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Characterization of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Biosynthesized by Mixed Microbial Consortia Fed Fermented Dairy Manure

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ABSTRACT: The bioplastic poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV), was isolated from a bioreactor using mixed microbial consortia fed volatile fatty acids (VFA), from fermented dairy manure, as the carbon source. The molar fraction of 3-hydroxyvalerate (3HV) amounted to 0.33 mol mol^{-1} for two isolated PHBV samples as determined by GC-MS and ^{1}H-NMR spectroscopy. The chemical, thermal, and mechanical properties were determined. The PHBVs had relatively high M_w (~790,000 g mol^{-1}). Only a single glass transition temperature (T_g) and melting point (T_m) were observed. Isolated PHBVs exhibited good flexibility and elongation to break as compared with commercial PHBVs with lower HV. The diad and triad sequence distributions of the monomeric units were determined by ^{13}C-NMR spectroscopy and followed Bernoullian statistics suggesting that the PHBVs were random. The PHBV sequence distribution was also characterized by electrospray ionization-mass spectrometry (ESI-MS^n) after partial alkaline hydrolysis to oligomers showing a random 3HV distribution. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40333.

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a class of renewable bioplastics, which can be produced by microbial biosynthesis as intracellular granules. Poly-3-hydroxybutyrate (PHB) is the most common PHA and as a consequence has been studied most extensively.[1,2] The primary limitation of PHB as a thermoplastic material is its brittleness upon crystallization due to large spherulites, which exhibit inter-spherulitic cracks.[3] However, the copolymer of 3-hydroxyvalerate (3HV) with 3-hydroxybutyrate (3HB) to form poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) has improved ductility as a result of being less crystalline and can be used in various applications such as flexible packaging materials.[2] The ductility of PHBVs is positively dependent on 3HV content which inhibits crystallization.[4]

Current commercial production of PHB and PHBV uses pure culture bacteria and expensive refined substrate, for example, glucose, resulting in high production costs. An alternative, the use of open, mixed consortia and waste feedstocks have been proposed as a potential technique to reduce these production costs. Recent work has shown that PHB and PHBV can be produced using mixed microbial consortia from organic waste streams such as crude glycerol from biodiesel production and volatile fatty acids (VFA).[5,6] The advantage of PHBV over PHB is that the chemical microstructures can be regulated (such as 3HV/3HB ratio and sequence distribution), which alters the physical and mechanical properties, melting and cocrystallization behavior, and biodegradability.[4,7–12] Regulating the feed carbon source/composition and the timing of its addition to the biosynthesis process has been shown to be one strategy of varying the 3HB and 3HV monomer distribution and sequence.[2,13] It has been suggested that the tensile properties of coblock PHBV films were different from the random copolymers if 3HV contents are comparable.[14,15] Therefore, it is important to be able to determine the composition and 3HB/3HV distribution in PHBVs during the biosynthesis as a function of bioreactor operating parameters to manipulate polymer properties.

This methods to analyze PHA, such as methanolysis and subsequent analysis by GC-flame ionization detection (FID), the first method used for PHB analysis, and GC-MS or directly by ^{1}H-NMR spectroscopy, provide limited information such as composition.[16–20] To obtain more detailed chemical information such as 3HV and 3HB sequence information in PHBVs ^{13}C-NMR

spectroscopy has been applied to look at diad (e.g., 3HB*3HV) and 3HV-centered triad (e.g., 3HB-3HV*3HV) sequences of 3HB and 3HV units and establish whether the copolymer is a co-block polymer or randomly distributed or not based on statistical models.[8,21–23] However, it is difficult for NMR spectroscopy to discern higher levels than the triad segmentation. Thus, in practice mass spectrometry (MS) provides an alternative technique for the analysis of oligomers formed by partial degradation of copolymers as compared with NMR analysis.[21,24] Partial degradation can be achieved by methanolysis, hydrolysis, pyrolysis, and aminolysis of copolymer chains.[25–28] Therefore, it is feasible to determine the sequence distribution of oligomers and construct the sequence in the copolymer.

Recently, bacterial PHBV copolymers were studied using electrospray ionization mass spectrometry (ESI-MS) for the determination of the comonomer sequence distribution of their corresponding oligomers obtained by controlled partial alkaline hydrolysis.[10,24] The PHBV oligomers with the same composition and sequence distribution as the starting copolymer contained carboxylic and olefinic end groups.[10,24] Controlled depolymerization of PHB and PHBV was achieved through partial saponification of ester linkage, which was catalyzed by a KOH/18-crown-6 ether complex.[29,30] ESI-MS was then used to characterize the oligomeric PHB/PHBV products by the analysis of the pseudomolecular ions.[10,24] MS-MS analysis provided further detailed chemical structural information of individual PHBV chains and mixtures of PHBV copolymers containing varying levels of 3HV and sequence distributions of 3HB and 3HV repeats.

The aim of this study was to characterize PHBV isolated from a bioreactor fed VFAs, from fermented dairy manure, using mixed microbial consortia. The PHBV samples were compared with commercial samples, for composition, 3HB and 3HV sequence, and mechanical and thermal properties.

EXPERIMENTAL

Materials

Laboratory PHBV samples (PHBV-1 and PHBV-2) were biosynthesized in a 20 L scale bioreactor inoculated with activated sludge (mixed microbial consortia) obtained from the Moscow, Idaho wastewater treatment plant. The aerated bioreactor was run continuously for 1 year and was fed a mixture of VFAs from clarified fermented diary manure (Table I) with a solid (SRT) and hydraulic retention times of 4 d. Diary manure was collected from the University of Idaho North Farm Dairy biweekly, stored at 4°C, and fed each day to the continuously operated 20 L anaerobic fermenter operated under a 24 h cycle time with a SRT of 4 d, and organic volatile solids loading rate = 10.8 g L^{-1} d^{-1}.[31] The laboratory PHBV samples were obtained on the 6 and 11 month of bioreactor operation and prewashed with acetone (24 h), and then dried followed by extraction from lyophilized biomass with $CHCl_3$ and concentrated to dryness.[6] The yields of crude PHBV-1 and PHBV-2 from biomass were 42 and 36%, respectively. The PHBV extract was dissolved in a minimum volume of $CHCl_3$ and then precipitated in cold petroleum ether (boiling point range 35–60°C), collected by filtration and dried under vacuum with an average recovery > 98%. 3HV/3HB ratio was determined by GC-MS (Finnigan PolarisQ) as their methyl ester derivatives as described by Hu et al. 2013.[6] Commercial PHBV-C1 (Biopol D300G, Monsanto), PHBV-C2 (Biopol D600G, Monsanto), and PHB-C (T_m = 172°C, Sigma-Aldrich) were used for comparison.

Table I. Average Composition of VFA Feed (mg L^{-1}) from Fermented Dairy Manure Used for the Production of PHBV-1 and PHBV-2

	Concentration (mg L^{-1})	
VFA	PHBV-1	PHBV-2
Acetic	3420	3740
Propionic	1320	1590
Butyric	746	1458
Isobutyric	108	120
Valeric	220	420
Isovaleric	153	157
Caproic	90	159
Odd : Even VFAs (unit less)[a]	0.388	0.396

[a] Odd VFAs: acetic, butyric, isobutyric, and caproic; even VFAs: propionic, valeric, and isovaleric.

