4HB content synthesized by the recombinant *Cupriavidus malaysiensis* USMAA1020 strain using different cultivation strategies were characterized by different M_w and M_n [66].

The results from the study of the effect of 4HB monomers on the degree of crystallinity of the P(3HB-*co*-4HB) copolymers are presented in Table 2. The inclusion of 4HB in the C-chain of 3HB affected the ratio of the crystalline and amorphous phases in all the studied copolymers. With an increase in the content of 4HB in the copolymer, the degree of crystallinity decreased. The minimum C_x value (59%) is shown for the copolymer with the

maximum inclusion of 4HB (22.4 mol.%). A decrease in the degree of crystallinity in copolymers with 4HB compared with P(3HB) was also shown by other authors [18,68,69]. Thus, Svafiq et al. [67] showed that the crystallinity of the copolymer with 20 mol.% of 4HB was 59%, which is comparable with our data. However, in the other papers, the C_x of copolymers with a similar content of 4HB (16–18 mol.%) was significantly lower (45%) [18,68]. The lower values of the degree of crystallinity (3–7%) for copolymers with 17–20 mol.% of 4HB were shown by Ramachandran and Amirul [36] and Ye et al. [70]. Contradictory data were also found for copolymers with a high content of 4HB (more than 60 mol.%). Pospilova et al., 2022 [27] found that the crystallinity of copolymers with 66 mol.% of 4HB was 43%. Lower crystallinity values (4–15%) for copolymers with close 4HB content (64–65 mol.%) were found by Saito et al. [18] and Iqbal and Amirul [61]. Apparently, not only the composition of the polymer determines the properties of the polymer but also other factors such as the distribution of monomers in the polymer chain (block copolymers or random distribution of monomers), molecular weight, which in turn depend on the physiological and biochemical properties of the producer strain and cultivation conditions. Thus, the degree of crystallinity of P(3HB-*co*-4HB) samples with a close inclusion of 4HB (92–99 mol.%) synthesized by the recombinant strain *Cupriavidus malaysiensis* USMAA1020 was 40–63% depending on the cultivation strategy [67].

The temperature characteristics of copolymers are given in Table 2 and Figure 5. The synthesized copolymers are most likely a mixture of highly crystalline P(3HB) and low crystalline P(3HB-*co*-4HB), except for the sample of the copolymer P(3HB-*co*-4HB) with the 16 mol.% of 4HB which is possibly a mixture of P(3HB-*co*-4HB) copolymers with various contents of 4HB monomers. This is evidenced by the high melting point (171 °C) which was recorded for copolymers with 11.3, 14.0, 18.6 and 22.4 mol.% of 4HB content and single melting peaks on the thermograms, which may indicate the prevalence of the crystalline phase of highly crystalline P(3HB) over the P(3HB-*co*-4HB). For a sample with an inclusion of 16 mol.% of 4HB, the presence of two melting peaks and a significant temperature decrease to 127.5 and 167.1 °C, respectively, was shown on the thermograms. It may indicate the prevalence of the crystalline phase of the P(3HB-*co*-4HB) copolymer. The presence of two melting peaks for P(3HB-*co*-4HB) copolymers was also shown in other papers [36,39]. The T_{melt} of P(3HB-*co*-4HB) copolymers with different contents of 4HB monomers have been studied by many authors; however, the published results are contradictory. A number of authors showed that T_{melt} varied depending on 4HB content: as 4HB content was increased from 0 to 65–83 mol.%, the T_{melt} dropped from 170–175 °C to 37–68 °C [22,64,65]. In contrast, other studies did not reveal the effect of the 4HB component at a range of 22–45 mol.% [38], 3–40 mol.% [71] and 23–66 mol.% [39] on the T_{melt} values of the copolymers.

