Synthesis and Characterization of Poly(3-hydroxybutyrate) and Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) Polymer Mixtures Produced in High-Density Fed-Batch Cultures of *Ralstonia eutropha* (*Alcaligenes eutrophus*)

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Received April 17, 1998; Revised Manuscript Received June 22, 1998

ABSTRACT: Alternate feeding of glucose and propionic acid to phosphate-depleted batch cultures of *Ralstonia eutropha* (reclassified from *Alcaligenes eutrophus*) produced poly(3-hydroxybutryate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] with thermal properties markedly different from random copolymers of similar monomer content. These polymers exhibited a single glass transition and a single melting peak that was significantly higher than expected for a random copolymer. The polymers were found, by solvent fractionation and NMR, to be a mixture of poly(3-hydroxybutyrate) [P(3HB)] and a random copolymer, P(3HB-*co*-3HV). When compared with random copolymers of similar monomer content, the polymers produced by alternate substrate feeding displayed no improvement in mechanical properties and possessed similar aging characteristics.

Introduction

Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) [P(3HB-*co*-3HV)] is a well-studied example of the many poly(hydroxyalkanoate)s (PHA) that can be produced by bacteria.[1] It is currently marketed as a biodegradable plastic (Biopol) by Monsanto. P(3HB-*co*-3HV) is produced from glucose and propionic acid in fed-batch culture by *Ralstonia eutropha* (reclassified from *Alcaligenes eutrophus*[2]) and is a random copolymer. P(3HB-*co*-3HV) has more commercial applications than poly(3-hydroxybutyrate) [P(3HB)] because it is substantially more flexible and can be melt-processed with less risk of thermal degradation. The thermal and mechanical properties of P(3HB-*co*-3HV) are well established.[3,4]

Yoshie et al.[5] have observed that bacterial P(3HB-*co*-3HV) does not have a narrow composition distribution but is composed of polymer chains containing differing proportions of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) monomers. These authors used solvent fractionation to separate a commercial sample of P(3HB-*co*-3HV) into several fractions. For example, P(3HB-*co*-21.8% 3HV) was fractionated into seven fractions ranging from 10.2 to 34.4 mol % 3HV. A wide composition distribution has also been observed for P(3HB-*co*-3HP)[6] produced by *Alcaligenes latus* and P(3HB-*co*-4HB)[7] produced by *R. eutropha*.

By varying the amounts of glucose and propionic acid supplied in fed-batch culture of *R. eutropha*, it is possible to produce P(3HB-*co*-3HV) containing 0−30 mol % 3HV.[8]

The objective of the present study was to assess the effect of alternate feeding of glucose and propionic acid on the composition and microstructure of PHA. Alternate feeding might produce either a mixture of P(3HB) and P(3HB-*co*-3HV), a random copolymer, or even a blocky polymer of the type poly[P(3HB)-*block*-P(3HB-*co*-3HV)]. In the latter case, a true block copolymer, composed of 3HB and 3HV blocks, cannot be synthesized by *R. eutropha* by alternate feeding of these substrates because both 3HV and 3HB monomer units are produced from propionic acid. The composition and properties of PHA produced by alternate substrate feeding are described and compared with those of a random copolymer produced by cofeeding glucose and propionic acid.

