Characterization of New Bacterial Copolyesters Containing 3-Hydroxyoxoalkanoates and Acetoxy-3-hydroxyalkanoates

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ABSTRACT: Two novel bacterial poly(3-hydroxyalkanoates) (PHAs) with either 3-hydroxy-7-oxooctanoate (HOO) and 3-hydroxy-5-oxohexanoate (HOH) or 8-acetoxy-3-hydroxyoctanoate (AHO), 6-acetoxy-3-hydroxyhexanoate (AHH), and 4-acetoxy-3-hydroxybutyrate (AHB) monomer units were produced at pilot scale. For the biosynthesis of these PHAs *Pseudomonas oleovorans* was cultivated at a 24 L scale in two-liquid-phase fed-batch processes using mineral salts medium and mixtures of 2-octanone/octane or *n*-octylacetate/octane as carbon sources. The bacterial accumulation of the polyesters was induced by nitrogen starvation and the addition of the substrate mixtures. Under these conditions, 26 and 45 g of PHA were isolated. The PHAs contained 10.3 and 3.3 mol % of oxo and acetoxy group monomers. Physical characterization was done with respect to their molecular weights, and thermal properties and similar results were found as for octane-based PHA. All monomer units have been identified by 2D ^{1}H and ^{13}C heteronuclear correlated NMR spectroscopy (HSQC, HMBC, and HSQC-TOCSY), and the composition of the copolyesters was quantified from ^{1}H NMR spectra.

Introduction

A variety of different poly(3-hydroxyalkanoates) (PHAs) have been described and characterized. The most prominent member of this group of biopolymers is a copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate (PHB/V), which was produced as BIOPOL in a multiton scale by ICI. Since PHB/V is not an elastomer, its use is limited to thermoplastic applications. A large set of medium-chain-length PHAs are accumulated as storage material by a variety of bacteria, of which *Pseudomonas oleovorans* was found to be one of the most versatile.[1] These mcl-PHAs can also contain 4- or 5-hydroxy acid repeating units,[2] different side chain lengths (C_3−C_{16}),[3] or additional functional groups.[4] These functionalities as well as the specific chain lengths were shown to influence the physical properties of the materials such as crystallinity, brittleness, and elasticity. Functional groups also allowed processing of the polyester as demonstrated with the production of biodegradable rubber from PHA-containing unsaturated groups.[5]

However, only a few of these functionalized mcl-PHAs have been produced in gram or kilogram amounts[6−8] with most of them containing olefinic functionalities.[5,9] The majority of the other functional poly(hydroxyalkanoates) reported[10] were only found in analytical amounts not sufficient for isolation, physical characterization, and application development. The reason for this bottleneck in availability is that the substrates used for the production of these functionalized PHAs are in most cases toxic to the cells. This makes the synthesis of many functionalized PHAs a difficult task.[11]

In this paper we report the laboratory-scale and the pilot-scale synthesis of gram amounts of two novel functionalized mcl-PHAs using *Pseudomonas oleovorans* grown in nutrient solutions containing 2-octanone or *n*-octyl acetate, respectively. The different monomer substructures were resolved completely from 1D and 2D NMR spectra.

Results and Discussion

Polyester Biosynthesis. The two copolyesters of 3-hydroxy-7-oxooctanoate (HOO), 3-hydroxy-5-oxohexanoate (HOH), 3-hydroxyoctanoate (HO), 3-hydroxyhexanoate (HH), 8-acetoxy-3-hydroxyoctanoate (AHO), 6-acetoxy-3-hydroxyhexanoate (AHH), 4-acetoxy-3-hydroxybutyrate (AHB), 3-hydroxybutyrate (HB), HO, and HH units (see Figure 1) were produced by *P. oleovorans* using the substrate mixtures octane and 2-octanone or octane and *n*-octylacetate as carbon sources. Both processes were first established at a 1 L level and were then scaled up to 24 L pilot-scale fermentations in order to obtain large quantities of the functionalized polyesters.