Size Exclusion Chromatography (SEC)

The number- and weight-average molar mass (M_n and M_w, respectively) and the dispersity (M_w/M_n) of all commercial PHB (PHB-C) and PHBV (PHBV-C1, PHBV-C2) and laboratory PHBVs (PHBV-1, PHBV2) were determined by SEC. A Jordi DVB linear mixed bed column (7.8 × 300 mm^2) column was used for the separation of polymers at 40°C; 100 μL samples (2 mg mL^{-1}) were injected on elution with HPLC grade $CHCl_3$ at 1 mL min^{-1} and detected with triple detector array [refractive index (RI), Waters model 2478], low- and right-angle laser light scattering (LALLS, RALLS), and differential viscometer (Viscotek model 270, Viscotek Corporation). Data were analyzed by OmniSEC v4.1 (Viscotek) software. The system was calibrated using a narrow polystyrene standard (Viscotek, M_w = 98,946 g mol^{-1}).

Differential Scanning Colorimetry (DSC)

DSC was performed on all samples (4–6 mg, in duplicate) using a TA Instruments model Q200 DSC with refrigerated cooling. The samples were (i) equilibrated at 40°C (3 min) then ramped to 180°C at 10°C min^{-1} to destroy any prethermal history, held isothermally for 3 min, (ii) cooled to −50°C at −10°C min^{-1} and held isothermally for 3 min and the cycles repeated. The T_m and the T_g were determined from the peak maximum and the inflection point of the second heating scan, respectively. The degree of crystallization (X_c) of samples were calculated from the ratio of the melting enthalpy (ΔH_f) of the sample to (ΔH_f^0) of 100% crystalline polymers (146 J g^{-1} for PHB).[6,32,33] Data were analyzed using TA Universal Analysis v4.4A software.

Tensile Properties

PHBV and PHB films were solvent cast from a $CHCl_3$ solution (10 mg mL^{-1}) using a Teflon mold, air dried and vacuum dried prior to use. Samples were cut into 10 × 3 × 0.05 mm^3 sized specimens. Tensile testing was performed on a DMA Q800 (TA

instruments) with controlled force of 3 N min^{-1} until yield. Tensile strength, Young's modulus, and elongation to break were determined from the constructed stress-strain curve using Universal Analysis v4.4A software.

NMR Spectroscopy

Samples were dissolved in $CDCl_3$ and ^{1}H- and ^{13}C-NMR spectra were recorded on an Advance Bruker 300 MHz spectrometer at 27°C. Spectra were analyzed using SpinWorks v3.1.7 software. The 3HV molar fraction was determined by the ratio of the integrated ^{1}H peak areas due to the 3HV methyl resonance and the sum of 3HB + 3HV methyl resonance corresponding to B_4 and V_5, respectively (Scheme 1).[18,34] ^{13}C-NMR spectra were obtained using a Lorentz-Gauss transformation of the FID with a line broadening of 1 Hz and Gaussian multiplication factor of 0.3.

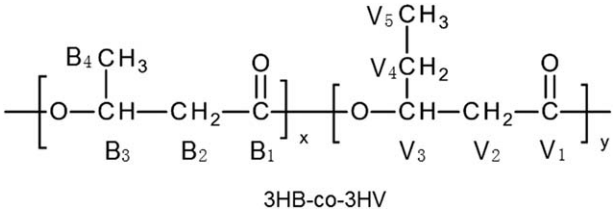
Partial Hydrolysis and Analysis by ESI-MS^n

PHB and PHBV samples were each dissolved in $CHCl_3$ (10 mg mL^{-1}) and subjected to controlled alkaline hydrolysis in 5*M* KOH containing 18-crown-6 ether complex (5 mg mL^{-1}) at 35°C with constant stirring for 16 h.[31] After filtering, the oligomers were recovered and dried over anhydrous sodium sulfate prior to analysis. Negative ion ESI-MS^n analysis was performed using a Finnigan LCQ-Deca ion trap mass spectrometer. The oligomers were dissolved in $CHCl_3$/methanol mixture (1 : 1, volumetric) to a concentration of 1–2 mg mL^{-1}. The sample was introduced to the ESI source at 5 μL min^{-1}. The ESI source was operated at 4.5 kV and capillary heater was set at 275°C. MS data were collected and analyzed using Xcalibur v2 software.

RESULTS AND DISCUSSION

Compositional Analysis

The ^{1}H-NMR spectrum of PHBV-2 is shown in Figure 1. The triplet CH_3-proton resonance at $\delta = 0.92$ ppm corresponds to the methyl groups of 3HV monomer unit [Scheme 1, Figure 1(a)], while the doublet CH_3-proton resonance at $\delta = 1.29$ ppm was assigned to methyl group of 3HB monomer [Scheme 1, Figure 1(a)]. The molar fraction of 3HV was determined from the integrated areas of V5 and B4, which were 0.34 and 0.335 mol mol^{-1}, for samples PHBV-1 and PHBV-2, respectively. These values were close to the result that was 0.341 and 0.324 mol mol^{-1} of 3HV for isolated PHBVs, respectively, as determined by GC-MS.[35] Replicate analyses on the same sample were within ±0.004 mol mol^{-1} 3HV and using the commercial PHBV sample for the compositional calibration with an R^2 of 0.99. All



Scheme 1. Structure of PHBV showing NMR assigned protons and carbons.

commercial PHB, PHBV-C1, and PHBV-C2 compositions determined by both methods were close (Table II). These PHBVs have relatively high 3HV fraction as compared with the literature using mixed microbial consortia.[36] As polymer composition is directly influenced by the carbon source, with even-carbon VFAs (e.g., acetic andbutyric) generating 3HB and odd-carbon VFAs (e.g., propionic and valeric) being the source of 3HV, the high 3HV fraction can be attributed to the substrate.[2]

While the VFA composition in the fermented dairy manure used to produce PHBV-1 and PHBV-2 varied in relative magnitude (Table I), the ratio of odd- to even-carbon VFAs was comparable between the two (0.388 and 0.396 for PHBV-1 and PHBV-2, respectively), which contributed to the similar 3HV molar fractions observed.

