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Structures and Properties of Polyhydroxyalkanoates Synthesized by *Methyloligella halotolerans* C2 and *Methylobacterium extorquens* G10 from a Methanol–Pentanol mixture

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Abstract—The structures and properties of polyhydroxyalconoates and their polyhydroxybutyrate copolymers synthesized with the use of the bacteria *Methyloligella halotolerans* C2 and *Methylobacterium extorquens* G10 from methanol and its mixture with pentanol are studied. The compositions and molecular masses of the copolymers are shown to depend on their synthesis conditions and may vary widely. All the polymers are found to have high levels of crystallinity, and two types of crystals are detected in the copolymers.

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Polyhydroxyalkynoates (PHA) are of great interest for application in medicine as well as environmentally friendly packaging material. Another reason to produce new polymers is the growth in oil consumption; the decrease in oil reserves; and the possibility of microbial biosynthesis of polymers with the use of renewable raw materials, such as methanol.

Satisfying the needs of various areas of biopolymers application requires the development and improvement of methods of their obtainment, a search for or simulation of new producer strains, and investigation of the influence of synthesis conditions and the properties of the resulting polymers.

Polyhydroxyalkonoates $[-O-C(C_xH_{2x+1})H-(CH_2)_y-C(O)-]_n$ ($x = 1–13$, $y = 1–4$) constitute a class of biocompatible, biodegradable polymers [1]. Widely studied compounds are poly-3-hydroxybutyrate ($x = 1$, $y = 1$) (PHB), poly-3-hydroxyvalerate ($x = 2$, $y = 1$) (PGV), and their copolymers P(HB-co-HV).

These polyesters, which have high levels of biocompatibility, are degraded in the body and the environment into harmless fragments metabolized to water and carbon dioxide, and in some cases, they are natural metabolites of the body [2]. Unlike the obtainment of a number of other known biodegradable polymers of hydroxycarboxylic acids, such as polylactides, the obtainment of PHA does not require the preparation of cyclic monomers and their thorough purification, a circumstance that significantly reduces overall cost and simplifies production. The raw materials used to obtain PHA may be a variety of materials derived from renewable sources; synthesis may be performed with the use of various strains [3]. Therefore, a fundamental problem is to study the effects of the producer strains and biosynthesis conditions on the molecular (statistical, block copolymer, homopolymer, or a mixture of homopolymers) and crystalline (isomorphic crystallization of homopolymers and copolymers, homopolymers mixtures) structures and properties of these polymers, the characteristics of the crystallization kinetics, and the production of composite materials based on them.

The most promising prospective materials with different properties may be crystalline–amorphous random copolymers, such as hydroxybutyrate (HB) and hydroxyvalerate (HV). They are interesting because, owing to the crystal–chemical similarities of PHB and PHV, under certain biosynthesis conditions, the PHB crystalline component of the resulting copolymers consists of macromolecules where HB units are replaced with HV units and/or, vice versa, the PHV crystalline component is made of macromolecules where some the HV units are replaced with HB units. When the unit replacement is observed throughout the range of molar concentrations, G. Natta proposed to name this phenomenon in crystalline-amorphous random copolymers “isodimorphism” [4].

The degrees of crystallinity of the copolymers usually depend weakly on the molar concentration of HB

or HV units and remain about 50–55%. The melting point of the copolymers with a predominant content of hydroxybutyrate units, 180°C, is greater than that for a copolymer containing about 40 mol % HV, 60°C. The melting point increases from 60 to 120°C for the PHV homopolymer with a further increase in the content of hydroxyvalerate units in the copolymers [5].

In mixtures of pure PHB and its copolymer with hydroxyvalerate, cocrystallization is possible, depending on the HV content of the copolymer [6, 7]. Thus, for mixtures of PHB and the copolymer where there is less than 13 mol % HV units, the content in the crystalline phase of PHB is the same as that in the total mixture; i.e., in these mixtures, there is full cocrystallization and the components of the mixture are equally included in the crystalline phase.

However, if there is more than 15 mol % hydroxyvalerate in a copolymer composition, the PHB chains form mainly the crystalline phase and it has more PHB than the mixture does. Thus, the crystalline-phase composition in a mixture varies with the copolymer composition, and the cocrystalline-phase content decreases with an increase in the number of HV units in the copolymer. These results show that the composition of a crystalline phase is determined by the competition between phase segregation and crystallization.

It is necessary to know the content of HV units in the crystalline phase to completely describe the phase composition of a mixture.

