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Miscibility of Natural Polyhydroxyalkanoate Blend with Controllable Material Properties

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ABSTRACT: Polyhydroxyalkanoate (PHA) copolyesters were synthesized by *Cupriavidus necator* cells in continuous feeding of cosubstrates. During the PHA accumulation phase, the composition of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 4-hydroxyvalerate (4HV) of the copolyesters changed with time, resulting in a change in their miscibility. The as-produced PHA finally became a miscible blend of copolymers with a broad chemical composition distribution. The good miscibility and low crystallinity of the natural P(3HB-*co*-3HV-*co*-4HV) blend lead to a remarkable increase in ductility and elongation at break. It indicates that the material properties of copolyesters can be tailored via feeding control of cosubstrates. It was also found that the fractions of natural PHA blend exhibited distinctive thermal behavior and the overall behavior of the as-produced PHA blend was primarily dependent on a fraction of high 3HB content. The material properties of a PHA blend are therefore not determined by its overall chemical composition but more likely by the combined effect of individual copolyesters or fractions. Moreover, the degree of X-ray crystallinity of random P(3HB-*co*-3HV-*co*-4HV) blend declined significantly with the increase of 3HV and 4HV content, in contrast to the high crystallinity of well-known P(3HB-*co*-3HV) copolyesters. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 2004–2016, 2013

KEYWORDS: polyhydroxyalkanoates; bacterial co-polyesters; blend miscibility; biopolymer physical properties

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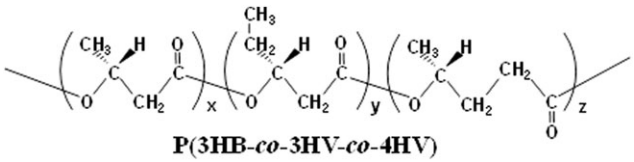
INTRODUCTION

Blends of polymers of distinctly different composition can be miscible, immiscible, or partially miscible, depending on phase segregation and interactions between the component polymers.[1,2] Good miscibility can lead to a uniform polymer blend with desired physical properties. This blending phenomenon also occurs during biosynthesis and purification of polyhydroxyalkanoates (PHAs), a family of biopolyesters that are synthesized and accumulated by many bacteria as carbon and energy storage materials.[3–5] PHA synthesis occurs when a surplus of carbon source is present and bacterial growth is hindered by deficiency of essential nutrients in the medium.[6] The biopolyesters can be biodegraded completely via microbial activities and can be melted and molded like conventional thermoplastics and elastomers.[7–10] The biobased polymers have raised great interest in industry because they can be made from renewable recourses including industrial and household wastes.[11] In comparison with petrochemical polymers, PHA production via microbial fermentation consumes less amount of energy with a lower carbon footprint.[12]

The material properties of biopolymers synthesized by microbial cells are determined by many factors including microbial species, carbon source, nutrient supply, and fermentation conditions. When the PHA-producing cells are fed with cosubstrates, they may produce copolyesters of narrow chemical composition with random, alternative, or block arrangements of monomers, or a mixture of copolyesters with a broad chemical composition distribution, depending on the biosynthesis conditions. Most bacterial PHA copolymers fall in the latter category. The as-produced PHA is actually a natural mixture or blend of different copolyesters, and its material properties depend on the miscibility and cocrystallization of the macromolecules.[13] Specifically, the basic thermal and mechanical properties of the PHA copolyesters may be affected to a great extent by the overall chemical composition, and/or the chemical composition distribution of copolymers, and/or the arrangement of monomers in individual polymer backbones. PHA macromolecules with different chemical composition often exhibit poor or moderate miscibility and hence partial phase segregate during crystallization, resulting in inconsistent changes in mechanical properties.[14,15] Obviously, a broad application of PHA bioplastics depends on control of their material mechanical properties that are affected by compositional distribution, crystallinity, microstructure, blend composition, and molecular weight.[5,16]

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



Scheme 1. General chemical structure of a terpolymer of P(3HB-*co*-3HV-*co*-4HV).

Glycerol and levulinic acid are two inexpensive renewable carbon sources for PHA production by bacteria. Glycerol, a biodiesel by-product, is used by microbial cells to form poly(3-hydroxybutyrate), a rigid plastic exhibiting high crystallinity, high melting temperature, high tensile strength, but low ductility.[17,18] Levulinic acid is a platform chemical that can be derived from carbohydrates and cellulosic biomass via hydrolysis.[19,20] It can be used by bacteria to form short-chain-length (scl) PHA copolyesters consisting of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 4-hydroxyvalerate (4HV), as shown in Scheme 1.[21–23] The terpolyester, P(3HB-*co*-3HV-*co*-4HV), from sole levulinic acid substrate exhibits higher ductility with reduced melting temperature in comparison with P(3HB) and P(3HB-*co*-3HV). However, levulinic acid may inhibit cells at a high concentration level, which can be overcome by adding the acid gradually to avoid its accumulation in the culture medium.[24,25]

When PHA is produced from cosubstrates, the less toxic substrate such as glycerol is often used as a primary carbon source for cell growth, major monomer 3HB, reducing power (NADPH), and energy carrier (ATP) in polymer biosynthesis, whereas the inhibitive substrate such as levulinic acid is carefully introduced to provide alternative monomers of copolymers. It is a popular feeding strategy in fed-batch fermentation to achieve high cell density and high PHA productivity.[26] The as-produced PHA is therefore a complex mixture of random copolymers and/or homopolymers. It is therefore of great interest to understand whether or not the natural PHA blend is a miscible mixture, how its miscibility changes with composition during biosynthesis, and what is the effect of the miscibility and chemical composition on the thermal and mechanical properties of the as-produced PHA. It is also important to know how to control the biosynthesis conditions under which the miscibility of as-produced PHA blend can be controlled for desired material properties.

In this work, natural PHA blends were synthesized from glycerol and levulinic acid as a mixed carbon source by a mutated *Cupriavidus necator* strain (formerly *Ralstonia eutropha*) in a high-cell-density fed-batch fermentation. The as-produced P(3HB-*co*-3HV-*co*-4HV) blend was separated into several fractions with different chemical composition by using solvent fractionation to study the chemical composition distribution of the blend. We also prepared P(3HB) homopolymer on sole glycerol and a random P(3HB-*co*-3HV-*co*-4HV) terpolymer on sole levulinic acid and made artificial blends from them. The physical properties of the natural as-produced and artificial PHA blends were compared to clarify their miscibility and its effect on the material properties. In addition, the crystallinities of the random P(3HB-*co*-3HV-*co*-4HV) terpolymers were compared with those of P(3HB-*co*-3HV) to understand the effect of the minor 4HV monomer unit on the material properties.

EXPERIMENTAL

Bacterial Strain and Microbial Synthesis

A laboratory mutant strain of *Cupriavidus necator* (ATCC 17699) was used in this study. The strain was adapted in a glycerol-rich environment and effectively forms P(3HB) from glycerol. It was maintained by monthly subculture on 2.0% agar slants (pH 7.0) containing the following (per liter): 5 g of yeast extract, 2.5 g of meat extract, 5 g of peptone, 5 g of $(NH_4)_2SO_4$, 5 g of glycerol, and 1 mL of trace solution. An inoculum of *C. necator* was prepared by aseptically transferring the cells from a slant into 5 mL of YPM medium (the medium described above without agar) in a test tube and incubated for 48 h at 30°C. The cells were further cultivated in a 500-mL flask containing 200 mL of a mineral solution supplemented with 20 g/L glycerol and 2 g/L levulinic acid and shaken at 30°C and 200 rpm for 36 h on a rotary shaker. The mineral solution contained the following (per liter): 1.2 g of NaH_2PO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 2 g of $(NH_4)_2SO_4$, 7.34 g/L K_2HPO_4, and 1 mL of trace element solution, as described previously.[24] The flask culture was repeated two times to obtain highly active cells as the inoculum for fermentation in a bench-top bioreactor.