Polymer Properties

The physicochemical properties of PHBVs with lower 3HV molar fraction (<0.30 mol mol^{-1}) have been widely studied and reviewed.[3,36–38] However, the documentation on higher 3HV based PHBVs is limited, especially from mixed microbial consortia.[39] The properties of laboratory and commercial PHB and PHBVs, and PHBVs cited from the literature (denoted by PHBV-R's) with a variable molar fraction of 3HV as well, are given in Table II. The M_w and M_w/M_n of the laboratory PHBVs and commercial PHB and PHBVs were obtained by SEC. The M_w/M_n values of PHBV-1 and PHBV-2 were, respectively, 2.2 and 2.1. The M_w of PHBV-1 (790,000 g mol^{-1}) and PHBV-2 (770,000 g mol^{-1}) were higher than PHBV with 0.45 mol mol^{-1} HV prepared using mixed cultures fed on substrates of acetate, propionate and butyrate.[22] These M_w's are comparable to samples reported in the literature for PHBVs with higher 3HV molar fraction (see Table II, samples PHBV-R3, PHBV-R5, and PHBV-R6).[37,38] For comparison, the M_w for PHBV-C1 (0.056 mol mol^{-1} 3HV) and PHBV-C2 (0.197 mol mol^{-1} 3HV) were low at 300,000 and 420,000 g mol^{-1}, respectively. While PHBV-C1 and PHBV-C2 have M_w/M_n values of 3.3 and 2.2, respectively.

The thermal properties of commercial and isolated PHB and PHBVs are given in Table II. As expected, the T_m of PHBVs was lower than PHB homopolymer. The isolated PHBV-1 and PHBV-2 were shown to have higher 3HV molar fraction and lower T_m and T_g values than the commercial PHBV-C1, PHBV-C2 samples. An overall evaluation of PHBV samples shows that the thermal properties of copolymers highly correlate with the 3HV molar fraction, such as: (i) T_g decreases from 5.5 to −30.0°C with increasing 3HV fraction increases from 0.056 to 0.72 mol mol^{-1}; (ii) T_m decreases when 3HV fraction <0.34 mol mol^{-1}, which has similar trends as reported elsewhere; (iii) however, PHBV-1 and PHBV-2 with lower 3HV fraction were observed to have lower X_c than the samples PHBV-R5 and PHBV-R6, having 0.71 and 0.72 mol mol^{-1} 3HV, respectively, and this may be attributed to the differences in D and higher 3HV monomeric unit chain length of these laboratory isolated samples (Table II).[22,36–39] PHBV-1 and PHBV-2 were considerably more flexible (low Young's modulus) than the commercial PHB, PHBV-C1, and PHBV-C2 samples. If compared with solution casted films studied before (PHBV-R2, -R4, and -R5), the

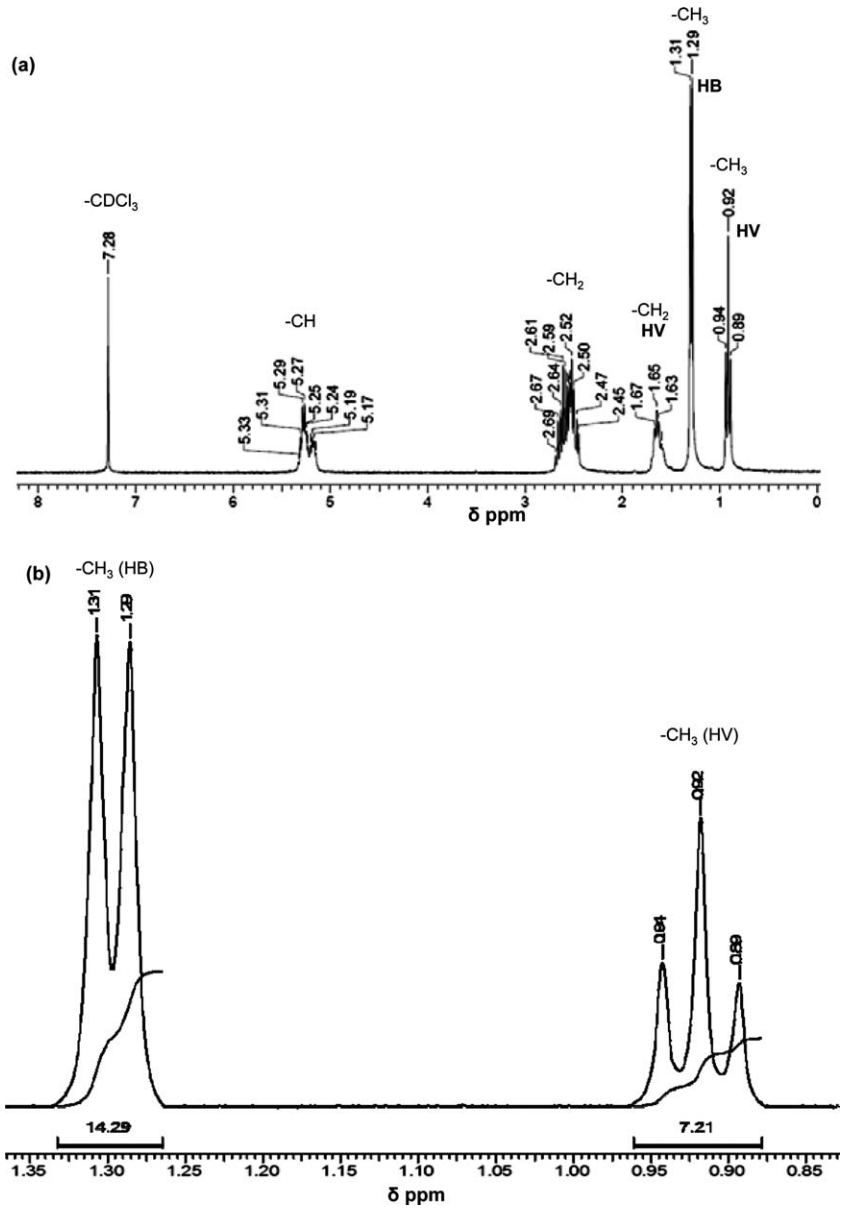


Figure 1. (a) 300 MHz ^{1}H-NMR spectrum of PHBV-2, and (b) —CH3 groups for HB (3HB) and HV (3HV).

elongation to break also increases as the molar fraction of 3HV increases from 0 to 0.55 mol mol^{-1}; however, when the 3HV reaches to 0.71 mol mol^{-1}, the PHBV-R5 copolymer becomes as brittle as the PHB homopolymer.[38] Values of Young's moduli and elongation to break of PHBV-1 and PHBV-2 were lower than the samples PHBV-R1 and PHBV-R2, respectively, with comparable 3HV molar fraction (0.34 mol mol^{-1}), and this may be due to differences in sample dimensions and preparation.[36,38] Comparison of tensile properties of isolated PHBVs with other PHB, PHBV and commodity plastic indicates these copolyesters have comparable strength and flexibility to LDPE, and therefore these copolyesters obtained from diary manure fermentation could be used as packaging materials, such as sealed and carry bags.[34,40]

^{13}C-NMR Comonomer Sequence Distribution Analysis

The ^{13}C-NMR spectrum of PHBV-2 is shown in Figure 2. The peak assignments are in close agreement with those reported previously.[41] Multiple peaks [Figure 2(b)] are assigned to carbonyl (B_1 and V_1) and methylene (B_2, V_2, and V_4) Cs from different sequence distributions of 3HB and 3HV units (Table III). The carbonyl region is split into four peaks at $\delta = 169.69$, 169.51, 169.50, and 169.32 ppm, being assigned to different diad sequences: 3HV*3HV, 3HV*3HB, 3HB*3HV, and 3HB*3HB, respectively. The triad sequences were determined from a resonance of 3HV side-chain methylene C (V_4 and V_2) composed of four peaks assigned to 3HV-centered triads (3HV-3HV*3HV, 3HB-3HV*3HV, 3HV-3HV*3HB, and 3HB-3HV*3HB) and consistent with those reported by Kamiya