Traditionally used bacterial strains synthesize low-molecular-mass polymers ($M_w = (60–150) \times 10^3$). We were able to isolate a culture of obligate (grown only on methanol and methylamine) colorless methylobacteria of the new genus *Methyloligella* gen. nov., which synthesize macromolecular biopolymers with $M_w \geq 2000 \times 10^3$, a circumstance that dramatically increases the range of possibilities of their practical application [8]. Currently, the conditions to cultivate *Methyloligella* sp. C2 with laboratory bioreactors are optimal. Along with this, work has been done and the conditions to obtain polymers and copolymers with the rose-colored producer *Methylobacterium extorquens* G10 are optimal. In particular, it has been possible to obtain the mutant strain G10, for which carotinoid pigment synthesis is excluded, as well as the transformant with an increased multicopy of PHA synthase [8]. Application of such strains has a positive effect on the efficiency of production of biosynthesized polymers. (In the former case, it simplifies product purification from the carotinoid pigment and in the latter case, it increases the rate of the polymer accumulation, that is, one of the key characteristics of the production process as a whole.)

The purpose of this study is to investigate the structures and properties of homo- and copolymers of polyhydroxyalkanoates produced via bacterial synthesis from methanol and its mixtures with pentanol.

EXPERIMENTAL

In this study, the objects of investigation are polyhydroxybutyrate and the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate synthesized with the use of the methylobacteria *Methylobacterium extorquens* G10 and *Methyloligella halotolerans* C2 grown in methanol or in its mixtures with pentanol.

Medium Composition and Cultivation Conditions

M. extorquens G10 was grown in a mineral medium with the composition 1.0 g/L KH_2PO_4, 1.0 g/L $Na_2PO_4 \cdot 12H_2O$, 1.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, and 10 mL of a trace-element solution. The contents of the trace-element solution were as follows (g/L): $CoCl_2 \cdot H_2O$, 0.5; $CaCl_2 \cdot 6H_2O$, 0.5; $MnSO_4 \cdot H_2O$, 0.2; $ZnSO_4 \cdot H_2O$, 0.5; Na_2MoO_4, 0.02; $CuSO_4 \cdot 5H_2O$, 0.1; $FeSO_4 \cdot 7H_2O$, 1.0. The required pH (7.0) was adjusted with 10% NaOH.

Cultivation was performed in a 750-mL Erlenmeyer flasks containing 100 mL of the medium with methanol (0.5 vol %) on a rotary shaker (180 oscillations per minute) at 29°C for two days. The culture in the mid logarithmic growth phase (an optical density of $OD_{600} = 1.0$) was used as inoculum in a bioreactor. Batch cultivation in an Ancum-2M bioreactor (Biopribor, Russia) was performed during automatic maintenance of a temperature of 30°C and a pH of 6.85. To the bioreactor, 4.0 L mineral medium and 200 mL inoculum were added. The required pH was maintained with 25% NH_4OH or 10% NaOH.

When the culture reached $OD_{600} = 30$ (15 g/L dry biomass), 50 mL concentrated medium containing 270 g/L H_3PO_4, 80 g/L $MgSO_4 \cdot 7H_2O$, 2.3 g/L $FeSO_4 \cdot 7H_2O$, 0.6 g/L $CaCl_2 \cdot 6H_2O$, 0.5 g/L $MnSO_4 \cdot H_2O$, 0.6 g/L $ZnSO_4 \cdot H_2O$, and 0.4 g/L$CuSO_4 \cdot 5H_2O$ was added.

With consumption of methanol by a growing culture, methanol was added in portions of 5 to 20 mL. The lack of methanol in the medium was determined from a sharp increase in the level of dissolved oxygen (pO_2). The values of pO_2 were maintained at 20–30% saturation via increases in the stirrer speed up to 1000 oscillations per minute and in the air-flow rate to 6 L/min.

Because the PHB biosynthesis using methylobacteria was performed when the culture growth was limited in nitrogen, when the optical density of the cul-

ture medium reached 70, we maintained the pH with 10% NaOH.

For the biosynthesis of P(HB-co-HV), a cosubstrate should be added to the medium. The most efficient cosubstrate for methylobacteria is pentanol [8].

Pentanol was added to the mixture with methanol until the optical density reached 70, and 5, 10, 15, and 20 vol % mixtures of pentanol in methanol were used until the end of the cultivation. The gradual (fractional) introduction of pentanol to the medium helped lower its toxic effects on the growing culture.

The cultivation time of *M. extorquens* G10 was 92 h. The amount of dry biomass varied from 25 to 48 g/L with a bioplastic content from 30 to 53%, depending on the composition of the methanol–pentanol mixture.

M. halotolerans C2 was grown in a mineral medium of the composition 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 1.0 g/L $(NH_4)_2SO_4$, 0.15 g/L $CaCl_2 \cdot 6H_2O$, 2.0 g/L $NaH_2PO_4 \cdot 2H_2O$, and 2.0 g/L $KH_2PO_4 \cdot 3H_2O$ as well as 5 mL yeast autolysate because the culture needs vitamins. Preparation of inoculums and cultivation in an Ancum-2M bioreactor were performed at pH 7.1, as they were for the culture *M. extorquens* G10. The cultivation time was 84 h. The dry biomass content reached 48 g/L containing 17% PHB. To cultivate methylobacteria, we used raw methanol, which is more than an order of magnitude cheaper than pure methanol.