An increase in 4HB conten leads to a decrease in the enthalpy of melting for all polymeric samples. Furthermore, an increase in 4HB content from 0 to 22.4 mol.% leads to a decrease in the glass transition temperature from 4.7 to −5.7 °C. It is known that, with a lower T_g value, the P(3HB-*co*-4HB) copolymers are tougher and more flexible than P(3HB). Thus, the polymer with a T_g value lower than room temperature exhibits a soft and flexible morphology. Other researchers have also shown a decrease in T_g with an increase in the molar fraction of 4HB [15,39,61,70]. However, the T_g values for copolymers with a similar content of 4HB can differ significantly. The T_g of the P(3HB-*co*-4HB) copolymers with 23–24 mol.% of 4HB was −5–−7 °C, as reported in studies by Chanprateep et al., and Vigneswari et al. [15,39], which is consistent with our data. However, the T_g of the P(3HB-*co*-4HB) copolymers of a similar composition was −16–−19 °C [61,70]. This may be due to the use of different strategies of bacterial cultivation for the synthesis of 3(HB-*co*-4HB) copolymers [15].

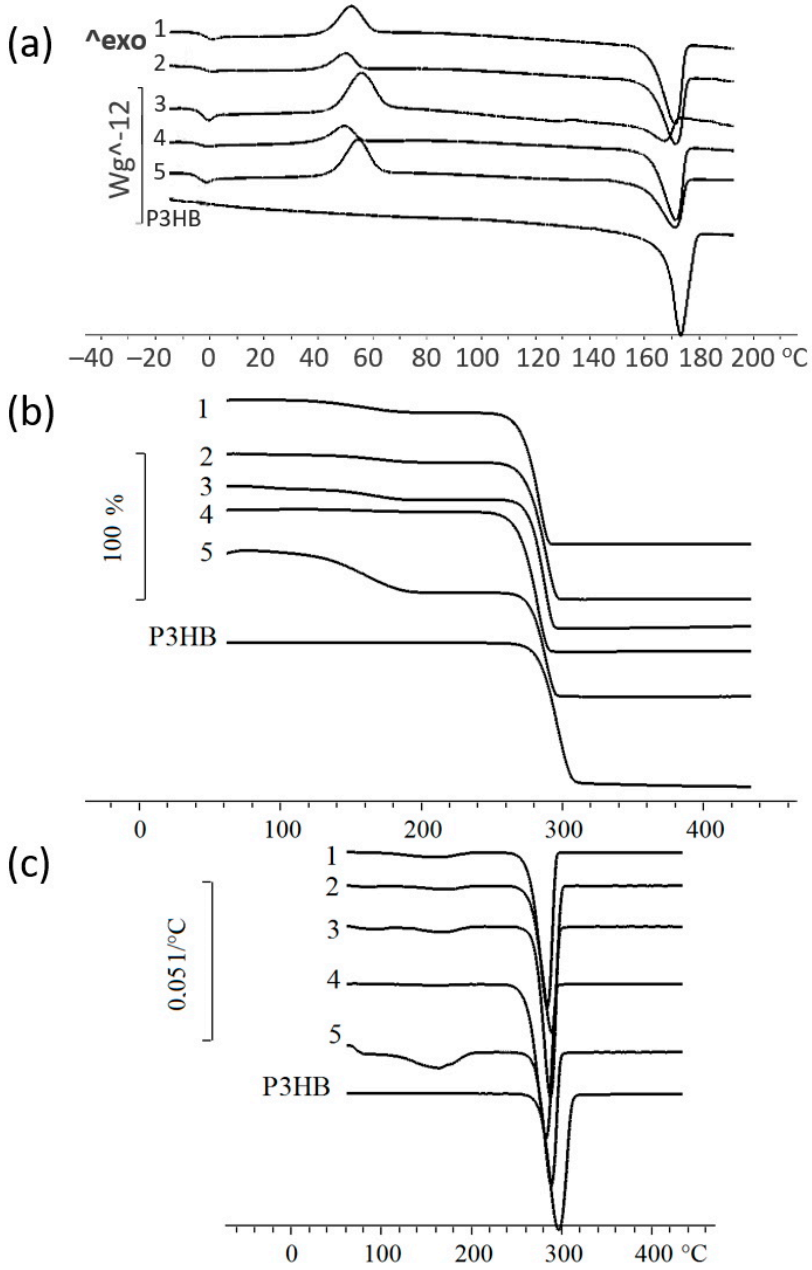

Figure 5. Temperature characteristics of P(3HB-*co*-4HB) samples with different sets of monomers: (**a**) DSC curves with glass transition temperature (T_g), crystallization temperature (T_c) and melting point (T_{melt}) regions: (**b**) crystallization temperature; (**c**) thermal stability (TGA) (the numbering indicating the composition of the copolymer is the same as Table 2).