The developed fed-batch process consisted of two phases: During the growth phase, octane was present as the sole carbon source. For growth control, a nitrogen stock solution was added pulsewise to the culture until sufficient amounts of biomass were produced (from 12.3 to 13.5 g L^{-1} biomass). The subsequent PHA accumulation phase was induced by nitrogen starvation and the addition of the substrates (2-octanone/octane and *n*-octyl acetate/octane) which provided the precursors for the formation of the functionalized polyesters (see Figure 2, arrows). Both substrate mixtures were dissolved in the carrier solvent hexadecane, a long chain alkane, which is not metabolized by *P. oleovorans* and which has been shown to reduce the toxicity of medium chain length alkanes to bacterial cultures.[12] In the present experiments the use of hexadecane allowed undisturbed growth of *P. oleovorans* whereas cell viability was

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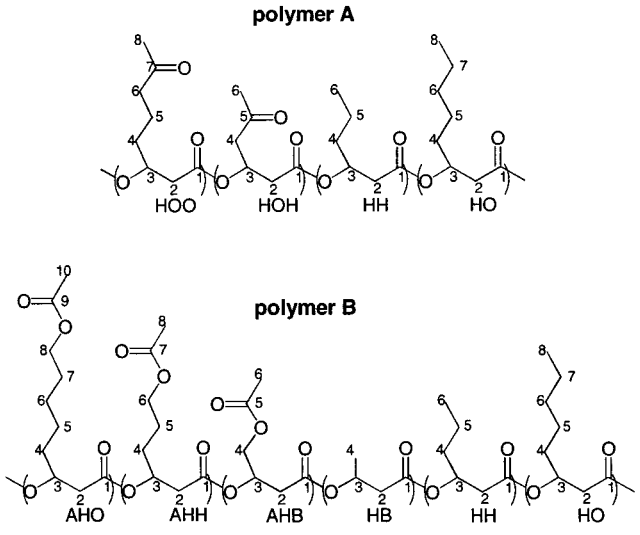

Figure 1. Structures of monomer units for polymer A and B with the notation used for the ^{1}H and ^{13}C chemical shift assignments. HOO = 3-hydroxy-7-oxooctanoate, HOH = 3-hydroxy-5-oxohexanoate, HH = 3-hydroxyhexanoate, HO = 3-hydroxyoctanoate, AHO = 8-acetoxy-3-hydroxyoctanoate, AHH = 6-acetoxy-3-hydroxyhexanoate, AHB = 4-acetoxy-3-hydroxybutyrate, and HB = 3-hydroxybutyrate.

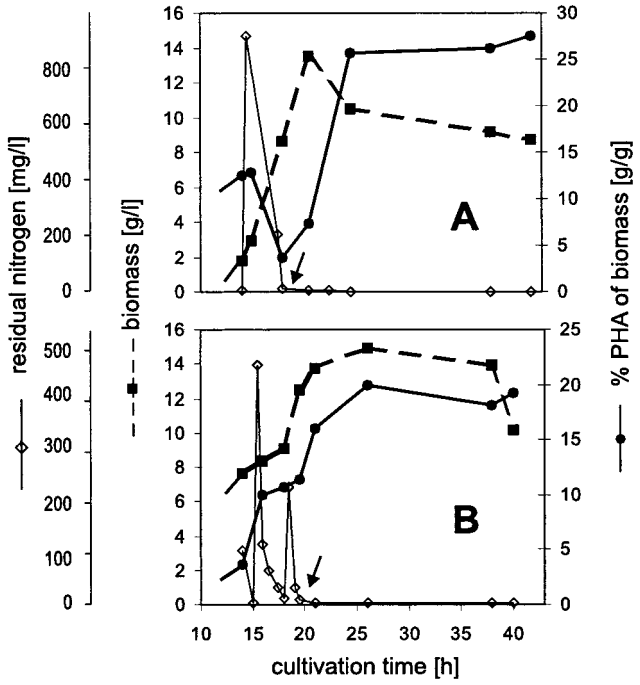

Figure 2. Time course of the pilot-scale (24 L) productions of functionalized polymers from mixtures of 2-octanone/octane (A) and *n*-octylacetate/octane (B) using *Pseudomonas oleovorans* in two-liquid-phase fed-batch processes. The arrows indicate the addition of the substrate mixtures.

markedly reduced when only the functionalized substrates were present (data not shown). After the addition of the 2-octanone and *n*-octylacetate mixtures, biomass increased for a period of several hours; all of the biomass increase could be accounted for by the accumulation of functionalized PHA (Figure 2).