The biomass content was determined via measurement of the optical density (OD_{600}) in a 0.5 cm cell on a Specol 221 spectrophotometer (Carl Zeiss, Germany), and the dry biomass was calculated with the use of a calibration curve. To plot the calibration curve, we took 50 mL of the cell suspension with a known optical density and centrifuged it (10000 *g*, 20 min), and the resulting precipitate was dried at 105°C to a constant mass.

Contents of NH_4^+ in the culture medium was analyzed on an MA-130 ion meter (Mettler Toledo GmbH, Switzerland) with an ammonium sensor.

To isolate the PHB and P(HB-co-HV) from the biomass, a culture liquid was centrifuged (10000*g*, 30 min) and the precipitate was dried lyophilically. A sample of the dried biomass was stirred with six volumes of methanol for 1 h and centrifuged at 5000*g* for 20 min. The resulting precipitate was extracted with chloroform under stirring for 3 h. The suspension was filtered through decalcified filter paper. The chloroform extract was clarified with grade-A activated charcoal, which was separated on a Buchner funnel under vacuum. The clarified extract was concentrated 5–6 times in a rotary evaporator (to the liquid jelly state). The concentrate was poured in a 6-fold volume of methanol under vigorous stirring. The precipitate PHB or P(HB-co-HV) was isolated on a Buchner funnel and dried at 105°C.

Table 1. Assignment of the signals in the proton spectra of hydroxyalkanoates

Polymer	Chemical shift of the protons, ppm			
	αCH_2, m	βCH, m	$\gamma CH_3/\gamma CH_2$	δCH_3
PHB	2.41–2.68	5.15–5.35	1.28 (d, $J = 6$ Hz)	–
PHV	2.48–2.60	5.07–5.21	1.55–1.72 (m)	0.89 (d, $J = 6$ Hz)

NMR, viscosimetric, X-ray, and DSC methods were used to characterize the polymers.

X-Ray diffraction measurements were performed on a DRON-3M diffractometer in the transmission mode (asymmetric, focusing on the detector, crystal monochromator on the primary beam). CuK_α-radiation was used. Scanning of the diffraction pattern was done in the step-by-step mode with increments of $\Delta 2\theta = 0.04°$ and an acquisition time of $\tau = 10$ s. Diffractograms were treated with the program fityk.

Thermal characteristics (temperature and heat of fusion) were determined on a DSC-30 calorimeter with a TS-15 processor and the program Star SW 9.30 (Mettler). Measurements were performed under argon in the heating mode at a rate of $q^+ = 10$ K/min.

The method of high-resolution ^1H NMR was applied to find the HB–HV unit ratios in the synthesized polymer compounds and mixtures. Determination was performed on a Bruker MSL-300 spectrometer in solutions at a temperature of 24°C. Samples were prepared in $CDCl_3$. Proton spectra were obtained under the following conditions: a frequency of 300.13 MHz, 200 scans at 11 905 Hz (39.7 ppm), 90°, an impulse of 3 μs, a delay of 1 s, and the impulse program PAPS.PC followed by Fourier transformation. The signal of residual protons of chloroform at 7.27 ppm was used as an internal reference standard for chemical shifts.

Analysis of the samples for the quantitative contents of PHB and PHV units was performed with the use of the signals of the protons of the methyl groups (Table 1). NMR spectra of the samples are shown in Fig. 1.

Quantitative determination of polymer composition was calculated with the use of the ratio of the chemical-shift intensity integrals, $\gamma CH_3/\gamma CH_2$. The precision of determining composition was ±5 mol %.

The widths of the spectral lines depend on the intramolecular and intermolecular interactions, i.e., on the chain lengths of the polymers, conformations, crosslinks, and other conditions.

The molecular masses of the polymers were determined through the viscometric method via measurement of the viscosities of solutions of PHB and P(HB-co-HV) in chloroform at 30°C, and they were calculated according to the equation $[\eta] = 7.7 \times 10^{-5} M^{0.82}$ [9].