The highest T_c (84.7 °C) was exhibited by pure P(3HB). The T_c of the P(3HB-*co*-4HB) copolymers was about 49.5–65.0 °C. A decrease in the T_c with an increase in 4HB content was also noted by Chanprateep et al. [15]. The lower T_c of the copolymers compared to P(3HB) suggests that the copolymers remain highly ductile for longer time periods and, thus, are more readily processable. Samples with 4HB monomer contents of 11.3, 14.0, and 18.6% showed two crystallization peaks: one peak was upon cooling and the other peak was upon reheating. Samples with 4HB contents of 16.0 and 22.4 mol.% did not crystallize upon cooling. Their crystallization occurred upon subsequent heating. This is explained by a decrease in the mobility of molecules with an increase in 4HB content, which leads to vitrification of a part of the sample upon cooling; however, upon reheating, the energy of the system increases and the mobility of the molecules is restored, which leads to secondary

crystallization, as in the case of the samples containing 11.3, 14.0, and 18.6 mol.% of 4HB, or primary crystallization as in samples with 4HB inclusions of 16.0 and 22.4 mol.%.

The results obtained are in good agreement with the paper of Jo et al., 2022 [72]. In that paper, the thermal behavior and rheological properties of the P(3HB-*co*-4HB) copolymer and mixtures of P(3HB-*co*-4HB) with P(3HB) were studied. The authors also noted a decrease in the glass transition temperature with the 4HB increase, as well as double crystallization peaks associated with steric hindrances created by 4HB. The authors also noted the absence of a significant effect of 4HB content (up to 30 mol.%) on the melting point both for P(3HB-*co*-4HB) samples and for a mixture of P(3HB-*co*-4HB) with P(3HB). A decrease in the melting point was noted for the samples and mixtures with a 4HB content of more than 30 mol.%.

The thermal stability of the samples was studied by the TGA method. The highest thermal stability was demonstrated by the sample with a 4HB content of 18.6 mol.%, and degradation temperature of 269.3 °C. The other samples showed lower thermal stability; their thermograms showed two zones of mass loss upon heating. The first zone is in the temperature range of 124–154 °C and the second (main) in the range of 269–271 °C. It should be noted that the degradation of samples with the inclusion of 11.3–16.0 mol.% 4HB in the temperature range of 124–154 °C may be characterized as insignificant from 5 to 9%. A more significant weight loss (about 26.3%) was found for the sample with the inclusion of 22.4 mol.% 4HB. The data on the T_{degr} temperature of thermal degradation were given for a few samples in the available literature. In a study by Iqbal and Amirul [61], the T_{degr} of copolymers with a 4HB content from 10 to 65 mol.% practically did not differ and amounted to 290–310 °C.

4. Conclusions

The synthesis of P(3HB-co-4HB) copolymers by the new wild-type strain Cupriavidus necator IBP/SFU-1 on fructose as the main C-substrate with the addition 1,4-butanediol, 1,6-hexanediol and ε-caprolactone as precursors of the 4HB monomer was studied. It was found that ε-caprolactone was the best precursor for the synthesis of the P(3HB-co-4HB) copolymers. By varying the bacterial cultivation conditions, including the concentration and number of doses of the precursor supplemented into the bacterial culture, we studied the production parameters of the culture (bacterial biomass concentration and polymer content). The conditions that ensured the formation of 4HB monomers from the precursor and their incorporation into the C-chain of poly(3-hydroxybutyrate) were found. A set of copolymers with different contents of 4HB (from 11.3 to 22.4 mol.%) was synthesized and the physicochemical properties of the copolymers were studied. It was established that all the studied samples of P(3HB-co-4HB) had reduced values of molecular weight parameters, as well as the degree of crystallinity. An increase in the 4HB content (from 0 to 22.4 mol.%) led to a decrease in the T_g from 4.7 to −5.7 °C. In addition, the effect of the 4HB monomer units on the temperature properties of the copolymers was shown as the lowering of the T_{degr} and T_c.

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