Experimental Section

Fed-Batch Cultures. *R. eutropha* NCIMB 40529 was grown in a fermentor (working volume 5 L) equipped with baffles and three Rushton turbine impellers. The temperature was maintained at 34 °C. The P-limiting medium (2.5 L) contained glucose (0.33 M), H_3PO_4 (1.63 mL of H_3PO_4 [85% w/w, specific gravity (sg) 1.69] per L), K_2SO_4 (16.15 mM), $MgSO_4 \cdot 7H_2O$ (8.86 mM), $(CH_3COO)_2Ca$ (2.23 mM), Na_2SO_4 (0.63 mM), $FeSO_4 \cdot 7H_2O$ (0.53 mM), $ZnSO_4 \cdot 7H_2O$ (0.33 mM), $MnSO_4 \cdot H_2O$ (0.24 mM), and $CuSO_4 \cdot 5H_2O$ (18 μM). The medium was sterilized by filtration (0.2 μm), adjusted to pH 6.8 within the fermentation vessel by addition of ammonia solution (7 M), and inoculated with a 200 mL culture grown in a shake-flask culture with glucose (20 g L^{-1}) as the sole carbon source.[9] The pH was maintained at 6.8 by automatic addition of sulfuric acid (2 M) or ammonia solution (7 M), the latter also providing a source of N. The dissolved oxygen concentration was measured by a polarographic probe (Ingold) and maintained above 20% air saturation by manually increasing the agitation and aeration rates from 500 rev min^{-1} and 2.5 L min^{-1}, respectively. Small doses (0.2 mL) of poly(propylene glycol) (2000 grade; Fisher) were added automatically at 15 min intervals during exponential growth to prevent foaming. The feeding phase was initiated as soon as glucose was no longer detectable in the culture supernatant. Glucose (600 g L^{-1}) and propionic acid (250 g L^{-1}) were fed (50 mL h^{-1}) alternately for 40 h, as indicated in Table 1. Bacteria were recovered by centrifugation (8000*g*, 15 min, 4 °C), washed with water, and lyophilized.

Analytical Methods. Inorganic phosphate in culture supernatant was determined by the method of Chen et al.,[10] and glucose was determined using a commercial kit (GOD-

S0024-9297(98)00606-8 CCC: $15.00
Published on Web 08/05/1998

Table 1. Production of PHA by Alternate Substrate Feeding

PHA	substrates[a] (g h^{-1})	feeding regime[a] (h)	biomass (g L^{-1})	PHA content (% w/w)	3HV (mol %)	$10^{-5}M_w$ (g mol^{-1})	M_w/M_n	$[\eta]$ (dL g^{-1})
F3	G(15)/P(6.25)	G(5)/P(20)/G(15)	82.1	50.5	10.2	6.7	4.38	3.69
F4	G(15)/P(6.25)	G(10)/P(10) etc.	58.2	52.6	18.1	5.6	3.95	4.48
F5	G(15)/P(6.25)	P(8)/G(8) etc.	81.1	58.9	7.5	6.9	4.36	4.19
F8	G(15)/P(6.25)	G(5)/P(5) etc.	89.2	78.2	7.3	7.1	3.70	5.00
R[b]		G+P(40)	118.1	70.8	7.3	4.6	2.51	2.37

[a] G = glucose, P = propionic acid. Figure in parentheses indicates feeding period for substrate specified. [b] Random copolymer.

Perid; Boehringer Mannheim). Propionic acid was determined by gas chromatography (GC). Samples (2 mL) of supernatant or propionic acid standards (0.1−5 g L^{-1}) were mixed with butyric acid (0.1% v/v, 1 mL) as internal standard, acidified with H_2SO_4 (5 M, 0.5 mL), and shaken with ether (1 mL) in screw-capped tubes. The tubes were centrifuged (1000*g*, 5 min) to break the emulsion. Samples of the ether layer were analyzed by GC, using a column (4 mm × 1.4 m) packed with 10% SP 1000/1% H_3PO_4 on Chromosorb 100/120 mesh (Supelco) and operated at 180 °C with N_2 as carrier gas (flow rate 30 mL min^{-1}).

The PHA content of bacteria was determined by GC of methyl-3-hydroxy acids produced by methanolysis[11] of lyophilized cells, using a BP20 capillary column (0.25 mm × 25 m; SGE) operated at 145 °C, with N_2 as carrier gas.