After the period of PHA accumulation, biomass dropped slightly when *n*-octylacetate was present and more significantly when 2-octanone was the substrate. The fermentations on 2-octanone and *n*-octylacetate yielded 228 and 264 g of biomass from which 26.2 and 45.0 g of PHA (polymers A and B) were extracted, resulting in PHA recovery yields of 0.43 and 0.89 g g^{-1} for polymer A and B, respectively. Part of polymer A was lost during the precipitation step, which led to a cloudy suspension while polymer B formed larger aggregates leaving a clear mixture. However, comparable recovery yields could most likely be achieved for both polymers by adapting the process to the specific needs of each polymer. The cultivation data, the physical properties, and the molar monomer composition of both functionalized polyesters are summarized in Table 1.

GC analysis was a useful tool for the determination of the cellular PHA content. However, for detailed analysis of polymer compositions the method was unsuitable due to missing monomer standards, which would be used especially for the unambiguous identification of the rather small amounts of the newly incorporated compounds. Detailed characterization of the polymer structures was therefore made by NMR analysis.

NMR Characterization of mcl-PHA Containing Oxo Groups (Polymer A). The ^{1}H NMR spectrum of polymer A is dominated by the strong signals of HO. In addition, there are weak resonances from the keto-functionalized monomer units HOO and HOH. All HOH proton resonances H-(2,3,4,6) are directly observable. The H-(8) resonance at 2.12 ppm indicates the occurrence of the HOO monomer unit; however, other ^{1}H resonances (H-(2−6)) of this monomer are covered by the strong signals of HO and HH. In contrast, in the 1D ^{13}C NMR spectrum all carbon resonances of HOO are visible whereas the corresponding signals of HOH are scarcely detectable due to the low amount of this monomer in the polyester. Therefore, 2D ^{1}H,^{13}C correlated NMR spectra were analyzed to unambiguously assign the ^{1}H and ^{13}C resonances from HOO and HOH in polymer A. The chemical shifts obtained from these experiments are summarized in Table 2. The polymer composition was determined from the ^{1}H NMR spectrum and is shown in Table 1. The observed chemical shifts and the incessant assignment of neighboring carbon atoms and protons along the carbon chains of HOO and HOH (see HMBC correlations, Table 2) confirm that the ketones are not present as starting compound impurities and must therefore result from metabolic activity.

No chemical shift dispersion of ^{13}C NMR signals as a result of copolymer repeat unit sequence effects was observed.[13,14] Also, no correlation signal between H-(3) of HOH and C-(1) of HO at 5.47/169.1 ppm to support a copolymer structure has been found. However, this signal may be too small to be detectable due to the low abundance of HOH in the polymer or be absent at all due to a H-(3)/C-(3)/O/C-(1) torsion angle near 90° and consequently $^{3}J(^{1}H,^{13}C) \approx 0$. More intense ^{3}J correlations signals between H-(3) and C-(1) of neighboring units might be present. Since the ^{1}H resonances H-(3) of HH, HO, and HOO overlap at 5.17 ppm, these signals between successive copolymer units were masked from H-(3)/C-(1) correlations within individual components.