Microbial fermentation was conducted in a 3-L bioreactor (New Brunswick Scientific, Edison, NJ). Temperature, pH, and dissolved oxygen were monitored and controlled during the fermentation; culture conditions were maintained at 30°C and pH 6.8. The dissolved oxygen concentration was kept at 10% of air saturation or above, with an automatic increase in agitation speed. The fed-batch fermentation began with 1.7 L of the mineral solution with 20 g/L of glycerol with an initial C/N ratio at 5, and 170 mL of *C. necator* inoculum. After the cell density reached the desired level ($\sim$ 5 g/L), a glycerol solution (500 g/L) was added via a manual pump to keep the residual glycerol concentration above 10 g/L. For the initial pH control and nitrogen feeding, a solution of 15% ammonia water was added to the culture to maintain an ammonia–nitrogen concentration at 3 g N/L and a C/N ratio of $\sim$ 5. When cell density reached the desired level (OD_{620} $\sim$ 80) and no additional nitrogen was needed, the ammonia nitrogen solution and the glycerol solution were replaced with a NaOH/KOH (5*M*/5*M*) solution and glycerol : levulinate solution (7 : 3 ratio) to initiate the nitrogen limitation condition for PHA copolymer accumulation.

Aliquots of 25–35 mL culture medium were dispensed into preweighed polypropylene centrifuge tubes. Microbial growth was monitored with a UV/Vis spectrophotometer (DU 530, Beckman, CA) by measuring the optical density of the culture medium at 620 nm. Fermentation was stopped when the OD_{620} began to decrease. The samples of the culture medium were centrifuged at 5000 $\times$ *g* for 10 min to separate the supernatant (culture solution) from wet pellets (PHA-containing cells). The wet pellets were subsequently washed with distilled water and lyophilized to determine the dry cell mass and PHA content.

To compare the physical properties of biopolyesters, P(3HB-*co*-3HV-*co*-4HV), random copolymers (named as Sample B, C,

and D) were prepared via fed-batch fermentation or flask cultures on levulinic acid as the sole carbon source (see Table II). A P(3HB) homopolymer (named as Sample E) produced on glycerol as the sole carbon source in our previous work[17] was also used in this study. All samples were purified before film casting.

PHA Purification

PHA polymers that accumulated in cells were extracted with chloroform (10 mL chloroform/1 g dry cells) for at least 48 h at 60°C and purified by two rounds of precipitation with cold methanol. Polymer films of ~ 150 μm thickness were prepared by casting the PHA–chloroform solution onto a Teflon Petri dish and allowing the solvent to evaporate at room temperature for 1 week to reach a constant weight. The solvent-casted films were further aged in a vacuum at 50°C for at least 1 week to reach equilibrium crystallinity and subsequently stored at room temperature for a month prior to further analysis.

Fractionation Procedures of As-produced PHA Blend

The original as-produced P(3HB-*co*-3HV-*co*-4HV) copolymer (named as Sample A) was fractionated by the following procedure[27]: the as-produced P(3HB-*co*-3HV-*co*-4HV) blend was first dissolved in chloroform at a concentration of 5 g/L, and a predetermined amount of *n*-hexane was carefully added to the solution under gentle agitation at ambient temperature. After the precipitate was visually observed, the mixed solution was kept at ambient temperature for 24 h, and the precipitated fraction was obtained by filtration and dried in a vacuum desiccator at ambient temperature. This procedure was repeated until the addition of a large amount of *n*-hexane caused no appreciable precipitation. The residual polymer in the supernatant solution with an excess amount of *n*-hexane was recovered as the final fraction by solvent evaporation. The fractionated P(3HB-*co*-3HV-*co*-4HV) films (named as Samples A-1, A-2, A-3, and A-4) were prepared by solution casting from the chloroform solution, as described previously.

Preparation of Artificial P(3HB)/P(3HB-*co*-3HV-*co*-4HV) Binary Blends

P(3HB) (named as Sample E) and P(3HB-*co*-3HV-*co*-4HV) (named as Sample B) were dissolved in chloroform (5 wt % solvent) at different P(3HB)/P(3HB-*co*-3HV-*co*-4HV) ratios (100/0, 80/20, 60/40, 40/60, and 0/100 by weight). Blend films were prepared by casting the solution onto a Teflon Petri dish and allowing the solvent to evaporate, as described previously.

Analytical Procedures

Glycerol and levulinic acid in the medium solution were measured using a Shimadzu LC-10AD HPLC system equipped with a RI detector (Shimadzu, Japan) and an organic acid column (C-610H, Supelcogel). The column was maintained at 30°C and eluted with a water–phosphoric acid solution (0.1% H_3PO_4) at 1 mL/min. The ammonium nitrogen concentration was determined using Hach H260G-BNDL ammonia ISE analysis.

The PHA content in cells was determined with a gas chromatographer (Varian 450-GC) equipped with a flame ionization detector. Methanolysis of PHA in the lyophilized cells was performed in a methanol solution (3% sulfuric acid) with octanoic acid as the internal standard.[17]

The distribution of molecular weights of PHA macromolecules was measured with size exclusion chromatography (SEC) equipped with an RI detector and two Shodex mixed-bed K805L columns in series (Showa Denko, Tokyo, Japan). Samples dissolved in hot chloroform were eluted with chloroform at 1 mL/min. The molecular weights were calibrated with narrow-cut polystyrene standards (Sigma-Aldrich, Saint Louis, MO), and the average molecular weights were calculated from the distribution curves using the manufacturer's SEC software (Shimadzu, Japan). All data taken were confirmed by repeated measurements.

PHA polymers were analyzed using ^1H and ^{13}C NMR spectroscopy (Varian Unity Inova 500 and 125 MHz) in a $CDCl_3$ solution to determine monomer composition, chemical structure, and polymer-sequence information. Samples were prepared for NMR analysis by dissolving solvent-cast film segments in $CDCl_3$ solution (25 mg/mL) via mixing and mild heating.

Differential scanning calorimetry (DSC) analysis of PHA films (~ 5 mg) encapsulated in aluminum pans was performed with a TA Instruments Q2000 Modulated DSC equipped with a refrigerated cooling system. Scanning was conducted under a nitrogen flow of 50 mL/min from −50°C to 200°C at a heating and cooling rate of 10°C/min. The melting temperature (T_m), glass transition temperature (T_g), cold-crystallization temperature (T_{cc}), and enthalpy of fusion (ΔH_m) were determined from the DSC thermograms. The T_g was taken as the midpoint of the heat capacity change. All data taken were confirmed by repeated measurements.

Wide-angle X-ray diffraction patterns of P(3HB-*co*-3HV-*co*-4HV) samples were recorded at 27°C on a Rigaku MiniFlexII system using nickel-filtered Cu Kα radiation ($\lambda = 0.154$ nm; 30 kV; 15 mA) in the 2θ range 4–60° at a scan speed of 2.0°/min. The degree of crystallinity (X_c) for PHA films was calculated from diffracted intensity data using Vonk's method.[28]

A stress–strain test of solution-cast films (20 × 5 × 0.15 mm^3) was performed at room temperature using a TestResources universal testing machine with a strain rate of 5 mm/min.