Extraction, Purification, and Fractionation of PHA. Lyophilized bacteria were refluxed (1 h) with redistilled chloroform (10 mL/g) and recovered by filtration (Whatman GF/B). The solution was concentrated by rotary evaporation to approximately 50 g of PHA L^{-1}. The polymer was precipitated by addition of hexane (4 vol), recovered by filtration, and then washed with hexane. Solvent-cast films were prepared by slow evaporation of chloroform from a PHA/chloroform solution containing approximately 20 g of PHA, in a glass casting dish (15 × 15 cm). PHA was solvent-fractionated with heptane[5] and acetone[12] according to the published methods, except that fractions were recovered by filtration rather than centrifugation.

Analysis of PHA. NMR analysis was carried out as previously described.[13]

The weight average molecular mass (M_w) of purified polymers was determined by gel permeation chromatography and viscometry using four Waters Ultrastyrogel columns (mean permeability 10^5, 10^4, 10^3, and 10^2 nm), with $CHCl_3$ as eluant. A differential refractive index detector and a single capillary viscometer were used, and M_w was estimated by the universal calibration method.

Differential scanning calorimetry (DSC) measurements were performed on a Perkin-Elmer DSC7 instrument and calibration was performed with indium and zinc. Samples (5−10 mg) were heated (20 °C min^{-1}) from 20 to 200 °C and then cooled to 20 °C at a rate of 20 °C min^{-1}. To determine the glass transition temperature (T_g), samples were cooled from +200 to −70 °C at a rate of 50 °C min^{-1} and then heated to 200 °C at a rate of 20 °C min^{-1}. Crystallization rates were determined by DSC. Samples (5−10 mg) were heated (20 °C min^{-1}) from 20 to 200 °C, held for 1 min and then quench-cooled (100 °C min^{-1}) to temperatures in the range 60−90 °C. The crystallization half-time ($T_{1/2}$) was taken as the fastest rate of crystallization.

Compression-molded films (25 × 25 cm) were made by molding the polymer powder at T_m + 10 °C under a pressure of 4.4 MPa. After molding, the films were annealed at 75 °C for 15 min in a vacuum oven with nitrogen bleed to facilitate crystallization. Samples were aged at constant temperature (22 °C) and humidity (50%). Tensile test pieces (0.1 in. width, 0.4 in. gauge length) were cut from polymer films using steel ASTM regulation punches. Mechanical testing was carried out using a Monsanto tensometer, using a crosshead speed of 10 mm min^{-1}. Samples were analyzed according to ASTM D638.[14]

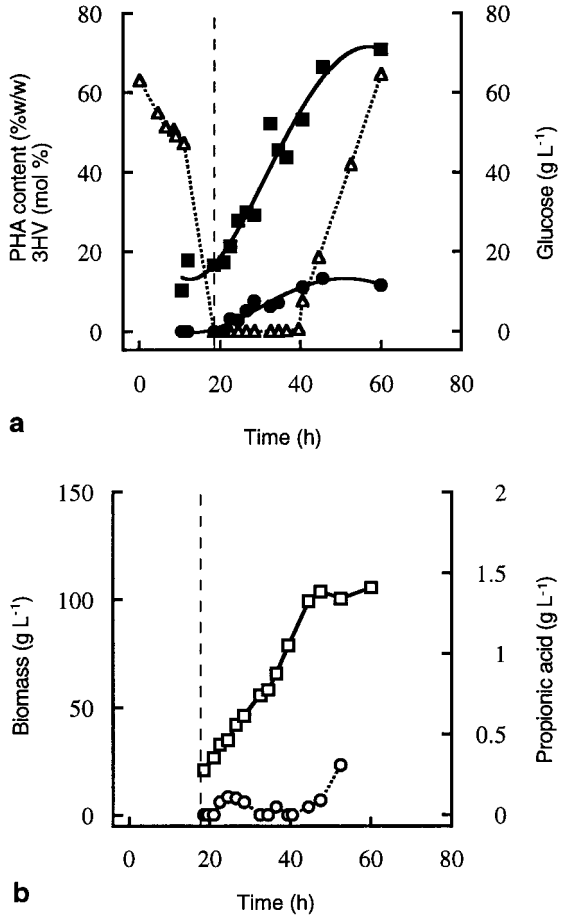

Figure 1. Production of a random copolymer by cofeeding glucose and propionic acid. Shown are (a) PHA content (■), mole fraction of 3HV (●), and residual glucose concentration (△) and (b) biomass (□) and residual propionic acid concentration (○). The vertical dotted line represents initiation of feeding at the time of glucose and phosphate exhaustion.