In contrast to the results from physical characterizations (see below), NMR experiments cannot provide a positive proof that polymer A is indeed a copolymer with random distribution of the monomer units and not a blend of homopolymers. Furthermore, Figure 2 shows that a significant amount of polymer was already present in the cells before addition of octanone. Mcl-PHA metabolized from this substrate could therefore accumulate in newly formed or already existing polymer

Table 1. Process Data, Characteristics, and Composition of Functionalized PHAs from *Pseudomonas oleovorans* Grown on Mixtures of *n*-Octane and 2-Octanone (Polymer A) or *n*-Octyl Acetate (Polymer B)

polymer	C source	biomass (g L^{-1})	% PHA[a] (wt %)	isolated PHA (g)	T_m (°C)	M_w (kg mol^{-1})	M_w/M_n	copolyester composition[b] (mol %)							
								AHO	AHH	AHB	HB	HH	HO	HOO	HOH
A	2-octanone/ octane	9.5	26.5	26.2	48.4	228.5	2.94					5.6	84.1	9.7	0.6
B	*n*-octylacetate/ octane	11.0	19.1	45.0	49.7	299.3	3.41	1.2	1.2	0.9	2.9	11.1	82.7		

[a] PHA content of biomass. [b] Determined from ^{1}H NMR data.

Table 2. Assignment of the ^{1}H and ^{13}C Chemical Shifts and $^nJ(^1H,^{13}C)$ (n = 2, 3) Correlations Observed for the Incorporated Comonomer Units 3-Hydroxy-7-oxooctanoate (HOO) and 3-Hydroxy-5-oxohexanoate (HOH) in Polymer A

position	HOO			HOH		
	$\delta(^1H)$[a]	$\delta(^{13}C)$[b]	$^nJ(^1H,^{13}C)$ correlations	$\delta(^1H)$[c]	$\delta(^{13}C)$	$^nJ(^1H,^{13}C)$ correlations
1		169.2	*d*		168.9[e]	H-(2, 3), C-(1)
2	2.52	39.0	*d*	2.63	38.6[a]	H-(4), C-(2)
3	5.17[f]	70.9	*d*	5.47	67.2[e]	H-(2, 4), C-(3)
4	1.57	33.1	H-(5, 6), C-(4)	2.82	46.6[a]	H-(2, 3, 6), C-(4)
5	1.58	19.2	H-(3, 4, 6), C-(5)		204.7[e]	H-(3, 4, 6), C-(5)
6	2.42	42.9	H-(4,8), C-(6)	2.16	30.4[a]	
7		207.6[e]	H-(5, 6, 8), C-(7)			
8	2.12[c]	29.9				

[a] From ^{1}H,^{13}C HSQC correlations. [b] From 1D ^{13}C{^{1}H} spectrum and ^{1}H,^{13}C HSQC correlations. [c] From 1D ^{1}H spectrum. [d] No correlations resolved due to overlapping with strong signals from HO and HH. [e] From ^{1}H,^{13}C HMBC correlations. [f] From ^{1}H,^{13}C HSQC-TOCSY correlation.

Table 3. Assignment of the ^{1}H and ^{13}C Chemical Shifts and $^nJ(^1H,^{13}C)$ (n = 2, 3) Correlations Observed for the Incorporated Comonomer Units 8-Acetoxy-3-hydroxyoctanoate (AHO), 6-Acetoxy-3-hydroxyhexanoate (AHH), and 4-Acetoxy-3-hydroxybutyrate (AHB) in Polymer B

position	AHO			AHH			AHB		
	$\delta(^1H)$	$\delta(^{13}C)$	$^nJ(^1H,^{13}C)$ correlations	$\delta(^1H)$	$\delta(^{13}C)$	$^nJ(^1H,^{13}C)$ correlations	$\delta(^1H)$	$\delta(^{13}C)$	$^nJ(^1H,^{13}C)$ correlations
1		~169.3			~169.3			~169.3	
2	2.53	39.0		2.55	39.0		2.62	35.8	H-(4), C-(2)
3	5.17	70.7		5.18	70.3	H-(4), C-(3)	5.37	68.2	H-(2), C-(3)
4	1.60	33.9		1.65	30.3	H-(2, 6), C-(4)	4.11/4.28	64.1	H-(2), C-(4)
5	1.27	24.7	H-(3, 7), C-(5)	1.65	24.3	H-(4, 6), C-(5)		170.4	H-(4, 6), C-(5)
6	1.33	25.6	H-(8), C-(6)	4.02	63.8		2.04	20.6	
7	1.60	28.4	H-(8), C-(7)		170.9	H-(6, 8), C-(7)			
8	4.02	64.3	H-(7), C-(8)	2.02	20.8				
9		170.9	H-(8, 10), C-(9)						
10	2.02	20.8							

chains, which then result in different types of homo- or heteropolymers. Investigations in continuous culture and further chemical and physical analysis may resolve this problem, leading to a more detailed understanding of polymer formation from different substrates in bacterial cell cultures.