RESULTS AND DISCUSSION

Biosynthesis of Natural PHA Copolyester Blends

Levulinic acid and glycerol were used as cosubstrates by *C. necator* to synthesize PHA biopolyesters with a broad range of chemical compositions. A fed-batch culture of mutated *C. necator* grown on glycerol and levulinic acid was conducted in a 3-L laboratory fermenter. The culture consisted of an initial cell growth phase on glycerol under nutrient-rich conditions, followed by PHA formation from glycerol and levulinic acid under nutrient limitation. Figure 1 shows time-dependent changes in dry cell density, PHA content and concentration, monomeric composition, residual glycerol and levulinic acid concentrations, and C/N ratio. When the cell mass reached the desired level, the ammonia solution was replaced with a base solution to apply nitrogen limitation. Early nitrogen limitation results in a low

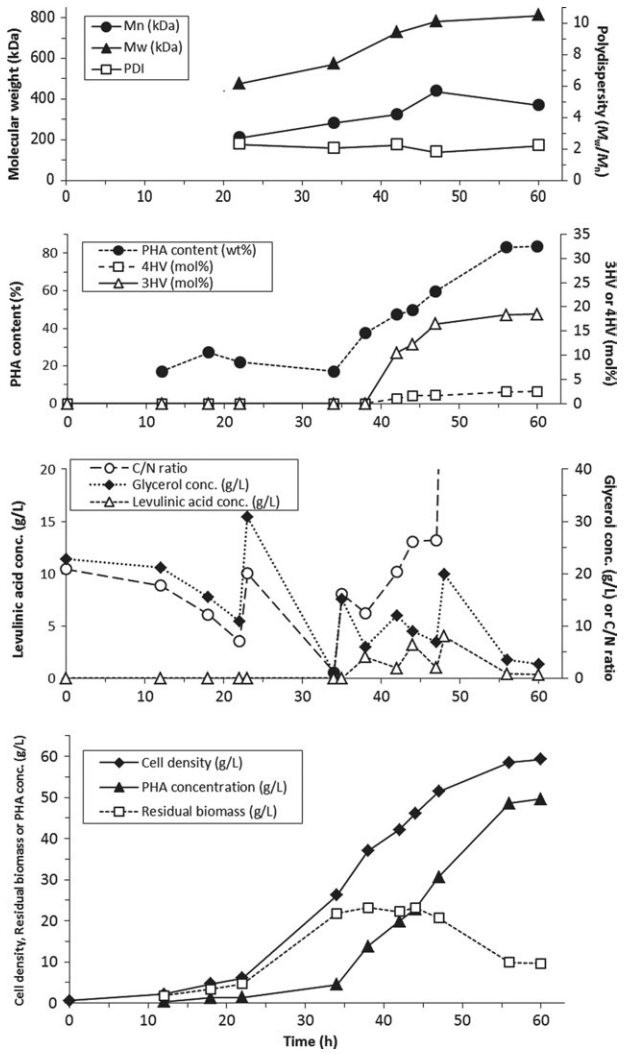


Figure 1. Time courses of PHA biosynthesis: cell growth, PHA formation, substrate and nutrient control, and *in vivo* molecular weights in fed-batch fermentation of glycerol/levulinic acid cosubstrates.

cell density, whereas late limitation leads to low PHA content; both give rise to low PHA concentration or productivity. We found that final PHA content increased when nitrogen limitation was applied at a cell density of ~ 36 g/L (OD_{620} ~ 105). After the ammonia nitrogen feeding stopped at 38 h, it took 8 h for the C/N ratio to approach infinity (i.e., N –> 0). The PHA content increased from 40% to 80% of dry cell density, and the PHA composition shifted from PHB homopolymer to P(3HB-*co*-3HV-*co*-4HV). The overall chemical composition of the copolyesters after 18 h of PHA accumulation was as follows: 79% 3HB, 18.5% 3HV, and 2.5% 4HV, as shown in Table I. The time-dependent change in 3HV and 4HV content during PHA accumulation phase indicates clearly that the chemical composition of copolyesters changes with time. The content of 3HV and 4HV after 60 h biosynthesis and accumulation was approximately twofold higher than after 42 h.

The amount of PHA produced per volume per time is a technical and economical indicator of microbial fermentation that depends on substrates (including nutrients), microbial strain, and fermentation technology. The PHA productivity after 60-h cultivation was ~ 0.87 g/L/h, close to that with glycerol as a sole carbon source (0.9–1.1 g/L/h).[17,18] As shown in Figure 1, the molecular mass and polydispersity of biopolyesters *in vivo* was around 810–950 kDa and 2.2–3.1 (similar to commercial PHA), appropriate for further polymer processing.[17,18] It can therefore be concluded that P(3HB-*co*-3HV-*co*-4HV) biosynthesis from glycerol and levulinic acid is able to meet the desired yields of cell mass and PHA with appropriate molecular size and chemical composition for large-scale PHA production.

Here, the reaction mechanism of P(3HB-*co*-3HV-*co*-4HV) formation from glycerol and levulinic acid via the well-established PHA biosynthesis pathway in *C. necator* is described as reported in the previous study.[17,24,25] In brief, glycerol is first converted into pyruvate via partial glycolysis and acetyl-CoA that is condensed into 3-hydroxybutyryl-CoA with a *β*-ketothiolase (*phaA*) and a NADPH-dependent reductase (*phaB*). The 3HB precursor is incorporated into a growing PHA backbone with a PHA synthase (*phaC*). A two-site working PHA synthase for chain elongation via transesterification is responsible for P(3HB) formation. In the presence of levulinic acid, the organic acid is first activated to form 4-ketovaleryl-CoA and then split into propionyl-CoA and acetyl-CoA. The two intermediates are either used via the main metabolism pathway for cell growth or condensed into 3-ketovaleryl-CoA for 3HV and/or 4-hydroxyvaleryl-CoA for 4HV. P(3HB-*co*-3HV-*co*-4HV) is formed simultaneously with P(3HB). Therefore, blend of both types of polymers may

Table I. Composition of PHAs and Their Physical Properties at Different Cultivation Times During a Fed-Batch Fermentation on Cosubstrates of Glycerol and Levulinic Acid

	PHA composition[a]			Thermal properties[b]					Molecular weight[c]	
Sampling time (h)	3HB (mol %)	3HV (mol %)	4HV (mol %)	T_g (°C)	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	ΔH_m (J/g)	M_w ($\times 10^3$)	M_w/M_n
42	88.5	10.5	1	3.7, –3.4	83	164	176	64	730	2.3
47	81.8	16.5	1.7	3.3, –3.1	83	162	175	54	780	1.8
60[d]	79	18.5	2.5	–1.3	79	161	175	59	810	2.2

[a]PHA monomer composition of isolated P(3HB-*co*-3HV-*co*-4HV) samples was determined by 500 MHz ^{1}H NMR.
[b]T_m, melting temperature (three peaks observed); T_g, glass transition temperature; ΔH_m, enthalpy of fusion.
[c]Molecular weight of PHA copolyesters *in vivo*. M_n, number-average molecular weight; M_w, weight-average molecular weight; M_w/M_n, polydispersity.
[d]Sample A.

Table II. PHA Polyesters Produced from Different Biosynthesis Strategies with Glycerol and Levulinic Acid Used in This Study

Sample	Substrates	Biosynthesis	PHA composition[a] (mol %)			Molecular weight[b]	
			3HB	3HV	4HV	M_w ($\times 10^3$)	M_w/M_n
A	Glycerol and Levulinic acid	Fed-batch[c]	79	18.5	2.5	710	2.4
B	Levulinic acid	Fed-batch[d]	54	43	3.0	1,320	2.4
C	Levulinic acid	Batch[e]	53.5	41	5.5	1,250	2.2
D	Levulinic acid	Multiple feeding[f]	38	55.3	6.7	1,060	2.2
E	Glycerol	Fed-batch[g]	100	0	0	550	2.0

[a]PHA monomer composition of samples was determined by 500 MHz ^{1}H NMR.
[b]Molecular weight of PHA copolymer films as determined by GPC.
[c]Fed-batch fermentation of glycerol and levulinic acid cosubstrate for 60 h, as shown in Figure 1.
[d]Fed-batch fermentation of levulinic acid (single substrate) for 72 h.
[e]Flask batch culture for 48 h with initial 10 g/L of levulinic acid.
[f]Flask culture for 48 h with multiple feedings of levulinic acid (10 g/L = 4 × 2.5 g/L).
[g]Fed-batch fermentation on single glycerol substrate cultivated for 60 h.

be formed with different composition of P(3HB-*co*-3HV-*co*-4HV) copolymers, depending on a dynamic environment of carbon substrate feeding.