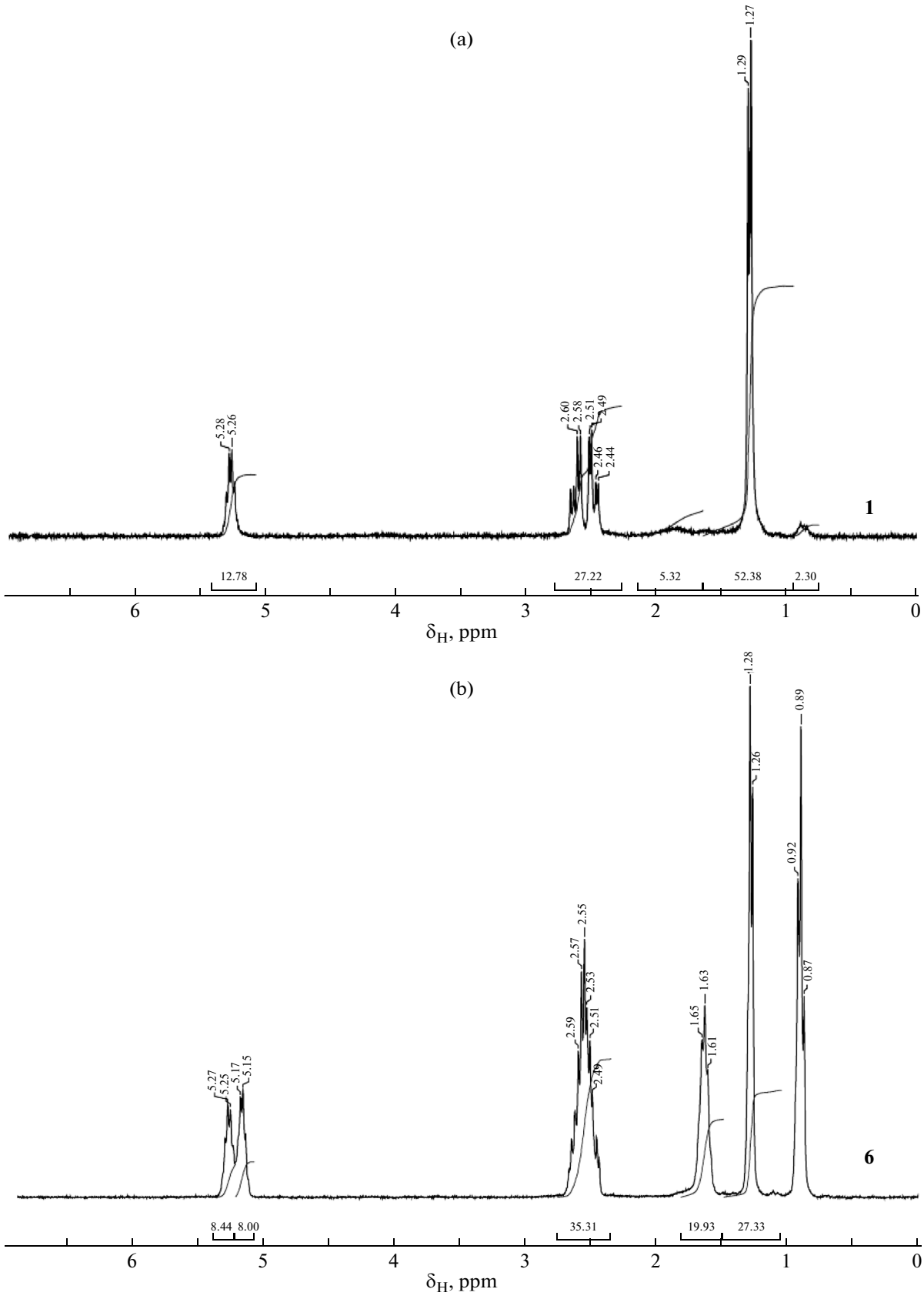

Fig. 1. The ^{1}H NMR spectra of samples **1**, **6**, and **7**. Here and in Figs. 2–4, the numbering of the curves corresponds to the numbers of the polymers in Table 2.