NMR Characterization of mcl-PHA Containing Acetoxy Groups (Polymer B). The chemical shift assignments of the monomer units AHO, AHH, and AHB are shown in Table 3, and the copolymer composition is summarized in Table 1. Although the ^{1}H NMR resonances of HB are partly hidden by overlapping signals of HO and HH, the observed correlation signals in the 2D ^{1}H,^{13}C NMR spectra indicate unambiguously the presence of HB in small amounts. This is an unusual finding for PHA formed by *P. oleovorans*.[15] In Figure 3 the ^{1}H,^{13}C HSQC-TOCSY spectrum of polymer B is shown, where H/C correlations of HB are assigned. This special NMR experiment has been performed since polymer B is a mixture of six rather similar monomer units in partly low concentrations and with strongly overlapping resonance signals (^{1}H and ^{13}C). After the gradient selected excitation of protons directly attached to ^{13}C nuclei, this ^{13}C edited proton magnetization is transferred to all ^{1}H nuclei in the same spin system by the TOCSY sequence. In this way otherwise rather confusing crowds of correlation signals become clearly interpretable. In Figure 3 also some crucial correlation signals to prove the presence of AHO and AHH are indicated. The HSQC-TOCSY spectrum was indispensable for the assignment of the signals of the monomer units AHO and AHH, whereas the NMR resonances of AHB are free from HO and HH overlapping, and the presence of this unit could be corroborated from the $^nJ(^1H,^{13}C)$ spectra (n = 1, 2, or 3) alone. Only the sum of the concentrations of AHO and AHH can be derived from the ^{1}H NMR spectra since NMR signals at 2.02 and 4.02 ppm from both components overlap. Their concentrations were set equal since the methyl carbon resonances at 20.84 and 20.79 ppm in the 1D ^{13}C NMR spectrum have comparable intensities.

Physical Characterization of the Polyester. Table 1 summarizes the production data and the physical properties of the copolyesters A and B. Melting temperatures (T_m) of 48 and 50 °C for polymer A and B were found. The incorporation of the oxo and acetoxy groups into the polyester side chain resulted in a decrease of T_m of about 12 °C compared to octane derived PHA (T_m = 61 °C, T_g = −29 °C[5]), which is the most prominent member of the polyesters synthesized by *Pseudomonas oleovorans*. Accordingly,[5] it was found that the incorporation of 5% olefinic monomer units into an octane