DSC analysis provided the basic thermal properties of P(3HB-*co*-3HV-*co*-4HV) samples obtained at 42, 47, and 60 h during the fed-batch fermentation. As listed in Table I, three melting temperature peaks were detected for all the samples. It is possible that the presence of multiple endotherms could arise either from compositional heterogeneity, multiple crystalline forms, or rearrangement of the original crystal morphology of solvent-cast copolyester films. More interestingly, the terpolyesters obtained at early PHA accumulation times (42 and 47 h) exhibited two glass temperatures (T_g) close to the glass temperatures of P(3HB) ($\sim$ 4°C)[8] and a random P(3HB-*co*-3HV-*co*-4HV) ($\sim$ −4°C),[25] respectively, while a single T_g was observed at −1.3°C for the polymer obtained at the end of the fermentation period (60 h). This phenomenon suggests that the early formed PHA polymers differ significantly in chemical composition such as P(3HB) versus P(3HB-*co*-3HV-*co*-4HV) and therefore poor miscibility results in two glass temperatures. In fact, each PHA granule in individual cells may have a unique history derived from the dynamic environment of the fed-batch culture. It is highly possible that these biomacromolecules have distinct chemical compositions and that their mixtures have different material properties. The miscibility of these copolyesters may improve with continual changes in their chemical composition. It is important to know under what conditions the PHA blend exhibits good miscibility and what material properties can be expected with this improved miscibility. To answer these questions, further investigation was conducted on the structure–property relationship of randomly distributed copolymers and the comparison to their artificial binary blends.

Fractionation and Sequence Distributions of As-produced P(3HB-*co*-3HV-*co*-4HV)

Table II lists the PHA samples used in this work, including the as-produced PHA blend (Sample A) from a fed-batch culture of glycerol and levulinic acid, three random P(3HB-*co*-3HV-*co*-4HV) terpolyesters (Sample B, C, and D) formed in fed-batch and batch cultures of levulinic acid only, and P(3HB) (Sample E) formed on glycerol in a fed-batch culture. The PHA blend (Sample A) harvested at 60 h was subjected to solvent fractionation as reported in the previous section. The composition of monomers in the copolymer fractions was determined from the relative integrated intensities of the proton resonances of 3HB, 3HV, and 4HV repeating units in the ^{1}H NMR spectrum, as shown in Figure 2(a). It can be confirmed that the intensity ratio of peaks (j), (i), (h), and (k) was 1 : 2 : 2 : 3, in good agreement with the ratio estimated from the structure of 4HV. In Table II, the molecular weights of P(3HB-*co*-3HV-*co*-4HV) terpolyesters from levulinic acid alone were higher than those of polymers formed on glycerol or glycerol cosubstrates. This difference may result from chain termination caused by a higher levulinic acid utilization rate via a shorter biosynthesis pathway compared to glycerol and higher PHA synthase activity for chain elongation via transesterification on levulinic acid.[17,24]

It has been reported that PHA copolyester obtained by microbial synthesis is usually a mixture of polymers with different chemical compositions.[13,27,29] The mixture can be separated into fractions with narrower composition distributions to examine the relationship between chemical structure, monomer composition, and physical properties of the bacterial copolyesters. The biosynthesized P(3HB-*co*-3HV-*co*-4HV) copolymer containing 18.5 mol % 3HV and 2.5 mol % 4HV (Sample A) was subjected to fractionation in a chloroform/*n*-hexane mixture. Four fractions (A-1 to A-4) with different monomer compositions were obtained from the original sample A when the concentration of *n*-hexane increased from 45 to 72.5 vol %. As shown in Table III, the 3HV and 4HV content of the fractionated copolymers increased gradually from the first fraction (3.5 and 0.5 mol %) to the last fraction (34 and 4 mol %). It should be noted that the final fraction (A-4) was obtained by solvent evaporation because its molecular weight was significantly lower than the other fractions and could not be precipitated out of the solution of chloroform ($\sim$ 27% v/v) and hexane ($\sim$ 73% v/v). The weight-averaged molecular weights (M_w) of the fractions (A1–A3) are quite consistent (M_w 510–850 kDa) and close to the molecular weight of the original sample (M_w 710 kDa). The

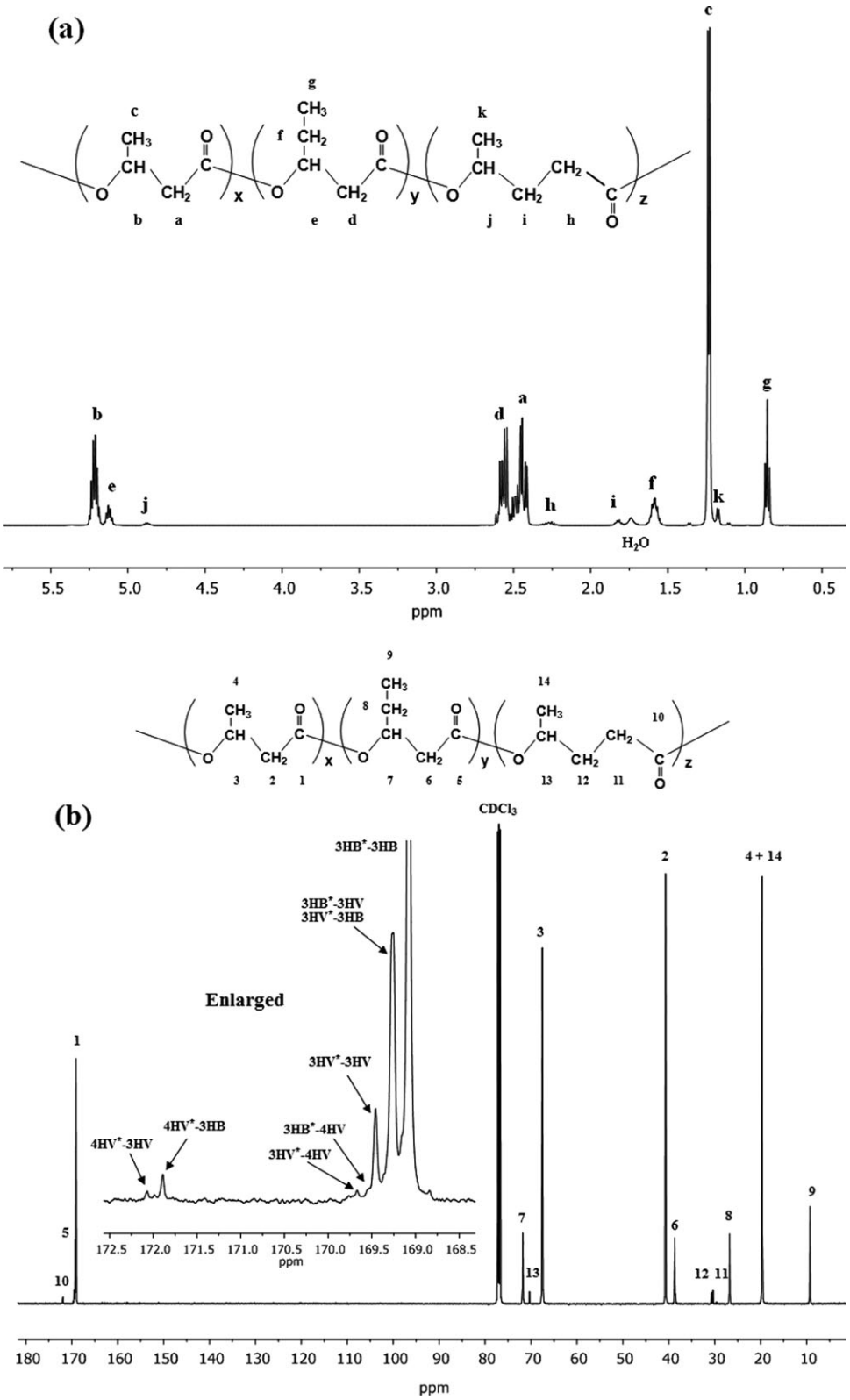


Figure 2. NMR spectra of extracted terpolymer of P(3HB-*co*-3HV-*co*-4HV) with 18.5 mol % 3HV and 2.5 mol % 4HV (Sample A) produced from mutated *C. necator* grown for 60 h using glycerol/levulinic acid cosubstrates: (a) ^{1}H NMR and (b) ^{13}C NMR.

low molecular weight (M_w 270 kDa) of the last fraction (A4, 15 wt %) may play a role in the miscibility of the blend. The broad distribution of chemical composition and molecular weight of the fractions clearly indicate that the as-produced PHA is actually a blend of copolyesters. Its material properties are therefore determined not only by individual fractions but also

Table III. Fractionation of As-produced P(3HB-*co*-3HV-*co*-4HV) Blend with Chloroform/*n*-Hexane

Fraction	Concentration of *n*-hexane (vol %)	Mass fraction (wt %)	PHA composition[a] (mol %)			Molecular weight[b]	
			3HB	3HV	4HV	M_w ($\times 10^3$)	M_w/M_n
A[c]		100	79	18.5	2.5	710	2.4
A-1	45	33	96	3.5	0.5	790	2.5
A-2	62.5	14	81	15.8	3.2	850	1.6
A-3	65	38	72	24.4	3.6	510	1.7
A-4	72.5	15	62	34.0	4.0	270	2.1

[a]PHA monomer composition of samples determined by 500 MHz ^{1}H NMR.
[b]Molecular weight of PHA film as determined by GPC.
[c]As-produced original PHA obtained after 60 h fed-batch biosynthesis.

by the miscibility of these fractions. As shown in Table I, this as-produced PHA blend has good miscibility, exhibiting a single glass temperature (T_g −3.0°C).