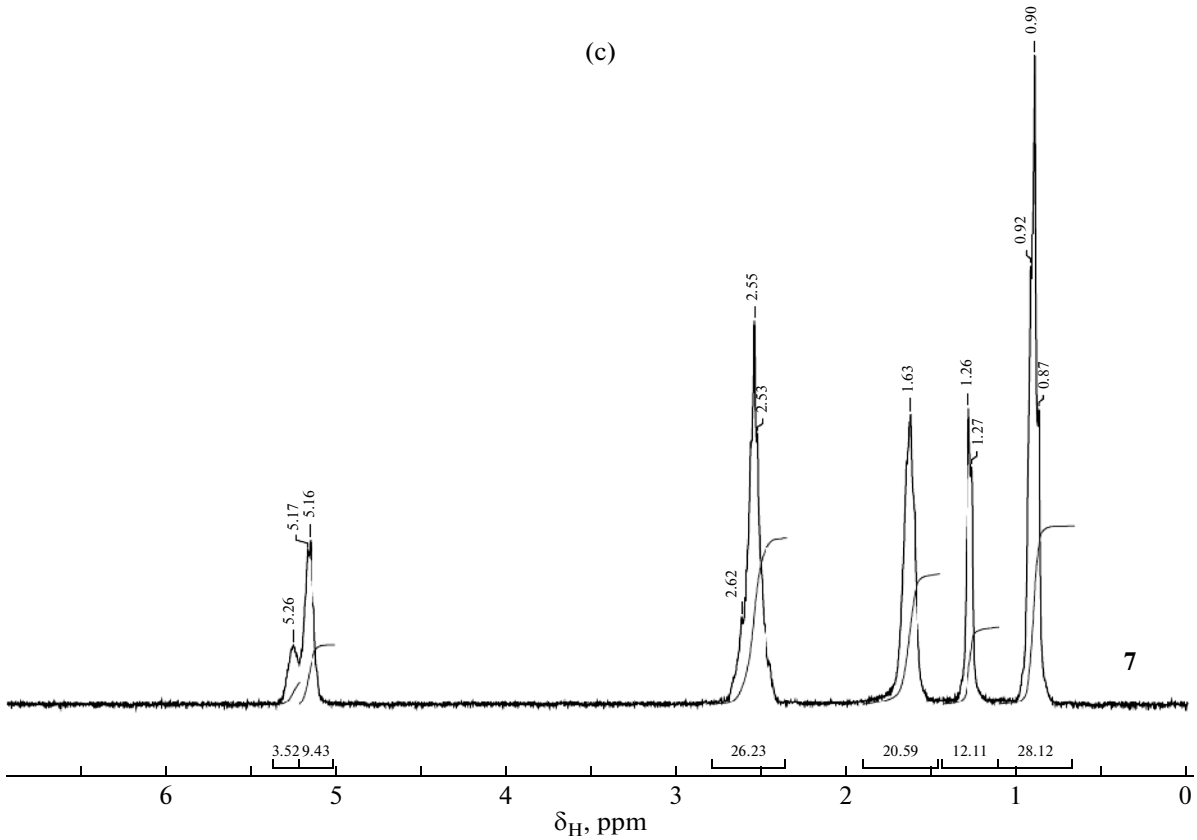

Fig. 1. (Contd.)

The study of the mechanical characteristics of the films was performed on a TIRA test 2200 test machine. Samples were tested in the form of vanes with a size of the working part of 10 mm × 3 mm × 0.2 mm, and the tensile speed was 5 mm/min. The test temperature was 20°C.

RESULTS AND DISCUSSION

Conditions of bacterial synthesis and the results of determining the compositions and molecular masses of the obtained polymers are shown in Table 2, where the copolymers with various contents of PHB and PHV units are shown to be synthesizable in a wide

Table 2. Conditions of bacterial synthesis and the results of determination of the compositions and molecular masses of the obtained polymers

Sample no.	Producer	Conditions of synthesis (% pentanol in a mixture with methanol)	Content of hydroxybutyrate, mol %	Content of hydroxyvalerate, mol %	Molecular mass, $\times 10^3$
1	*M. halotolerans* C2	0	98	2	7000
2	*M. halotolerans* C2	5	98	2	2300
3	*M. halotolerans* C2	10	95	5	1800
4	*M. halotolerans* C2	10	84	16	3000
5	*M. halotolerans* C2	15	73	27	1000
6	*M. halotolerans* C2	5	49	51	2700
7	*M. extorquens* G10	20	30	70	370
8	*M. extorquens* G10	20	28	72	250