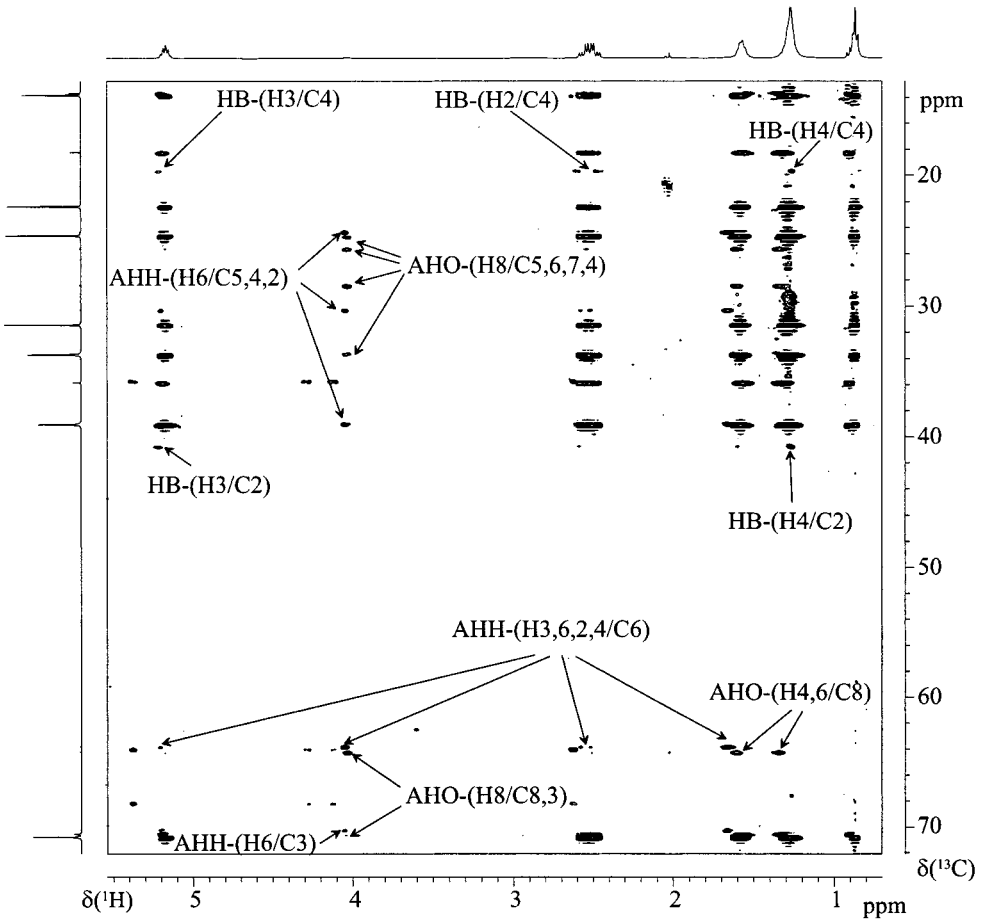

Figure 3. $^{1}H,^{13}C$ HSQC-TOCSY spectrum of polymer B with the assignment of selected correlation signals of the minor monomer units AHO, AHH, and HB (see materials and methods for experimental conditions).

derived PHA decreased T_m by 6 °C. The authors suggested that the lower T_m was due to decreased crystallinity of the functionalized polymer. The weight-average molar mass (M_w) of the PHAs isolated were in the range 230−300 kg/mol for both polymers (Table 1). Thus, the incorporation of the functional groups obviously resulted in increased molecular weights compared to octane derived PHA (M_w of 193 kg/mol).[5]

The incorporation of functional groups into polyester in order to design improved polymer properties can be done for two reasons: Either the functional groups modify the material characteristics or they can be a handle for polyester processing via chemical derivatization. The physical properties of the newly synthesized materials are generally similar to those found for mcl-PHA from *P. oleovorans*; the major differences affect thermal properties and molecular weights. The functional groups, however, offer a series of interesting possibilities to process these materials further to achieve specific material requirements as discussed for poly(*β*-malic acid) derivatives with drug and cross-linking pendant groups.[16] Ongoing investigations in our lab are therefore focusing on modification of the polymer properties by chemical or biological treatment of the polyesters and on the analysis of the properties of these postprocessed materials.

Materials and Methods

Bacterial Strain and Media. *Pseudomonas oleovorans* Gpo1 (ATCC 29347) was used in all experiments. The mineral salts medium (batch medium) contained 28 mM $(NH_4)_2SO_4$, 15 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mL/L trace element solution,[17] and 0.5% (v/v) PPG2000 antifoam. Stock solutions of all aqueous medium components and the carbon sources octane, 2-octanone/octane/hexadecane, and *n*-octylacetate/octane/hexadecane were prepared and sterilized separately and added to the culture as indicated in the text. All chemicals used were purchased from Fluka (Fluka, Switzerland) with purities >97%.