Figure 2(b) shows a typical 125 MHz ^{13}C NMR spectrum for the as-produced P(3HB-*co*-3HV-*co*-4HV) (Sample A) containing 18.5 mol % 3HV and 2.5 mol % 4HV. All signals split into several peaks and were assigned by comparison to previously reported data.[30,31] As shown in the expanded spectrum of the carbonyl region at 168.8–172.2 ppm, the carbonyl resonances of P(3HB-*co*-3HV-*co*-4HV) split into three main peaks arising from 3HB*–3HB, 3HB*–3HV/3HV*–3HB, and 3HV*–3HV diad sequences, and the other four minor peaks of the 3HB*–4HV, 3HV*–4HV, 4HV*–3HB, and 4HV*–3HV diad sequences. The carbonyl resonances of random terpolymer with higher 4HV content (Sample C) are given in Figure S1 of the Supporting Information. The diad sequence distribution was calculated from the ratios of the peak intensities of carbonyl carbon resonances. The relative intensities of the main peaks in the carbonyl resonances of as-produced and fractionated samples are given in Table S1 of Supporting Information. It is important to note that the relative 4HV minor diad fractions were neglected for these calculations because the proportion of resonances with the main diad sequences are very small due to low 4HV content. In addition, the randomness in the sequential distribution was estimated using the parameter D defined as $D=(F_{3HV-3HV}F_{3HB-3HB})/(F_{3HV-3HB}F_{3HB-3HV})$, where F_{X-Y} indicates the mole fraction of the X–Y diad sequence.[32] The D value of the completely random copolymer is equal to 1.0, whereas blocky or alternating copolymers displayed values much larger than 1 or very close to 0, respectively. As listed in Table IV, the D values for a series of well-fractionated terpolyester samples and the P(3HB-*co*-3HV-*co*-4HV) samples synthesized with a single carbon substrate (levulinic acid) closely approached 1.0 [varying between 1.0 and 1.5 (except Sample A-1)], indicating that these terpolymer

Table IV. Thermal Properties, X-ray Crystallinity, and Randomness of P(3HB-*co*-3HV-*co*-4HV) Samples

Sample	PHA composition[a] (mol %)			Thermal properties[b]					Lattice parameter (nm)			Crystal structure	X_c[c] (%)	Randomness[d] (D)
	3HB	3HV	4HV	T_g (°C)	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	ΔH_m (J/g)	*a*	*b*	*c*			
A	79	18.5	2.5	−1.3	79	161	175	59	0.583	1.297	0.579	PHB	42 ± 5	2.4
A-1	96	3.5	0.5	3.3	-	162	176	83	0.583	1.365	0.582	PHB	58 ± 5	ND[e]
A-2	81	15.8	3.2	0.4	75	116	-	59	0.592	1.329	0.590	PHB	40 ± 5	1.5
A-3	72	24.4	3.6	−1.2	80	106	-	43	0.582	1.314	0.584	PHB	30 ± 5	1.3
A-4	62	34.0	4.0	−3.6	74	100	-	32	0.580	1.312	0.586	PHB	23 ± 5	1.1
B	54	43	3.0	−4.2	61	78	-	40	0.589	1.339	0.591	PHB	31 ± 5	1.4
C	53.5	41	5.5	−5.8	64	75	-	23	0.594	1.334	0.596	PHB	26 ± 5	1.2
D	38	55.3	6.7	−8.3	60	-	-	36	0.932	0.984	0.570	PHV	34 ± 5	1.0
E	100	0	0	3.9	-	161	176	91	0.573	1.347	0.584	PHB	60 ± 5	ND[e]

[a]PHA monomer composition was determined by 500 MHz ^{1}H NMR.
[b]Measured by DSC at a heating rate of 10°C/min. T_g, glass transition temperature; T_m, melting temperature; ΔH_m, enthalpy of fusion.
[c]Degree of crystallinity was determined from X-ray diffraction patterns.
[d]Randomness parameter is as follows: $D = (F_{3HV-3HV}F_{3HB-3HB})/(F_{3HV-3HB}F_{3HB-3HV})$, where F_{X-Y} indicates the mole fraction of the X–Y diad sequence.[32] Relative intensities of the diad sequences were determined via solution-phase ^{13}C NMR spectroscopy (Table S1 in Supporting Information). The 4HV unit was excluded from the calculation because of their low intensities of the resonances from the other diad fractions.
[e]ND, not determined.

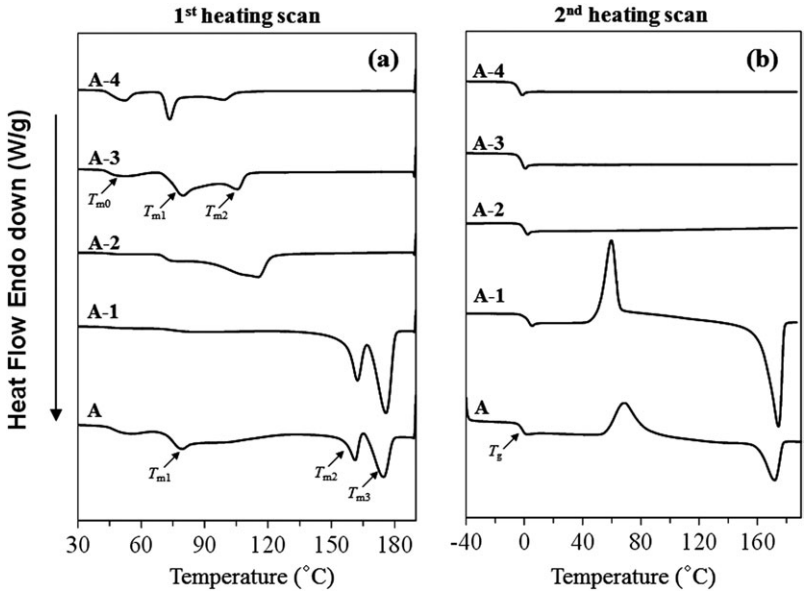


Figure 3. DSC thermograms of the as-produced and fractionated P(3HB-*co*-3HV-*co*-4HV) samples (a series of Sample A) at a heating rate of 10°C/min: (a) first heating scan and (b) second heating scan.

samples are random copolymers with a narrow distribution of monomer compositions. In contrast, the *D* values of the as-produced P(3HB-*co*-3HV-*co*-4HV) sample synthesized from glycerol/levulinic acid cosubstrates (Sample A) were much higher than 1.0, confirming that the original sample A is a copolymer blend containing of a mixture of random copolymers. In the as-produced sample, a fraction with very low 3HV content (<4 mol %) (Sample A-1) was obtained, and the resonance from 3HB*–3HB sequence may be overestimated due to the large proportion of resonance from the other diad sequences, resulting in an uncertain *D* value for sample A-1.