Results and Discussion

Production of PHA by Alternate Substrate Feeding. Polymers were produced by *R. eutropha* in high-density fed-batch culture, with phosphate as the growth-limiting nutrient. Various regimes for alternate feeding of glucose and propionic acid were used, and the polymers produced by alternate substrate feeding were coded according to the feeding program (Table 1).

The growth and accumulation phase of the control fed-batch culture, in which glucose and propionic acid were cofed over a 40 h period, is shown in Figure 1. Feeding was initiated at the time of glucose exhaustion, which occurred at approximately the same time as exhaustion of phosphate. At the end of the feeding period the cells contained 71% (w/w) P(3HB-*co*-3HV). The final 3HV content of the polymer was determined by NMR to be 7 mol %. The concentration of propionic acid in the culture remained low throughout the feeding phase and

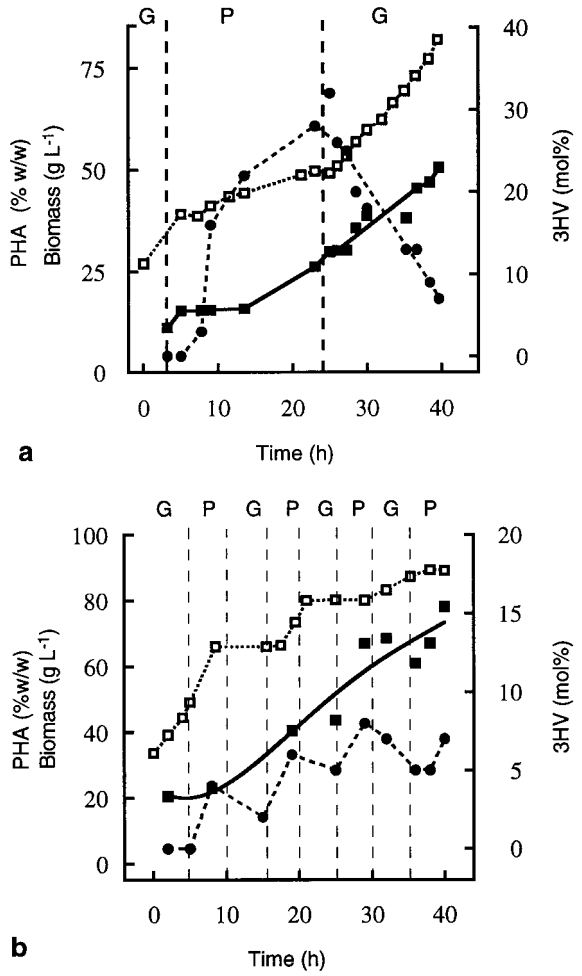

Figure 2. Production of (a) PHA F3 and (b) PHA F8 by alternate feeding of glucose and propionic acid. Shown are PHA content (■), mole fraction of 3HV (●), and biomass (□). Vertical dotted lines represent feeding changes from either glucose (G) or propionic acid (P).

increased slightly as the rate of PHA biosynthesis decreased (Figure 1). For alternate substrate feeding, glucose and propionic acid were fed sequentially, as specified in Table 1, but the 40 h feeding period and the total amounts of each substrate fed to cultures were unchanged.

Although the quantities of glucose and propionic acid supplied to fed-batch cultures were the same in each case, differences were seen in the final biomass concentration, PHA content of the bacteria, and mole fraction of 3HV in the polymer (Table 1). The yields of biomass and PHA from alternate substrate feeding were lower, although the mole fraction of 3HV in the polymer was higher than in the random copolymer produced by cofeeding of glucose and propionic acid. These changes are a result of the introduction of propionic acid feeding periods, where the yield of polymer is reduced (Figure 2a).