Polyester Biosynthesis. Cultivation of *P. oleovorans* at lab-scale was done with 1 L working volume in a stirred tank reactor.[18] For pilot-scale fermentation a 40 L bioreactor (MBR Bioreactor, Switzerland) with a working volume of typically 24 L was used. The standard culture conditions were 30 °C and pH 7. The pH was regulated automatically using 2 N sodium hydroxide. Stirring speed and aeration were 1500 rpm and 1 L air per minute in lab-scale and 700 rpm and 24 L air per minute in pilot-scale fermentations. The cultivations were carried out in 4-fold concentrated E2 containing 2% (v/v) octane.[17] For achieving increased biomass concentrations extra nitrogen was pulsed to the culture broth to a final concentration of 500−900 mg L^{-1} after the onset of nitrogen starvation. The last nitrogen pulses before polymer production started are shown in Figure 2. To start the formation of functionalized polyester, 1 L of hexadecane containing 20% (v/v) 2-octanone or *n*-octylacetate and 2% (v/v) octane was added when all nitrogen was used up (indicated with arrows, Figure 2). Biomass was harvested by centrifugation when constant PHA levels were reached. The polymer was extracted from the lyophylized biomass by Soxhlet extraction using CH_2Cl_2 and the raw product precipitated twice in ice cold methanol for purification.

Characterization of the PHAs. Cultivation data such as biomass and nitrogen concentration were determined as described elsewhere.[19] The cellular PHA content was determined by methanolysis of lyophilized biomass and by subse-

quent gas chromatographic analysis of the resulting hydroxyalkanoic acid methyl esters.[17]

Molecular weights were obtained by gel permeation chromatography (GPC) (Knauer, Germany). Samples were dissolved in tetrahydrofuran and injected in a PL-Gel mixed C column, 5 μm, 7.5 × 600 mm (Polymer Laboratories, UK). The flow rate was 1.1 mL min^{-1} at 45 °C. For detection a viscosity detector (H502, Viskotek, Germany) and for calibration narrow-dispersed polystyrene standards (Polymer Laboratories, UK) were used. Thermal properties were recorded using a Mettler TA4000 (Mettler Toledo, Switzerland) differential scanning calorimeter (DSC). For thermoanalysis the samples were heated from −60 to 100 °C (10 °C min $^{-1}$).

NMR spectra were recorded on a Bruker AMX-400 NMR spectrometer at 300 K using a 5 mm broad-band inverse probe with a *z*-gradient (100% gradient strength of 10 G/cm). Saturated solutions of the polymers in chloroform-*d* were filtered over Celite. Chemical shifts are given in parts per million (ppm) relative to the remaining signals of chloroform as internal reference (^{1}H NMR: 7.26 ppm; ^{13}C NMR: 77.0 ppm). The ^{1}H NMR (^{13}C NMR) spectra were recorded at 400.13 MHz (100.61 MHz) with the following parameters: 8.4 μs (10.2 μs) 90° pulse lengths, 128 (2048) transients, 8000 Hz (23 600 Hz) spectral widths, 32K data points, and 10 s relaxation delays. The ^{13}C NMR spectra were recorded in the inverse gated mode, using a proton decoupling field of 3.3 kHz during acquisition (WALTZ16[20]). 2D experiments were acquired as 1024 × 512 data matrices and zero filled to 1024 × 1024. The HSQC[21] (HMBC[22]) experiments were performed with the selection of ^{1}H,^{13}C coupling constants of 130 Hz (10 Hz), gradient strengths of −40:10 (15:9:12), and 16 transients per increment with a carbon decoupling field of 3.8 kHz for the HSQC experiments (GARP decoupling[23]). The HSQC-TOCSY[24] spectra were recorded with the selection of $^1J(^1\text{H},^{13}\text{C}) = 130$ Hz and a 29 μs 90° pulse length for the TOCSY transfer with a total mixing time of 192 ms, applying the above-mentioned carbon decoupling conditions. ^{1}H and ^{13}C NMR chemical shifts of the monomer units HO and HH were assigned by ^{1}H,^{13}C shift correlation experiments, and they agree with data based on empirical relations given by Doi and Abe.[14]

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