Table II. Molar Mass, Thermal, and Mechanical Properties of PHBV and PHB Samples

	3HV molar fraction (mol mol^{-1})		SEC		DSC			Tensile properties		
Sample	GC-MS	^{1}H-NMR	M_w ($\times 10^5$ g mol^{-1})	M_w/M_n	T_g (°C)	T_m (°C)	X_c (%)	Tensile strength (MPa)	Young's modulus (GPa)	Elongation to break (%)
PHB-C	0	0	4.1	1.9	2.5	175	60.2	22.5	1.31	2.80
PHBV-C1	0.064	0.056	3.0	3.3	5.5	160	44.3	17.0	0.990	10.7
PHBV-C2	0.189	0.197	4.2	2.2	0.9	156	36.2	20.6	1.07	8.77
PHBV-1	0.341	0.34	7.9	2.2	−3.2	147	2.1	15.0	0.299	58.8
PHBV-2	0.324	0.335	7.7	2.1	−2.1	148	1.9	14.8	0.289	57.6
PHBV-R1[36]	0.34		-	-	−9.0	97	-	18.0	1.20	970
PHBV-R2[38]		0.34	-	-	−8.0	97	-	18.0	-	970
PHBV-R3[37]	0.45		5.8	2.9	-	-	-	-	-	-
PHBV-R4[38]		0.55	-	-	−10.0	77	-	16	-	>1200
PHBV-R5[38]		0.71	5.1	2.0	−13.0	83	8.9	11	-	5
PHBV-R6[37]	0.72		3.5	1.4	−30.0	99	7.0	-	-	-

et al.[18] The relative peak intensities obtained for the V_2 and V_4 resonances and the V_1, B_1, and B_2 resonances were determined to estimate the mole, diad, and HV-centered triad sequence distributions. Relative peak intensities of PHBV-1 and PHBV-2 are listed in Table IV.

The parameters, statistical randomness (D) and coefficient R, were used to estimate the degree of randomness of the copolymer based on experimental diad and triad level, respectively.[10,18,41] As shown in Table V, D values were 1.25 and 1.40, whereas the R values were unity for PHBV-1 and PHBV-2, showing that the PHBVs were individual random copolymers not blocky or mixture of random copolymers. Within the experimental uncertainty of the diad and 3HV-centered triad fractions between these two samples are ±0.003 mol mol^{-1}. This result also supports that the copolymers exhibit only one T_m (Table II), while other researchers found samples that are a mixture of random copolymers will show two or three T_m's.[10]

The sequence distribution of diad and triad was calculated using three models.[18,19] The equations are summarized in Table VI. Model 1 used Bernoullian statistics, which is the simplest random copolymer model, and calculation were based on the experimental mole fraction of 3HV (F_V^E).[18] Model 2 is a first-order Markovian model that was used to examine the possibility of block, random and alternative copolymers. As shown in Table V, the four conditional probabilities P_{ij}'s (P_{VV}, P_{VB}, P_{BV}, and P_{BB}) of the probability matrix (P-matrix) were determined. From these P_{ij}'s, sequence distributions were derived.[13,41] It was reported that the biosynthesis distributions of 3HB and 3HV units may be interpreted in terms of a binary copolymerization as shown in four propagation steps[13]. Based on these steps, the selectivities of enzyme reactions of 3HB- and 3HV- terminals of PHBVs were evaluated by the reactivity index r_1r_2 calculated from P_{ij} values and listed in Table V. It can be seen the values of r_1r_2 are near to unity, which indicates that the sequence distribution of 3HB and 3HV units follows an ideal random copolymerization procedure.

Model 3 was a simulation of a mix of two Bernoullian random copolymers.[18] If two Bernoullian model copolymers with the 3HV mole fractions of A and B are mixed with a molar ratio of $X : (1 - X)$, then the three unknowns, A, B, and X can be calculated from the molar fractions of 3HV-centered triad sequences [A, B, $X \in (0,1)$] (Table V).[18]

Table VII gives the molar fractions of diad, and 3HV-centered triads of 3HB and 3HV in respective PHBV-1 and PHBV-2 samples. As stated above both PHBV samples with the D and R values close to 1, their experimental sequence distributions were found to be completely interpreted on the basis of model 1. Within the experimental uncertainty in the measured diad and triad fractions (±0.005) there was good agreement between the observed (experimental) values and calculated distributions based on Bernoullian model 1. Thus, it can be concluded that the comonomer distribution in PHBV produced by mixed microbial consortia was statistically random. Considering that studies on both laboratory and commercial bacterial PHBV samples were shown to be random copolymers, this conclusion seems to be reasonable.[4,7,8,10,41]

ESI-MS^n Analysis of PHBV and PHB

Controlled alkaline hydrolysis of PHB and PHBV samples was catalyzed by KOH/18-crown-6 ether complex. The comonomeric sequence distribution was characterized by ESI-MS^n analysis. Figure 3 shows the negative ion $[M - H]^-$ ESI-MS^n spectra of PH and PHBV oligomers corresponding to the 3HB/3HV oligomeric units and terminated by olefinic and carboxylic end groups from PHB after loss of the crown ether moiety. The PHB oligomers had differences of m/z 86 between 3HB units (Figure 3). The most abundant ion was m/z 515, which corresponds to $3HB_6$ (hexamer) for both commercial PHB and

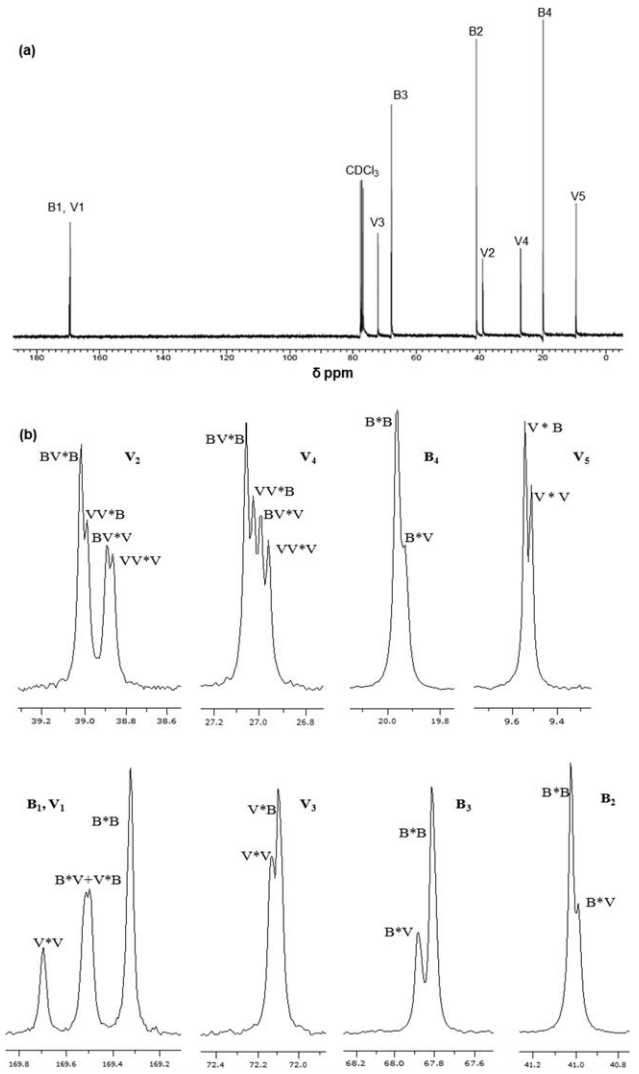


Figure 2. ^{13}C-NMR (a) full spectrum and (b) expanded region showing splitting of individual resonances of PHBV-2 sample.