The M_w of PHA produced by alternate substrate feeding were slightly higher than that of the random copolymer, and the polydispersities were significantly higher. The increase in polydispersity suggests that polymers produced by alternate substrate feeding may be a mixture of polymers. The intrinsic viscosities [η] of PHA produced by alternate substrate feeding were higher than that of the random copolymer.

A comparison between cofeeding and alternate feeding of glucose and propionic acid shows that during the propionic acid feeding periods the yields of both polymer and biomass were much lower than those observed during cofeeding. For production of PHA F3, the yield of polymer during the propionic acid feeding period was 0.11 g of PHA (g of propionic acid consumed)$^{-1}$. During subsequent feeding of glucose the yield of PHA was 0.22 g/g. The overall yield of 3HV from propionic acid (0.046 g/g) was approximately one-third of that observed during cofeeding of glucose and propionic acid (0.12 g/g).

During the production of PHA F3 (Figure 2a), the bacteria had accumulated approximately 15% (w/w) P(3HB) by the end of the initial glucose feeding phase (5 h). At this time the feeding was changed to propionic acid. Throughout the propionic acid feeding phase there was a continuous increase in the mole fraction of 3HV in the accumulated polymer. At the cessation of the propionic acid feeding phase (25 h), the bacteria had accumulated 30% (w/w) PHA containing 32 mol % 3HV. During the final feeding phase, when glucose was supplied, a decrease in the 3HV mole fraction was observed. This is to be expected as only 3HB monomers are synthesized from glucose by *R. eutropha*, and therefore the proportion of 3HV monomers in the PHA will decrease. Residual propionic acid was undetectable (<0.05 g L^{-1}) throughout the polymer accumulation phase, and glucose was present in small quantities (<0.3 g L^{-1}) only during the glucose feeding phases. Therefore there was negligible carryover of substrates into the subsequent feeding period. During the feeding of propionic acid, a random copolymer of 3HB and 3HV units is synthesized, and a 3HB homopolymer is produced during the glucose feeding phase. Changes in the PHA content and composition using more frequent feeding changes (the production of F8) can be seen in Figure 2b. It is again evident that the 3HV mole fraction of the polymer increases and subsequently declines during the feeding of propionic acid and then glucose, respectively.

NMR Analysis of PHA Produced by Alternate Substrate Feeding. Copolymers of 3HB and 3HV normally possess a statistically random (Bernoullian) arrangement of monomers.[15] This is due to the nature of their production, whereby the precursors of both monomers are available throughout the polymer accumulation phase.

To measure the degree of deviation from a random sequence of monomer units, the *D* parameter was defined[15] and can be calculated from the diad fractions using eq 1.

$$D = \frac{F_{BB}F_{VV}}{F_{BV}F_{VB}} \tag{1}$$

It is easily demonstrated that a copolymer containing a statistically random arrangement of monomers has a *D* value of 1.0. Deviations from this value may indicate that the polymer is an alternate copolymer (−ABABAB−), a block copolymer (−AAAABBBB−), or a mixture of polymers with different monomer compositions. The observed diad fractions and those calculated for a random copolymer of the same 3HV content are shown in Table 2. Also shown is the *D* parameter and the number-average sequence length (NASL),[16] which is another indication of the sequence distribution that can be calculated from the diad fractions.

Table 2. NMR Analysis of of PHA Produced by Alternate Substrate Feeding

PHA	3HV (mol %)	diad intensities[a]			NASL		
		VV	VB + BV	BB	3HB	3HV	*D*
F3	10.2	0.0326	0.1391	0.8283	12.9	1.5	5.6
		[0.0121]	[0.1958]	[0.7921]	[9.1]	[1.1]	[1.0]
F4	18.1	0.0622	0.2370	0.7008	6.9	1.5	3.1
		[0.0328]	[0.2964]	[0.6708]	[5.2]	[1.2]	[1.0]
F5	7.5	0.0257	0.1093	0.8650	16.8	1.5	7.4
		[0.0056]	[0.1388]	[0.8556]	[13.3]	[1.1]	[1.0]
F8	7.3	0.0341	0.1191	0.8468	15.2	1.6	8.6
		[0.0053]	[0.1353]	[0.8594]	[13.7]	[1.1]	[1.0]
R[b]	7.3	0.0047	0.1365	0.8588	13.6	1.1	0.9
		[0.0053]	[0.1353]	[0.8594]	[13.7]	[1.1]	[1.0]