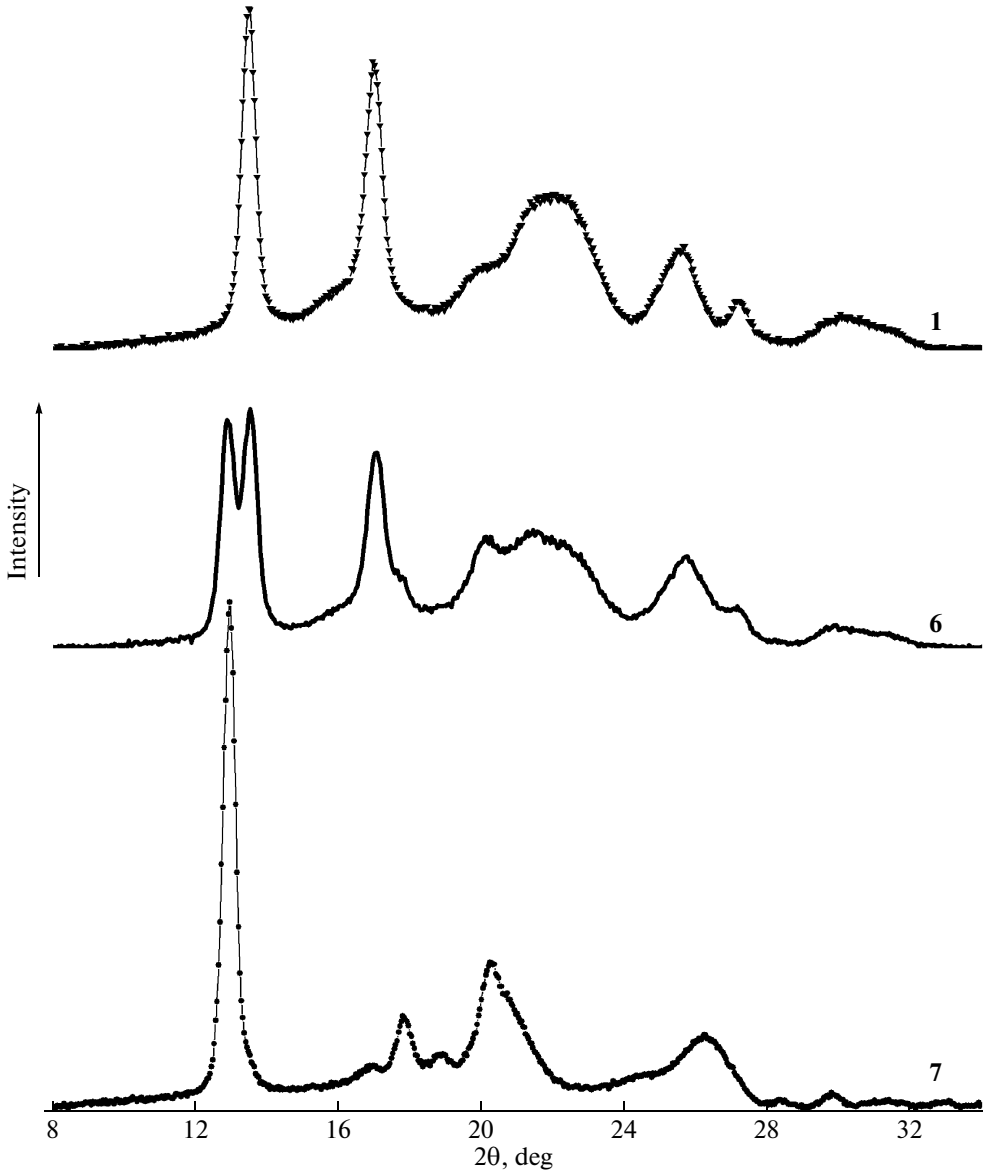

Fig. 2. Diffractograms of samples **1**, **6**, and **7**.

range of molecular masses from methanol and its mixtures with pentanol with the use of *M. halotolerans* C2 and *M. extorquens* G10. Note that the molecular masses of the polymers can reach 7000.

X-Ray Diffraction Study of the Polymers

Analysis of diffractograms of all of these polymers showed that they can be divided into three groups (Fig. 2).

The diffractograms of samples 1–5 of the first group are identical: the two most intense diffraction reflections, with diffraction angles of $2\theta = 13.50°$ ($d = 0.656$ nm, a Miller index of (020)) and $2\theta = 16.98°$ ($d = 0.522$ nm, a Miller index of (110)), and a diffraction reflection from the diffraction angle $2\theta = 27.19°$ ($d = 0.328$ nm, a Miller index of (040)). The diffractograms are typical of crystalline amorphous PHB [5]. The degrees of crystallinity of the samples are the same, $68 \pm 5\%$ (Fig. 2, sample 1).

The second group comprises the diffractograms of samples **7** and **8**, which are likewise identical. They present diffraction reflections at diffraction angles of $2\theta = 12.89°$ ($d = 0.687$ nm, a Miller index of (110)) and $2\theta = 17.83°$ ($d = 0.498$ nm, a Miller index of (020)), which are typical of crystalline amorphous PHV [5]. The crystallinity values of the samples are identical, $68 \pm 5\%$ (Fig. 2c, sample **7**).

Finally, the third group comprises the diffractogram of sample **6**, containing a reflection with an angle of diffraction of $2\theta = 12.90°$, present in the diffractograms of the samples **7** and **8**, which are assigned to

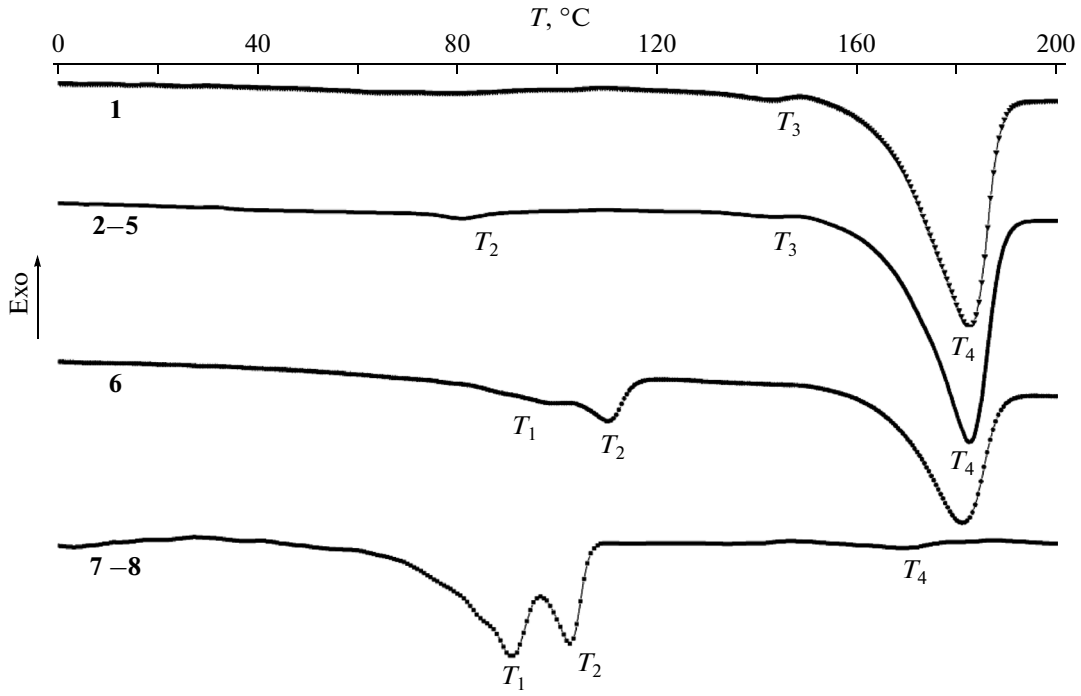

Fig. 3. Thermograms of samples **1**–**8**.