Thermal Properties of P(3HB-*co*-3HV-*co*-4HV)s

The thermal properties of solvent-cast films made from the as-produced and fractionated P(3HB-*co*-3HV-*co*-4HV) samples were measured using DSC. Figure 3(a) exhibits the DSC thermograms of the original sample (A) and its fractions (A1–A4) during the first heating scan, indicating the effect of chemical composition distribution on thermal behavior. Multiple endothermic peaks were observed in the thermograms of all P(3HB-*co*-3HV-*co*-4HV) samples, and two major peaks (T_{m2} and T_{m3}) were detected for the as-produced sample (A) and the first fraction (A-1). The melting peaks gradually shifted to lower temperature with increased 3HV and 4HV content (A-2, A-3, and A-4), and the heat flow intensity also declined. Only the first fraction (A-1) had a sharp melting point in the high temperature range, which was almost identical to the original. The fractionated copolymers with high 3HV and 4HV content (fraction A-3 and A-4) showed very broad endothermic behavior starting from the temperature around 40°C (T_{m0}); it was difficult to specify the peak melting temperatures. The presence of two separate melting peaks for the samples may be caused by the occurrence of various crystalline regions with different crystal sizes and orders of chain-packing in the solution-cast films. The smaller and/or disordered chain-packing crystalline regions may rearrange during heating to form larger thermally stable and/or ordered chain-packing crystals.[33] During the primary crystallization process at a given crystallization temperature, relatively thick crystalline lamellae may grow into a spherulitic morphology.[34–36] Thinner crystalline lamellae may be subsequently formed at room temperature. From the DSC results recorded at different heating rates (data not shown), the higher T_m should be the melting of the original crystals.

The three main endothermic peaks (T_{m1}, T_{m2}, and T_{m3}) were observed for the original as-produced P(3HB-*co*-3HV-*co*-4HV) (Sample A). The melting behavior of the original sample A exhibited combined characteristics of the fractions and can be separated into two ranges: a low temperature broad range for the A-2, A-3, and A-4 fractions (T_{m1}) and the high melting peaks from A-1 fraction (T_{m2} and T_{m3}). These results indicate that the various crystalline regions from copolymer fractions with different crystal sizes were presented in the as-produced sample A. As shown in Table II, fraction A-1 accounts for 33 wt % of the as-produced original PHA but determines the thermal behavior of the PHA blend (Sample A) at high temperatures. Obviously, fraction A-1 has very high 3HB content (96%), and its thermal behavior is primarily determined by P(3HB) crystalline. It is also interesting to note that fraction A-2 has a similar chemical composition (81 mol % 3HB, 15.8 mol % 3HV, and 3.2 mol % 4HV) to as-produced sample A (79 mol % 3HB, 18.5 mol % 3HV, and 2.5 mol % 4HV) but very different thermal behavior. This fact indicates again the important contribution of other copolyesters to the material properties of a PHA blend. In another word, the material properties of a PHA blend are not predominantly determined by the overall chemical composition but by the combined effect of individual copolyesters or fractions. The miscibility of the copolyesters is therefore important to the overall material properties.

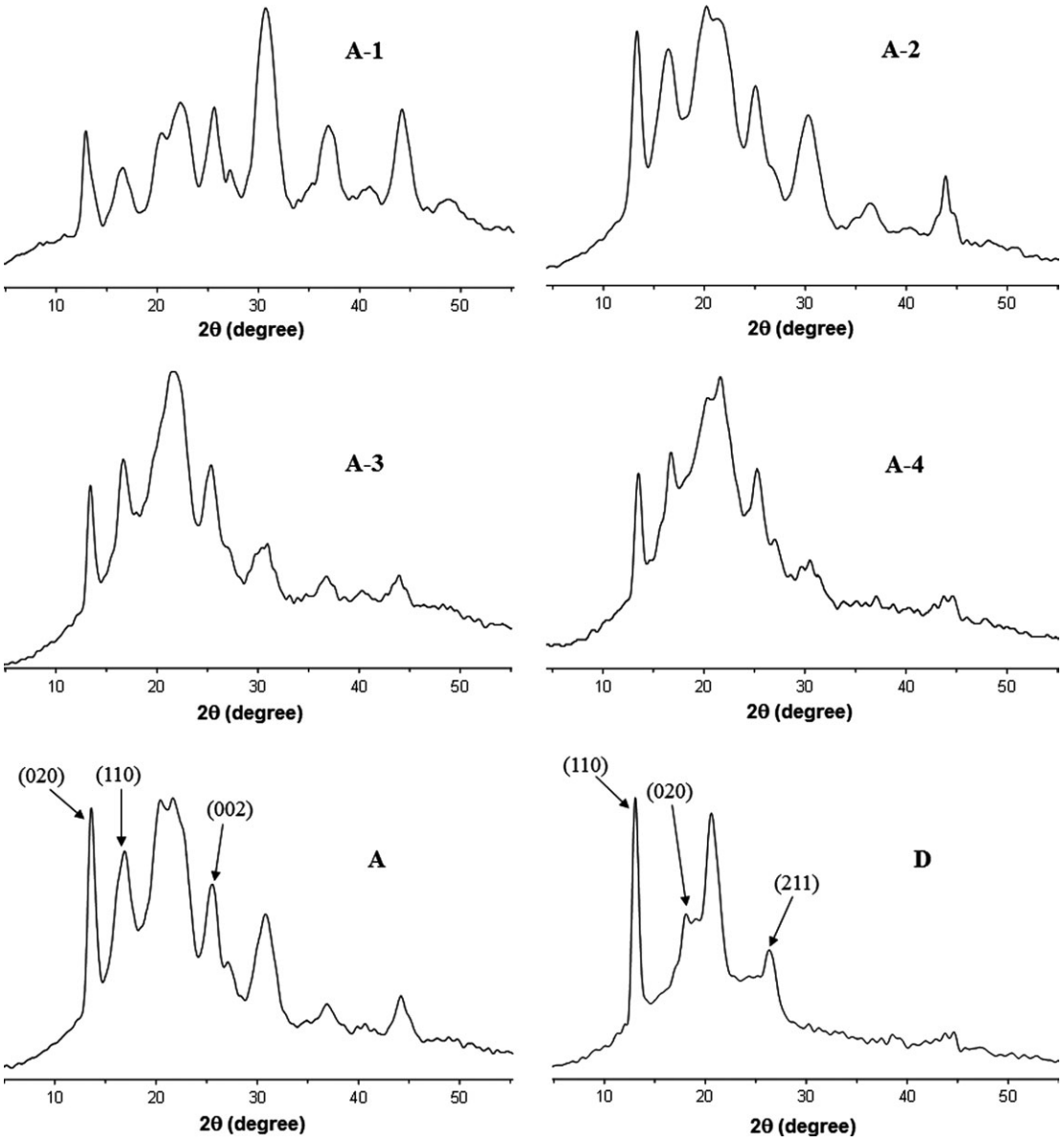


Figure 4. Wide-angle X-ray diffraction patterns for the as-produced and fractionated P(3HB-*co*-3HV-*co*-4HV) samples (a series of Sample A) and P(3HB-*co*-55.3 mol % 3HV-*co*-6.7 mol % 4HV) (Sample D).

Table IV summarizes the values of T_g, T_m, and ΔH_m of as-produced and fractionated P(3HB-*co*-3HV-*co*-4HV) samples. For the fractionated samples, the T_m and ΔH_m values dramatically decreased from 176°C to 100°C and from 83 to 32 J/g, respectively, as 3HV and 4HV contents increased. As expected, T_g values ranged between −3.6°C and 3.3°C and tended to decrease gradually with higher 3HV and 4HV content, as detected in Figure 3(b). For the randomly distributed terpolyesters (Sample B, C, and D) produced from levulinic acid only, the T_g value declined from −4.2°C to −8.3°C with increasing 3HV and 4HV contents. This trend indicates that the segmental mobility of the copolymer chain in the amorphous phase is highly dependent on their 4HV content. The T_m value also declines to ~ 60°C when 55.3% 3HV and 6.7% 4HV were incorporated into the copolymer. We suggest that the T_m values of P(3HB-*co*-3HV-*co*-4HV) terpolymers evaluated in the original crystals of the random copolymers are very close to those for P(3HB-*co*-3HV) copolymers with similar 3HV content.[37]