[a] From the carbonyl region of the ^{13}C-NMR spectrum. Numbers in brackets were calculated using Bernoullian statistics. [b] Random copolymer.

Table 3. Calculated[a] Composition of PHA Mixtures Produced by Alternate Substrate Feeding

PHA	3HV (mol %)	P(3HB) fraction (mol %)	P(3HB-*co*-3HV) fraction (mol %)	3HV content of copolymer fraction (mol %)
F3	10.2	69.1	30.9	33.0
F4	18.1	47.5	52.5	34.0
F5	7.5	69.5	30.5	24.6
F8	7.3	73.5	26.5	36.0

[a] Assuming that a mixture of P(3HB) and copolymer is produced(see text).

^{13}C NMR analysis of PHA produced by alternate substrate feeding revealed a significant increase in the VV diad fraction and NASL over those expected for a random copolymer of the same 3HV content (Table 2). The *D* parameter was considerably greater than 1 for all PHA produced by alternate substrate feeding. It has been found that P(3HB-*co*-3HV) with very high *D* values are usually mixtures of random copolymers.[15] In a study of the microstructure of bacterially produced P(3HB-*co*-3HV) the authors concluded that some PHA are random copolymers (*D* value 0.7−1.5), whereas others, with *D* values above 1.5, are mixtures of compositionally different copolymers.[15] The observed *D* values of PHA produced by alternate substrate feeding are well in excess of 1.5 (Table 2), indicating that a mixture of polymers was the most likely product. The VV and BV + VB diad fractions are attributable solely to the copolymer produced during the propionic acid feeding periods. In this case, the BB diad fraction of the copolymer can be calculated using eq 1, assuming a *D* value of 1.0. The calculated composition of each polymer produced by alternate substrate feeding is detailed in Table 3. The polymer produced in a feeding period can be estimated from the changes in PHA content and composition during that period. For example, during the propionic acid feeding period in the production of PHA F3, the PHA content of the bacteria increased by 15% (w/w) (Figure 2a). Overall, the PHA F3 accounted for 50% (w/w) cell dry weight at the end of the prescribed 40 h feeding period. It is therefore calculated that the copolymer produced from propionic acid constitutes approximately 30% of the total polymer, with P(3HB) making up the remainder (Table 4). This figure is in very good agreement with the composition measured by NMR, which shows a copolymer fraction of 31% (Table 3). Similar calculations provide excellent correlation between the composition measured by NMR and that calculated from analysis of the polymers produced during the feeding period (Tables 3 and 4).

Table 4. Composition of PHA Produced by Alternate Substrate Feeding[a]

PHA	PHA content (% w/w)	PHA from propionic acid (% w/w)	fraction of PHA from propionic acid (% of total)
F3	50.5	15.0	29.7
F4	52.6	26.4	50.2
F5	58.9	18.0	30.5

[a] As determined from gc analysis. Insufficient data for analysis of F8 PHA.

Table 5. Polymers Produced[a] during Accumulation of F4 PHA

feeding period	net PHA production (% w/w)	polymer synthesized
1, glucose	15	P(3HB)
2, propionic acid	10	P(3HB-*co*−33% 3HV)
3, glucose	11	P(3HB)
4, propionic acid	17	P(3HB-*co*−32% 3HV)

[a] Breakdown was calculated from PHA content and composition of bacteria harvested at the end of each feeding period.