PHV, and the diffraction reflections with diffraction angles of $2\theta = 13.52°$ and 17.02°, present in the diffractograms of samples 1–5 (PHB). The degree of crystallinity of sample **6** is $66 \pm 5\%$.

Comparing the data of X-ray diffraction studies with the data of ^{1}H NMR investigation and viscometry (Table 2), we note that the degree of crystallinity of a polymer does not depend on the molecular mass and the concentrations of HB and HV units.

Thermal Characteristics of Polymers

Figure 3 shows typical thermograms of samples, and Table 3 summarizes the thermal characteristics of the polymers. The thermograms of all the polymers have double melting points: Either temperatures T_1 and T_2 for samples **6**–**8** or T_3 and T_4 for samples **2**–**5**. These appear as melting and recrystallization (reorganization and recrystallization) of the crystallites with different morphologies (in the distribution of size, improvement, and stability of the crystallites) with the same molecular structure that form as a result of the preparation of crystalline–amorphous polymers [10].

Note that the double melting of the polyhydroxybutyrate crystallite is observed for the samples with predominant contents of hydroxybutyrate units from 98 to 50 mol %, and double melting of the polyhydroxyvalerate crystallite is found for the samples with contents of hydroxyvalerate over 50 mol % (Table 2, 3).

The thermogram of sample **1** (98 mol % HB) has two melting peaks at $T_3 = 141$°C and $T_4 = 183$°C and heats of fusion of $\Delta H_3 = 2$ J/g and $\Delta H_4 = 104$ J/g,

Table 3. Thermophysical properties of the polymers

Sample no.	T_1, °C	ΔH_1, J/g	T_2, °C	ΔH_2, J/g	T_3, °C	ΔH_3, J/g	T_4, °C	ΔH_4, J/g
1	–	–	–	–	141	2	183	104
2	–	–	82	1	138	2	181	97
3	–	–	81	3	140	6	180	90
4	–	–	80	3	142	2	181	98
5	–	–	81	3	140	2	182	103
6	99	13	110	15	–	–	181	65
7	90	36	105	17	–	–	172	4
8	91	52	103	19	–	–	169	4