Crystalline Structures of P(3HB-*co*-3HV-*co*-4HV)s

The crystalline structures of the as-produced and fractionated P(3HB-*co*-3HV-*co*-4HV) samples were characterized by wide-angle X-ray diffraction (WAXD). Figure 4 shows typical X-ray diffraction patterns of solution-cast films from the original (A) and fractionated P(3HB-*co*-3HV-*co*-4HV) samples (A-1, A-2, A-3, and A-4). For comparison, a random terpolyester (Sample D: 38 mol % 3HB, 55.3 mol % 3HV, and 6.7 mol % 4HV) formed on levulinic acid only is also presented. The peak at $2\theta = 17°$ corresponds to the *d*-spacing of the (110) reflection of the P(3HB) homopolymer-type lattice, whereas the peak at

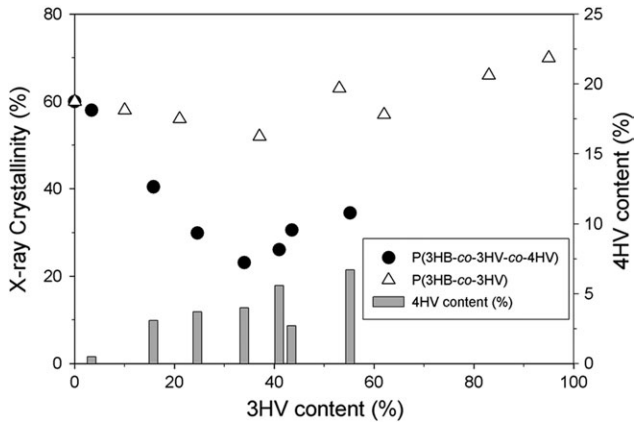


Figure 5. Effect of 4HV content on degrees of crystallinity with different random P(3HB-*co*-3HV-*co*-4HV) terpolymers compared to P(3HB-*co*-3HV) copolymers.

$2\theta = 18°$ corresponds to the (020) diffraction of P(3HV) lattice.[38–40] The diffraction patterns of all P(3HB-*co*-3HV-*co*-4HV) samples (except Sample D) showed reflections arising from the P(3HB) crystalline lattice, whereas the pattern of sample D reflected the P(3HV)-type crystalline structure. The two types of crystalline lattices cannot coexist in the as-produced PHA blend.

The crystallographic (lattice) parameters (*a*, *b*, and *c*) of P(3HB-*co*-3HV-*co*-4HV) samples were calculated from the reflections; values are listed in Table IV. The crystallographic parameters of P(3HB-*co*-3HV-*co*-4HV) terpolymers with 3HV composition from 0 to 43 mol % were not influenced by the presence of 3HV and 4HV units. It has been reported that lattice parameters and *d*-spacing of (110) reflections in the X-ray diffraction of P(3HB-*co*-3HV) copolymers extends as the 3HV content of the copolymer increases due to the incorporation of the 3HV unit in the P(3HB) crystalline structure.[38] However, the lattice parameters for random P(3HB-*co*-3HV-*co*-4HV) copolymers were constant, which can be attributed to the low amount of 4HV. As shown in the WAXD patterns for fractionated P(3HB-*co*-3HV) copolymers from a previous study, the transformation range of crystalline lattice types occurs in a very narrow composition range of 47–52 mol % 3HV content.[37] Therefore, sample D, containing 55.3 mol % 3HV, crystallizes solely in the P(3HV)-type crystalline lattice.

The crystallinity of the solution-cast films of P(3HB-*co*-3HV-*co*-4HV) terpolyesters was also determined from the typical X-ray diffraction patterns. As shown in Table IV, the X-ray crystallinity of random terpolymers decreased from 60% to 23% as the 3HV and 4HV fractions increased to 34% and 4%, respectively. Figure 5 shows the effect of copolymer composition on the degree of X-ray crystallinity for the random P(3HB-*co*-3HV-*co*-4HV) terpolymers as compared with P(3HB-*co*-3HV).[39] It has been reported that P(3HB-*co*-3HV)s show high degrees of crystallinity (>50%) over a wide range of copolymer compositions due to cocrystallization, which is known as isodimorphism.[38] As shown in Figure 5, the X-ray crystallinity of random P(3HB-*co*-3HV-*co*-4HV) decreases steeply with increasing 3HV and 4HV fractions, indicating that a small fraction of randomly distributed 4HV units may be excluded from the P(3HB) or P(3HV) crystalline phase and act as defects in the crystalline structure due to the presence of longer monomers (four carbons) in the PHA backbone, similar to the property of 4HB unit.[39] These results of crystallographic parameters and crystallinity suggest that isomorphic crystallization does not occur in these random P(3HB-*co*-3HV-*co*-4HV) copolyesters of narrow composition distribution.

Effect of Miscibility on Material Properties of PHA Blends

In the early stage of PHA accumulation from glycerol and levulinic acid, the as-produced P(3HB-*co*-3HV-*co*-4HV) mixtures exhibited two glass temperatures (Table I), indicating poor miscibility between newly formed P(3HB-*co*-3HV-*co*-4HV) and old P(3HB) formed on glycerol. The miscibility of a polymer blend greatly influences on its thermal properties, mechanical properties, and microstructural phase segregation. The miscibility of artificial P(3HB)/P(3HB-*co*-3HV-*co*-4HV) blends prepared at different compositions were investigated by measuring the glass transition temperature (T_g) of their solution-cast films. Artificial P(3HB)/P(3HB-*co*-3HV-*co*-4HV) blend films were prepared from mixtures of P(3HB) homopolymer (Sample E) formed on sole glycerol and a random P(3HB-*co*-43 mol % 3HV-*co*-3 mol % 4HV) copolymer (Sample B) formed on sole levulinic acid at predetermined weight ratios of 100/0, 80/20, 60/40, 40/60, 20/80, and 0/100. Figure 6(a) shows DSC thermograms of the second heating scan for the melt-quenched samples of the artificial P(3HB)/P(3HB-*co*-3HV-*co*-4HV) blends. It can be observed that the T_g shifts to lower temperature with P(3HB) weight fraction up to 80 wt %, whereas the glass transition splits into two T_gs for the artificial blends of sample B's weight fractions over 20 wt %. Table V summarizes the thermal properties of the artificial P(3HB)/P(3HB-*co*-3HV-*co*-4HV) blend samples. All the blends except 80/20 show multiglass transitions (double T_g) for each component polymer, indicating the immiscibility of the artificial binary blends. The melting behavior of the artificial blends was detected in three separate melting temperatures, suggesting the occurrence of two crystalline phases of thick P(3HB) and thin P(3HB-*co*-3HV-*co*-4HV) crystals caused by board or bimodal compositional distributions.

On the other hand, the as-produced P(3HB-*co*-3HV-*co*-4HV) blend harvested after 60 h of biosynthesis (Sample A) possesses a single T_g at $-1.3°C$, as shown in Figure 6(b) and Table I. This natural copolymer blend forms many crystalline structures in which the component polymers were thoroughly mixed, as seen in the melting thermogram of sample A in Figure 3(a). These results indicate that the as-produced P(3HB-*co*-3HV-*co*-4HV) copolymer is a miscible blend with a multicrystalline structure comprising the several semicrystalline PHA polymers. However, the artificial 60/40 blend with a similar average chemical composition to sample A and the as-produced P(3HB-*co*-3HV-*co*-4HV) copolymer obtained at the earlier time of PHA accumulation (42 and 47 h) possessed two glass transitions, indicating that they were poorly miscible, as shown in Figure 6. These results indicate that a miscible blend of PHA terpolyesters can be produced from cosubstrates in a fed-batch biosynthesis