PHBV samples [Figure 3(a,b)]. Sets of fragment ions at *m/z* 429, 343, 257, and 171 were formed due to successive loss of crotonic acid. This shows evidence that the fragmentation of copolymers occurred due to partial depolymerization. The ESI-MS^n results of isolated PHBV oligomers allowed the identification of their chemical structure up to 22-mer [Figure 3(c)].

The expanded ESI-MS^n spectra of PHBV oligomers as well as peak assignments (pentamer to decamer clusters) are shown in Figure 4. The mass difference between 3HB and 3HV units is *m/z* 14 and shows a distribution of 3HB-3HV oligomers. For example, the ions for the hexamer cluster gave peaks at *m/z* 515 (HB_6), 529 (HB_5HV_1), 543 (HB_4HV_3), 557 (HB_3HV_4), 571 (HB_2HV_5), 585 (HB_1HV_6), and 599 (HV_6) [Figure 4(c)] showing composition and sequence distribution. It can be seen that the highest intensity of the signals in the hexamer decreased with increasing the molar fraction of 3HV for PHBV-C1. For the PHBV-1 (and -2) samples, the intensity of signals in the same oligomer cluster was higher than commercial PHBV

Table III. ^{13}C-NMR Chemical Shifts for Carbonyl and Methylene Carbons in PHBV

Functional group	Chemical shift (δ, ppm)	Sequence
C=O (V_1, B_1)	169.69	3HV*3HV
	169.51	3HB*3HV
	169.50	3HV*3HB
	169.32	3HB*3HB
CH_2 (B_2)	41.02	3HB*3HB
	40.99	3HB*3HV
CH_2 (V_2)	39.02	3HB-3HV*3HB
	38.99	3HV-3HV*3HB
	38.89	3HB-3HV*3HV
	38.87	3HV-3HV*3HV
CH_2 (V_4)	27.06	3HB-3HV*3HB
	27.03	3HV-3HV*3HB
	26.99	3HB-3HV*3HV
	26.96	3HV-3HV*3HV

(PHBV-C1), which was due to the higher 3HV molar fraction [Figure 4(b,c)].

The sequence distribution in the copolymer chains was determined from the relative ion peak intensities in the MS, and the experimental values were used to compare with theoretical values calculated for random copolymers of similar compositions (0.34–0.40 mol mol^{-1} of 3HV units) according to Bernoullian statistics[24]:

$$P_{x,y} = \left(\frac{x+y}{y}\right) P_B^x P_V^y$$

where, P_B and P_v are the molar fractions of 3HB and 3HV in the oligomers.

The differences between experimental and calculated values have been expressed in terms of error by means of the Hamilton agreement factor (AF)[25]:

$$AF = \left[\sum (I_{exp,i} - I_{calcd,i})^2 / \sum I_{exp,i}^2\right]^{1/2}$$

where, $I_{exp,i}$ and $I_{calcd,i}$ are the normalized experimental and calculated abundances of partially degraded copolymers.[24,28] The AF between the calculated and experimental values for each oligomer cluster (from dimer up to 15-mers) as a function of the ratio of P_B to P_V. The results for both commercial and laboratory PHBV samples are given in Figure 5. The best fit of the composition value of laboratory PHBV estimated from the ESI-MS^n characterization was found for the composition ratio around 66/34 (3HB/3HV) calculated for the random copolymer [Figure 5(b)], which is in good agreement with that of ^{1}H-NMR and GC-MS analysis at 0.32–0.34 mol mol^{-1} of 3HV in PHBV-1 and PHBV-2. However, the AF of PHBV-C1 oligomers show a more apparent minimum at 95–94 mol % 3HB units [Figure 5(a)]. The results indicate that the PHBV-C1 was completely randomly distributed and consistent with ^{13}C-NMR spectroscopic data. For the PHBV-1 and −2 oligomers, the differences between the experimental and calculated oligomer

Table IV. Experimental Diad and 3HV-Centered Triad Relative Peak Intensities for PHBV-1 and PHBV-2

Sample	Carbon	HV[b] (mol mol^{-1})[a]	3HV	3HB	3HV* 3HV	3HV* 3HB	3HB* 3HV	3HB* 3HB	3HV-3HV* 3HV	3HB-3HV* 3HV	3HV-3HV* 3HB	3HB-3HV* 3HB
PHBV-1	V1,B1		0.360	0.640	0.134	0.226	0.221	0.419				
	B2			0.672			0.218	0.454				
	V2		0.328		0.132	0.196			0.195	0.208	0.244	0.352
	V4								0.191	0.222	0.247	0.340
	av	0.34	0.34	0.656	0.133	0.211	0.220	0.437	0.066	0.074	0.084	0.119
PHBV-2	V1,B1		0.352	0.648	0.145	0.208	0.193	0.455				
	B2			0.691			0.218	0.454				
	V2		0.309		0.118	0.191			0.205	0.177	0.257	0.360
	V4								0.239	0.236	0.210	0.314
	av	0.335	0.330	0.670	0.131	0.199	0.206	0.454	0.073	0.068	0.077	0.111

[a] Molar fraction of 3HV is determined by ^{1}H-NMR spectroscopy.