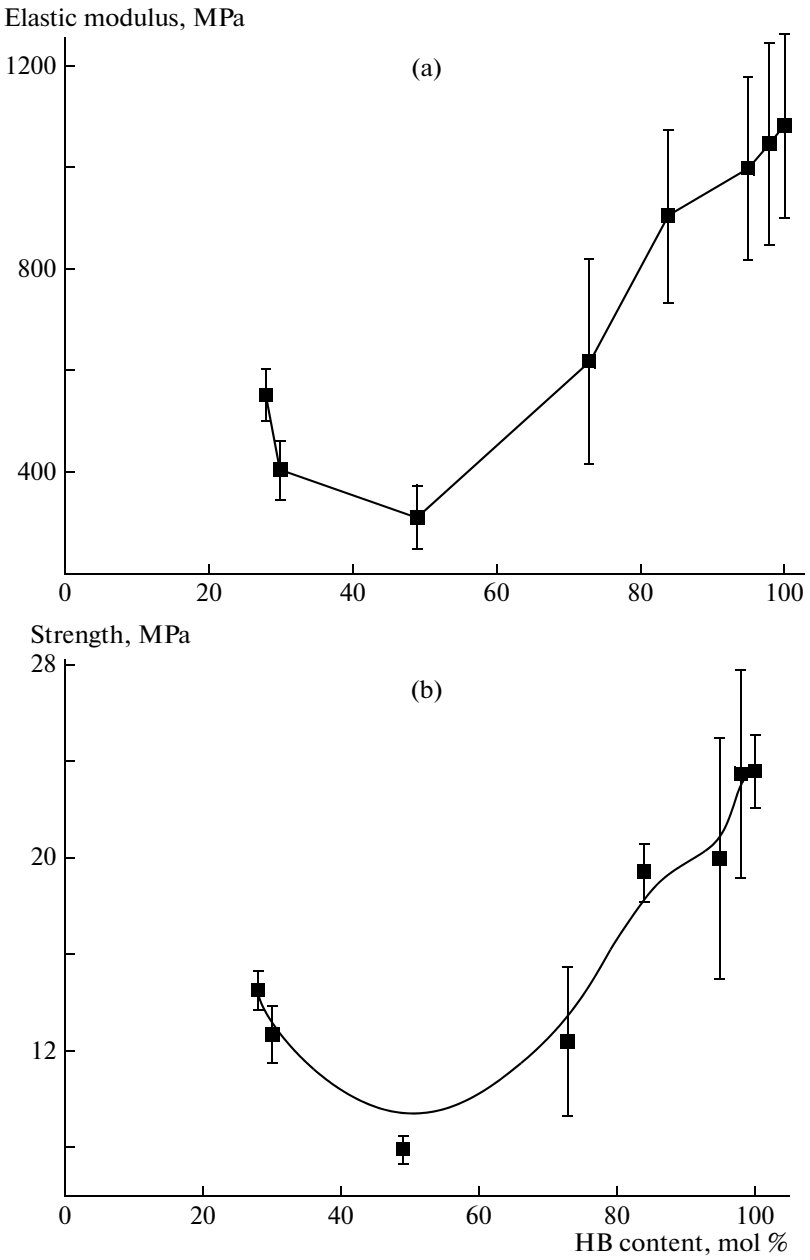

Fig. 4. Dependences of (a) elastic modulus and (b) strength on HB content in the samples.

respectively, which belong to PHB melting. Reduction of the HB content in the polymer to 73 mol % (samples **2**–**5**) does not lead to a noticeable change in the position of these peaks and their heats of fusion. However, endo peaks at $T_2 = 80$°C with an enthalpy of fusion $\Delta H_2 = 3$ J/g appear with the advent of a small number of HV units in the polymer. With allowance for the molar composition of these samples (Table 2) and the isodimorphism noted above, it is possible to relate these endo peaks to the melting of an insignificant amount of random copolymer P(HB-co-HV) with a predominant content of HV units that is present in samples **2**–**5** (except PHB). The absence of reflections of this phase on the diffractograms of samples **2**–**5** is due to its insignificant amount in the polymer.

On the thermograms of samples **7**–**8** (HV contents below 49 mol %), the melting point at which endo peaks occur corresponds to $T_1 = 90$–99°C and its heats of fusion increases with an increase in the HV content in the polymer. Also, on the thermograms, endo peaks T_2 shift to a region with the melting point at 103–110°C. This temperature corresponds to the melting of the PHV crystallites [5]. The crystallinity of these samples is determined mainly by PHV.

The decreases in the temperature and heat of fusion of PHB crystallites occur with the reduction of the

PHB content in samples **6**–**8**. Note that less than perfect polyhydroxybutyrate crystallites with a melting point of $T_3 \sim 140$°C do not form in these samples.

In samples **7**–**8**, the heat of fusion of PHB crystallites is low, which may explain the absence of reflections of this phase on the diffractograms of the mentioned samples.

The concentrations of HB and HV units are the same according to the ^{1}H NMR in sample **6**. The thermogram of the sample (Fig. 3) contains three endo peaks ($T_1 = 99$°C, $T_2 = 110$°C, and $T_4 = 181$°C), which are related to the melting of PHV and PHB. The diffraction pattern of the sample (Fig. 2) has reflections of both PHB and PHV. With a high probability, this sample consists of homopolymers of PHB and PHV.

Mechanical Properties of Polymers

Mechanical properties of the samples were determined in the tension mode. Although the degrees of crystallinity of the samples are approximately the same, the mechanical properties of the polymers of different compositions are substantially different. Dependences of elastic modulus and strength on the content of the HB units have an extreme character with a minimum at approximately equal content of HB–HV units. The elastic modulus and strength of the polymer of this composition are 310 and 7.9 MPa, respectively. Elastic modulus and strength increase with an increase in one of the components of the polymer (Fig. 4): The PHB homopolymer has an elastic modulus of 1100 and a strength of 23 MPa. The relative elongation of the polymers at break depends weakly on the composition and is approximately 10%. These significant differences in strength and rigidity of the polymers seem to be associated with the different structuring of the crystalline phase and especially with the sizes of the crystallites formed in the polymers of different compositions.

Thus, the compositions and crystal structures, molecular masses, and physicochemical properties of PHB, PHV, and P(HB-co-HV) depend on both the type of producer and the conditions of its cultivation. So, the selection of the various producers of bioplastics and the conditions of their cultivation is important for expanding the range of bioplastic application in various areas.

Thus, methylobacteria *M. extorquens* G10 and *M. halotolerans* C2 make it possible to obtain polymers of PHB and its copolymers in a wide range of compositions. The molecular masses of the polymers synthesized with the use of *M. extorquens* G10 and *M. halotolerans* C2 differ. The molecular mass of the polymer from *M. halotolerans* C2 is much higher than that from *M. extorquens* G10. Increasing the PHV content in the polymer synthesized with the use of *M. halotolerans* C2 leads to a reduction in its molecular mass. All synthesized polymers are crystalline–amorphous, and the degrees of crystallinity of the samples are about the same, ~70%. The thermograms of all the copolymers have double melting with a small endo peak before the main peak of the melting, which is characteristic for the polymers with crystallites of various morphologies. All copolymers have crystallites of two types of PHB and PHV. Depending on the polymer composition, the crystallites may consist of units of one type or copolymers.

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