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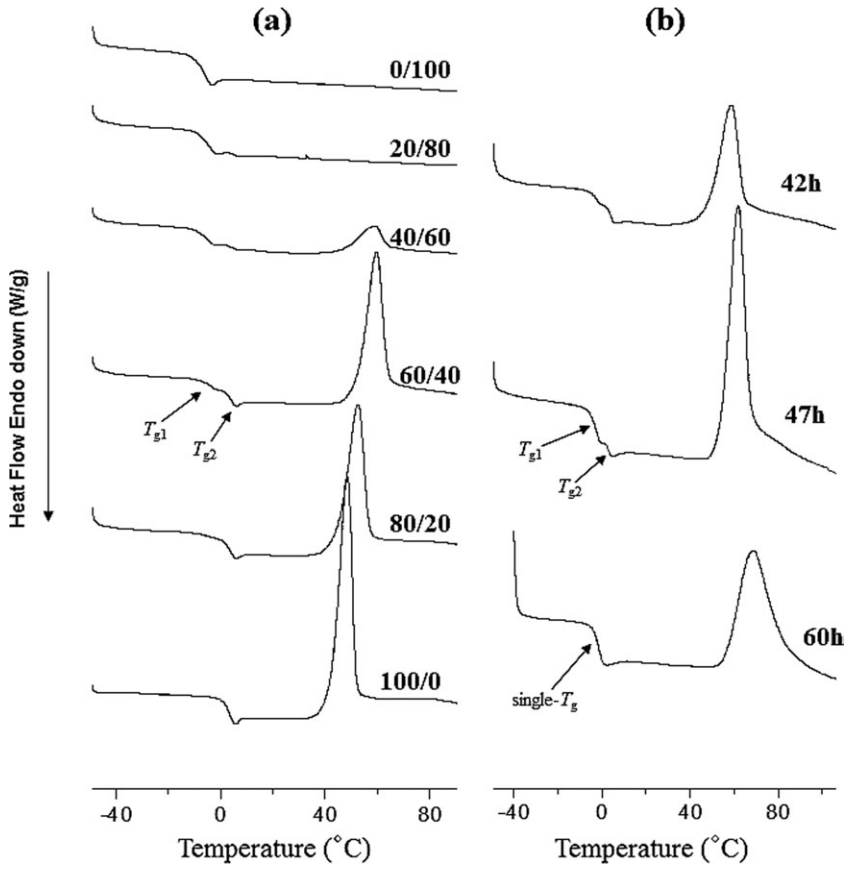

Figure 6. DSC second heating scan thermograms at a heating rate of 10°C/min after melt-quenched treatment of (a) P(3HB)/P(3HB-*co*-3HV-*co*-4HV) artificial blends of sample E and B at various E/B ratios and (b) P(3HB-*co*-3HV-*co*-4HV) blends isolated from cells harvested at 42, 47, and 60 h during the fed-batch fermentation.

but that the substrate feeding strategy is crucial to obtain the desired chemical composition distribution of copolyesters. As mentioned previously, each PHA granule contains different monomers depending on its history in the dynamic environment surrounding the cells. It has been reported that cocrystallization does not occur in the P(3HB)/P(3HV) blend because of the immiscibility of the two homopolyesters[41] but does occur in blends of P(3HB)/P(3HB-*co*-3HV).[42–44] The latter blends of P(3HB)/P(3HB-*co*-3HV) change in the order of complete cocrystallization, cocrystallization with partial phase segregation, and complete phase separation depending on the 3HB content in P(3HB-*co*-3HV).[45] Obviously, the 3HB content of the copolyester affects the miscibility of the homopolymer and copolymers. Similarly, P(3HB) has poor miscibility with a P(54 mol % 3HB-*co*-3HV-*co*-4HV) like sample B, but ter-polyesters with high 3HB content can also improve their miscibility. As mentioned above, the as-produced PHA (Sample A) is a blend of P(3HB-*co*-3HV-*co*-4HV)s with 3HB content ranging from 62 to

Table V. Thermal Data of Artificial P(3HB)/P(3HB-*co*-3HV-*co*-4HV) Blends

PHB/PHBVV[a] (ratio of E/B)	PHA composition[b] (mol %)			T_g (°C)	T_{m1} (°C)	T_{m2} (°C)	T_{m3} (°C)	ΔH_m (J/g)
	3HB	3HV	4HV					
100/0	100	0	0	3.9	-	161	176	91
80/20	89.5	9.3	1.2	3.8	-	161	175	92
60/40	80.5	17.7	1.8	3.8, −3.6	78	162	175	68
40/60	71.5	26.2	2.3	4.6, −4.6	75	162	175	59
20/80	62.4	34.9	2.7	4.6, −4.1	75	154	167	50
0/100	54	43	3.0	−4.2	61	78	-	40

[a]Polymer blends were mixtures of samples E and B.
[b]PHA monomer composition of the blending system was determined by 500 MHz ^{1}H NMR.

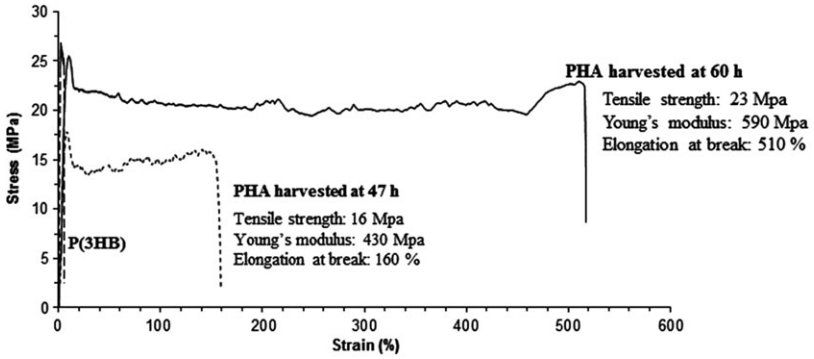

Figure 7. Typical stress–strain curves of P(3HB) (Sample E) and as-produced P(3HB-*co*-3HV-*co*-4HV) blend films harvested at 47 h (PHBVV47h) and 60 h (Sample A), respectively.

96 mol %. A continuous change in monomer composition seems necessary to form a miscible PHA blend.

A tensile test was performed to determine the effect of miscibility on the mechanical properties of PHA blends. Two thin films were prepared from as-produced PHA samples obtained at 47 h and 60 h (Table I), respectively. The former exhibited two glass transition temperatures and the latter exhibited a single glass transition temperature [Figure 6(b)]. The films were carefully aged in a vacuum oven for over a week and subsequently stored at room temperature for a month prior to measurement. Figure 7 shows typical stress/strain curves with a summary of mechanical data. For the as-produced P(3HB-*co*-16.5 mol % 3HV-*co*-1.7 mol % 4HV) immiscible blend harvested at 47 h (PHBVV47h), the measured tensile strength and the elongation at break were 16 MPa and 160%, respectively. The as-produced P(3HB-*co*-18.5 mol % 3HV-*co*-2.5 mol % 4HV) miscible blend harvested at 60 h (Sample A) exhibited greater mechanical strength and ductility than the PHBVV47h, with a tensile strength of 23 MPa and elongation at break of 510%. This is a much more ductile material than the previously reported glycerol-based P(3HB) homopolymer and P(3HB-*co*-20 mol % 3HV) copolymer, which possessed the elongations at break of 6% and 50%, respectively.[8,17] These results indicate that a longer biosynthesis time results in a more miscible PHA blend and that high 4HV content reduces the crystallinity of the blend, which not only improves the material's ductility but also the tensile strength. Indeed, the solution-cast film of the as-produced sample A was relatively transparent and did not show any visible changes even after storage for more than 5 months under ambient conditions (results not shown).

CONCLUSIONS

In this work, PHA copolyester blend was successfully biosynthesized from cosubstrates of glycerol and levulinic acid by a mutated *C. necator* with high PHA content and molecular size. The as-produced PHA blend was a natural miscible blend of copolymers with quite a broad chemical composition distribution. The low crystallinity and good miscibility result in a remarkable increase in mechanical strength and ductility of the natural P(3HB-*co*-3HV-*co*-4HV) blend, which could be controlled by feeding control of cosubstrates. With DSC, various crystalline regions from different copolymer fractions were observed in the as-produced PHA blend, and the overall behavior was primarily determined by the fraction of high 3HB content. The X-ray crystallinity of the random P(3HB-*co*-3HV-*co*-4HV) sample decreased considerably with increasing 3HV and 4HV content, distinct from the high crystallinity of P(3HB-*co*-3HV), indicating that a small fraction of randomly distributed 4HV units act as a defect in the P(3HB) crystalline structure and possibly preventing isodimorphism.

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