Table V. Parameters D, R, Experimental Number Average Sequence Lengths of 3HV Units (L_V^e), Number Average Sequence Length of Randomly Distributed 3HV Units in Copolymer (L_V^R), Ratio Between the Concentration of 3HV and 3HB Units (k), Four probabilities (P_{ij}'s) and the reaction index (r_1r_2) for PHBV-1 and PHBV-2

Sample	D[a]	R[b]	L_V[e]	L_V^R	k	P_{VV}[c]	P_{VB}[c]	P_{BV}[c]	P_{BB}[c]	r_1r_2[d]	A	B	X	Model[e]	SD[f]
PHBV-1	1.25	1	0.85	1.52	1.91									exptl	R
														1	
						0.39	0.61	0.34	0.67	1.25				2	
											0.19	0.55	0.63	3	
PHBV-2	1.40	1	0.85	1.49	2.03									exptl	R
														1	
						0.39	0.60	0.31	0.68	1.45				2	
											0.17	0.59	0.65	3	

[a] $D = F_{VV}F_{BB}/F_{BV}F_{VB}$ (F_{vv}, F_{BB}, F_{BV}, and F_{VB} are exptl values from Table VII.
[b] $R = L_V^R/L_V^E$ [$L_V^E = (F_{VVV} + F_{VVB} + F_{BVV} + F_{BVB})/(F_{BVB} + F_{VVB})$, F_{VVV}, F_{VVB}, F_{BVV}, and F_{BVB} are exptl values from Table VII].
[c] The estimated errors in the values of P_{ij}'s are <0.005.
[d] $r_1 = P_{BB}/P_{BV}$ and $r_2 = P_{VV}/P_{VB}$.
[e] Exptl represent experimental data; 1, 2, and 3 are calculated values by Model 1, Model 2, and Model 3, respectively.
[f] Sequence distribution; *R* indicates the copolymer is random.

Table VI. Model 1, Model 2, Model 3, and Equations Used to Calculate the Diad and Triad Distributions

Model	Calculation[a]	References
Model 1 (Bernoulian Model)	$F_{VV} = (F_V^E)^2$	18,21,23
	$F_{VB} = F_{BV} = F_V^E\ (1 - F_V^E)$	
	$F_{BB} = (1 - F_v^E)^2$	
	$F_{VVV} = (F_V^E)^3$	
	$F_{BVV} = F_{VVB} = (F_V^E)^2\ (1 - F_V^E)$	
	$F_{BVB} = F_V^E\ (1 - F_V^E)^2$	
Model 2 (First-order Markovian Model)	$P_{VV} = F_{VV}^E/F_V^E$	18,23
	$P_{VB} = F_{VB}^E/F_V^E$	
	$P_{BV} = F_{BV}^E/F_B^E$	
	$P_{BB} = F_{BB}^E/F_B^E$	
	$F_{VV} = P_{VV}P_{BV}/(P_{BV} + P_{VB})$	
	$F_{VB} = F_{BV} = P_{VB}P_{BB}/(P_{BV} + P_{VB})$	
	$F_{VVV} = P_{VV}^2P_{BV}/(P_{BV} + P_{VB})$	
	$F_{BVV} = F_{VB} = P_{VV}P_{VB}P_{BV}/(P_{BV} + P_{VB})$	
	$F_{BVB} = P_{VB}^2P_{BV}/(P_{BV} + P_{VB})$	
Model 3 (Mixture of two Bernoullian models or Second-order Markovian Model)	$F_{VVV}^E = A^3X + B^3(1 - X)$	18,19,23
	$F_{BVV}^E = F_{VVB}^E = A^2(1 - A)X + B^2(1 - B)(1 - X)$	
	$F_{BVB}^E = A(1 - A)^2X + B(1 - B)^2(1 - X)$	
	$F_V = AX + B(1 - X)$	
	$F_B = (1\text{-}A)X + (1 - B)(1 - X)$	
	$F_{VV} = A^2X + B^2(1 - X)$	
	$F_{VB} = F_{BV} = A(1 - A)X + B(1 - B)(1 - X)$	
	$F_{BB} = (1 - A)^2X + (1 - B)^2(1 - X)$	
	$F_{VVV} = A^3X + B^3(1 - X)$	
	$F_{BVV} = F_{VVB} = A^2(1 - A)X + B^2(1 - B)(1 - X)$	
	$F_{BVB} = A(1 - A)^2X + B(1 - B)^2(1 - X)$	

[a] Subscript *E* is values determined experimentally; F_X, F_{XY}, and F_{XVY} indicate mole fractions of sequence *X*, *XY*, and *XVY*, respectively, where *X*, *Y* = *V* or *B*; Four conditional probabilities P_{ij}'s (*i*,*j* = *V* or *B*) is conditional probability of *j* addition following the i unit at the propagation chain end with the relations that $P_{BV} + P_{BB} = 1$ and $P_{VB} + P_{VV} = 1$; *A*, *B*, *X* are following the convergence condition [F_{VVV}^E, F_{VVB}^E, F_{BVB}^E, A, B, X ∈(0,1)].

Table VII. Experimental and Calculated Mole, Diad, and 3HV-Centered Triad Sequence Distributions of PHBV-1 and PHBV-2

Sample	Model[a]	Fv[b]	F_V[c]	F_B[c]	F_{VV}[c]	F_{BV}[c]	F_{BB}[c]	F_{VVV}[c]	$F_{BVV/VVB}$[c]	F_{BVB}[c]
PHBV-1	exptl	0.34	0.34	0.66	0.13	0.22	0.44	0.06	0.08	0.12
	1		0.34	0.66	0.12	0.22	0.43	0.04	0.08	0.15
	2		0.36	0.64	0.14	0.22	0.43	0.05	0.08	0.13
	3		0.32	0.68	0.13	0.19	0.49	0.07	0.07	0.12
PHBV-2	exptl	0.335	0.33	0.67	0.13	0.20	0.45	0.07	0.07	0.11
	1		0.33	0.67	0.11	0.22	0.45	0.04	0.07	0.15
	2		0.33	0.67	0.13	0.21	0.45	0.05	0.08	0.12
	3		0.31	0.69	0.14	0.18	0.51	0.07	0.06	0.11

[a] Exptl represent experimental data; 1, 2, and 3 are calculated values by Model 1, Model 2, and Model 3, respectively.
[b] 3HV molar fraction (mol mol^{-1}) determined by ^{1}H-NMR spectroscopy.
[c] F_X, F_{XY}, and F_{XVY} indicate mole fractions of sequence *X*, *XY*, and *XVY*, respectively, where *X*, *Y* = *V* or *B*.

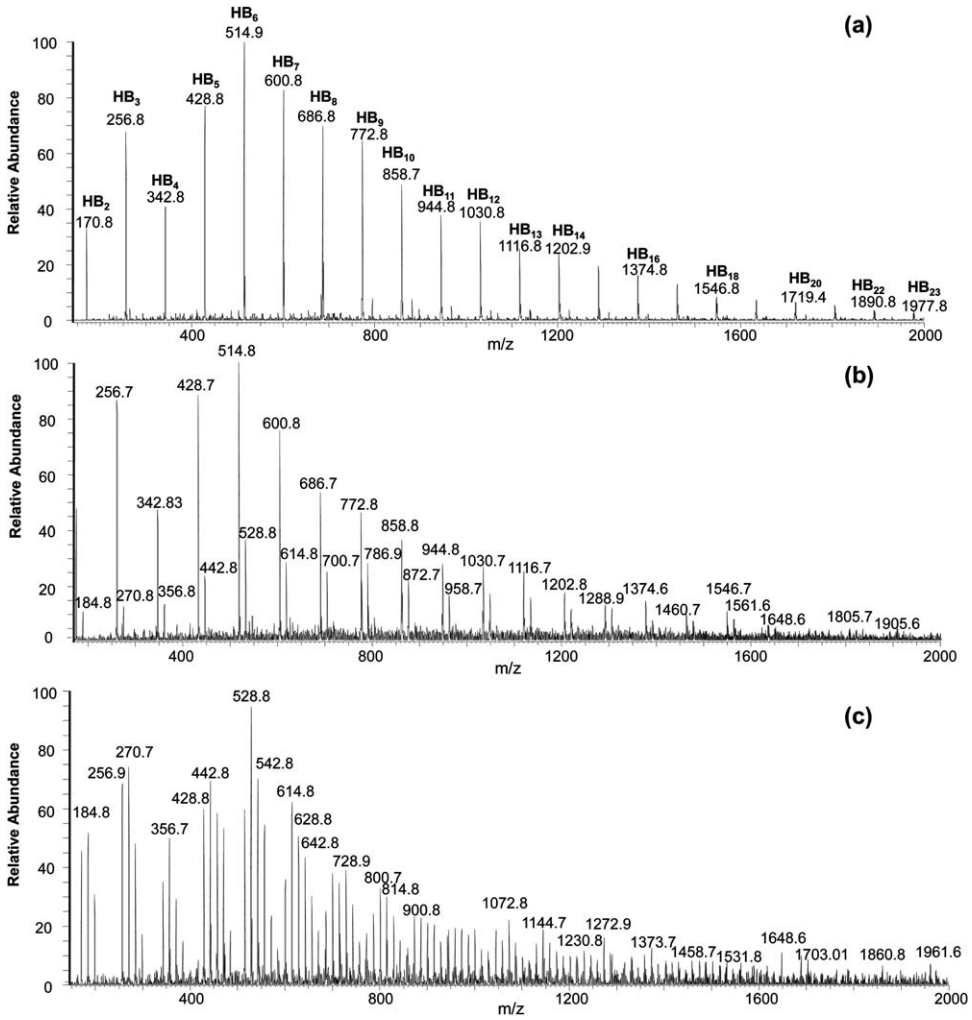


Figure 3. Negative ion ESI-MS spectra of partially hydrolyzed (a) PHB-C, (b) PHBV-C1, and (c) PHBV-1.

distributions (Bernoullian chain statistics model) reflected by AF were slightly larger than that of commercial PHBV oligomers. Thus, it could be concluded that the sequence distribution of PHBV copolymers determined by ESI-MS^n are consistent with those obtained by ^{13}C-NMR analysis.

To study the random composition and distribution of isolated PHBV comonomer units in each individual oligomer chain MS-MS fragmentation was conducted. The MS-MS experiments were on the ion at *m/z* 543 (HB_4HV_2) with two 3HV units being selected from hexamer cluster. This ion was selected since it was the most intense cluster (see Figure 3) and it has two 3HV units, which means it has 2 3HV units randomly positioned along the macromolecular chain. The MS-MS spectrum of *m/z* 543 showed that the fragmentation induces a set of clusters containing 2 fragment ions in the first stage while 3 fragment ions in the following steps with the same degree of oligomerization but different 3HV molar fraction by successive loss of a crotonic acid (*m/z* 86) or 2-pentenoic acid (*m/z* 100) from the carboxylic end (Figure 6). Figure 7 shows visually the fragmentation pathways during the partial depolymerization procedure, which showed that two pentamers were generated: HB_3HV_2 (*m/z* 457) and HB_4HV (*m/z* 443). In the following steps, the clusters containing three fragment anions were formed successively. For example, the cluster of fragment ions corresponding to 4-mer (Figure 6: *m/z* 371, *m/z* 357, and *m/z* 343) the ion *m/z* 371 was generated by the pentamer *m/z* 457 (HB_3HV_2) when losing a crotonic acid; while 2-pentenoic acid is eliminated the *m/z* 357 ion was formed, and this ion might be coming from *m/z* 443 (HB_4HV) by loss a crotonic acid. The smallest fragment ion *m/z* 171 (HB_2) was possibly formed by either the fragment ion *m/z* 271 (HB_2HV) through losing 2-pentenoic acid or the *m/z* 257 (HB_3) by expulsion of a crotonic acid. This pathway was consistent with the MS-MS spectrum in Figure 6. This result confirmed that PHBV samples with relatively high 3HV molar fraction of this work have a random distribution of 3HB and 3HV units along the copolymer chain, which was comparable with other researches on commercial PHBV sample containing low 3HV fraction (0.056 mol mol^{-1}).[24]

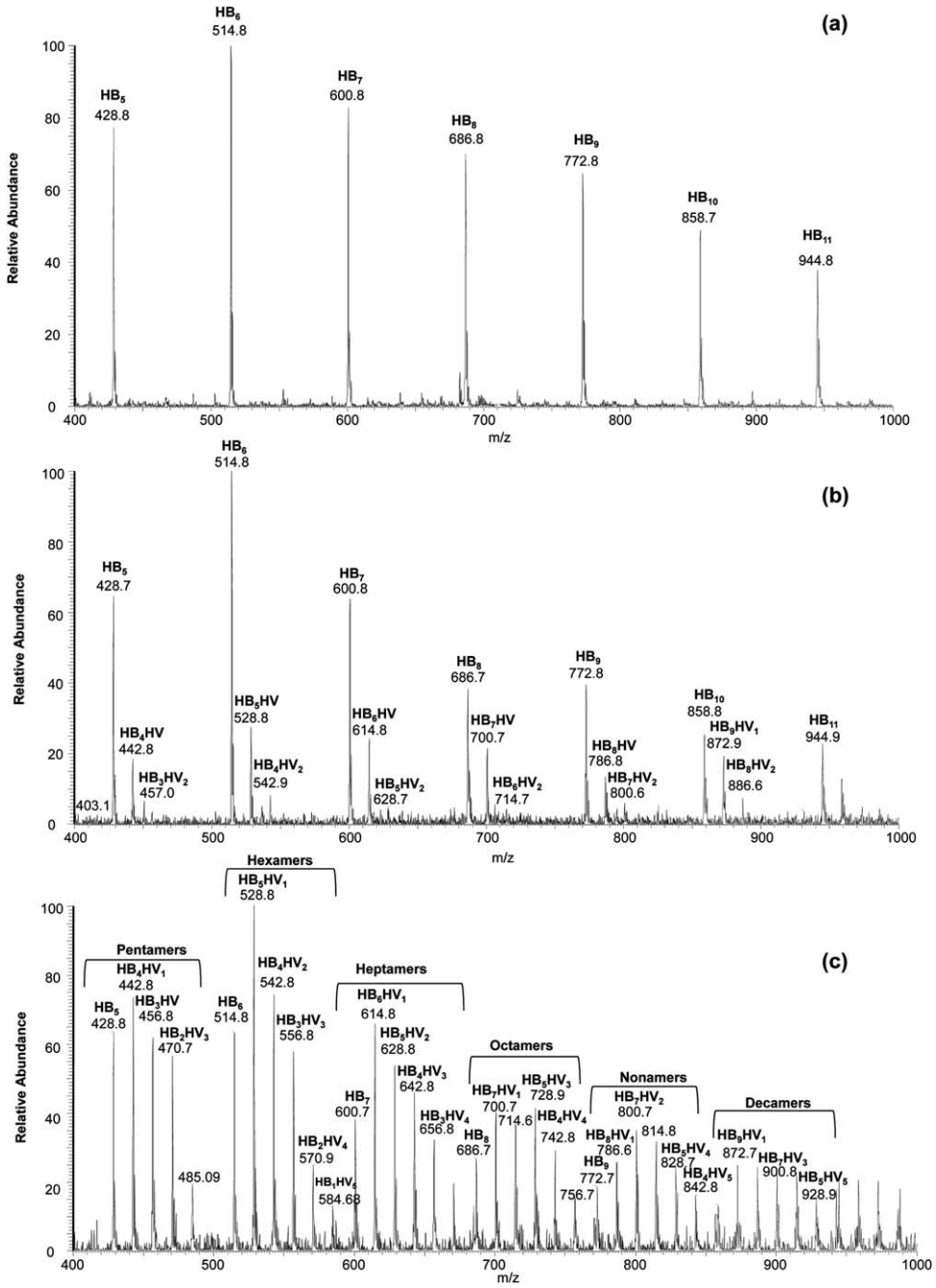


Figure 4. Expanded negative ion ESI-MS spectra of oligomers from partially hydrolyzed (a) PHB-C, (b) PHBV-C1, and (c) PHBV-1.

CONCLUSIONS

In this study we successfully biosynthesized PHBV continuously for 11 months from VFA, obtained from fermented dairy manure, using a mixed microbial consortia. A comprehensive characterization of the isolated PHBV by a combination of mechanical and thermal properties, NMR spectroscopy, and ESI-MS^n were done. The isolated PHBVs showed comparable mechanical properties as compared with other studies on PHBV copolymers with similar composition. The 3HV and 3HB sequence of isolated PHBVs were shown to be completely random copolymers based on Bernoullian and Markovian models using the diad and triad sequence distribution and comparable to commercial samples. These sequence distributions were interpretable on the basis of a model of individual random copolymers not a mixture of two or more random copolymers. This confirms that only a single T_m was observed for PHBVs. The NMR experiments required long acquisition times and sample throughput would be limited. While the controlled hydrolysis

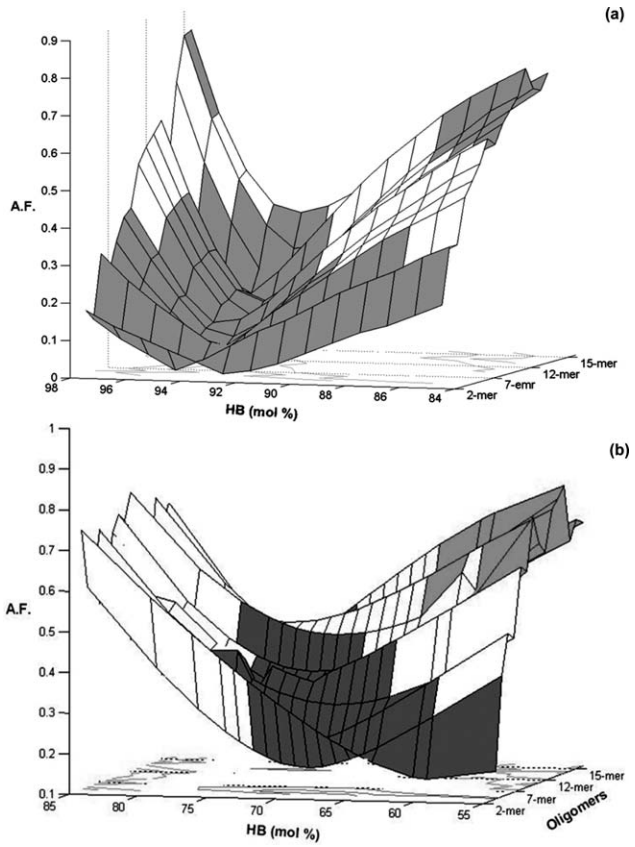


Figure 5. Calculated AF as a function of molar fraction of 3HB (mol %) corresponding to individual PHBV oligomers for (a) PHBV-C1 and (b) PHBV-1 sample.

followed by ESI-MS^n analysis approach was relatively rapid and it could be easily automated for routine analysis of PHBV copolymers. We have developed these analytical tools for the future analysis of tailored PHBV polymers currently undertaken in our laboratory by (i) controlling the feeding regimes of VFA and (ii) cross-linking during processing.

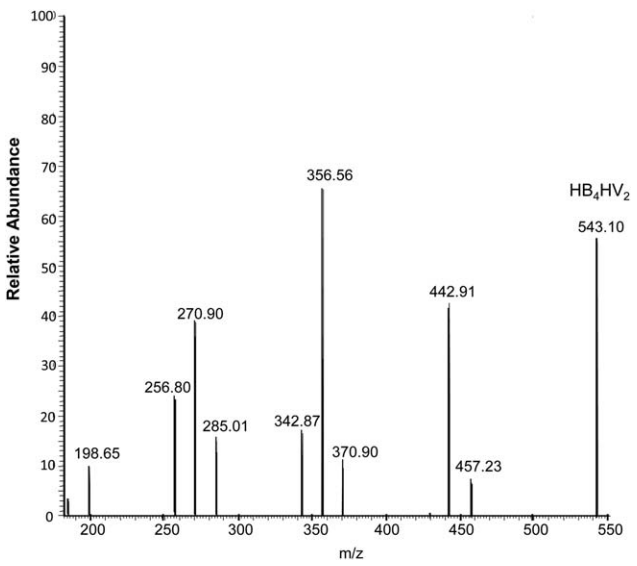


Figure 6. Sequence fragmentation spectrum (MS-MS, negative ion) obtained for isolated PHBV parent ion *m/z* 543.10 (hexamer cluster HB_4HV_2).

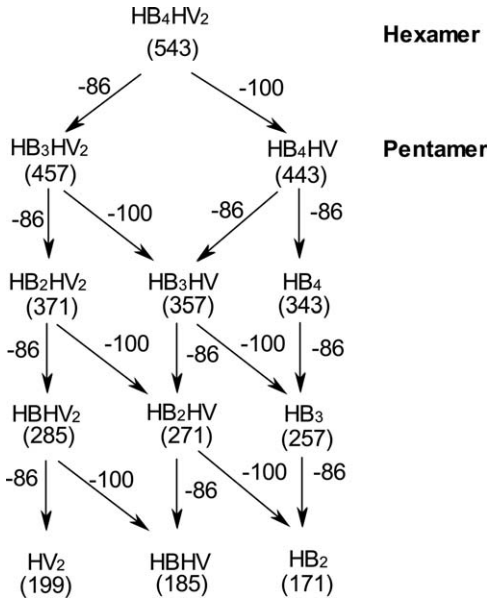


Figure 7. Fragmentation pathway as a result of MS-MS experiment for isolated PHBV parent ion *m/z* 543.10 (hexamer cluster HB_4HV_2).

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