The amount of PHA accumulated from propionic acid during production of PHA F4 was found to be 50% of the total by analyzing changes in the PHA composition throughout the feeding period (Table 5). NMR analysis of the F4 polymer revealed a copolymer fraction of 52.5 mol %.

During the production of PHA F4, P(3HB) had been accumulated to 15% (w/w) dry weight at the end of the first (glucose) feeding period. At the end of the first propionic acid feeding period the total polymer content of cells had increased to 25% (w/w), with an overall 3HV content of 13 mol %. It must be taken into account that 60% of the accumulated polymer at this time was P(3HB). The overall 3HV content of 13 mol % is the average 3HV content of the copolymer and P(3HB). The 3HV content of the copolymer was calculated to be 33 mol %. During the second propionic acid feeding phase, the PHA content of cells increased from 36% (w/w) to a final total of 53% (w/w), with a 3HV content of 15 mol % (determined by GC). During this period the net increase in the PHA content of the culture is 17% (w/w) and the overall increase in 3HV content of the polymer is 9 mol %. It was calculated that the 3HV content of copolymer synthesized in this period is 32 mol %. The complex nature of this polymer can be predicted from these observations (Table 5). It is clear that 3HB homopolymer accounts for 49% of the F4 polymer and the average 3HV content of copolymer, synthesized from propionic acid, is about 33 mol %. The actual composition of the F4 polymer, measured by NMR, revealed a P(3HB) fraction of 48% and the 3HV content of the copolymer to be 38 mol %.

Thermal Properties of PHA Produced by Alternate Substrate Feeding. The thermal properties of PHA produced by alternate substrate feeding were investigated by differential scanning calorimetry (DSC). Examples of the thermograms can be seen in Figure 3, and thermal data (T_m, T_g and ΔH_m) for all the polymers studied are given in Table 6. The mole fraction of 3HV had little effect on the T_m (Figure 4a). The ΔH_m values of PHA produced by alternate substrate feeding correlate well with data reported for random copolymers (Figure 4b). The polymers produced using longer propionic acid feeding periods (PHA F3 and F4) crystallized significantly faster than random copolymers of similar 3HV content, possessing crystallization half-times ($T_{1/2}$)

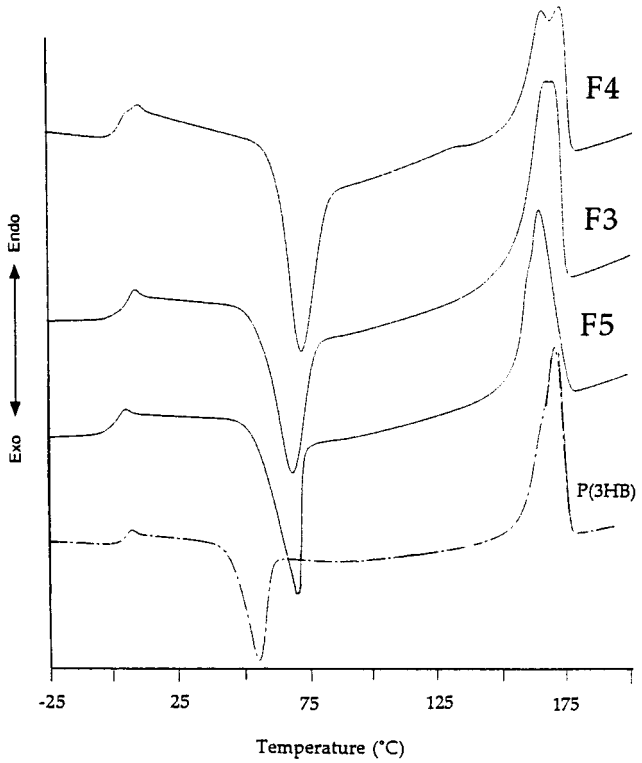

Figure 3. DSC thermograms of PHA produced by alternate substrate feeding and P(3HB).

Table 6. Thermal Properties of PHA Produced by Alternate Substrate Feeding

PHA	T_m (°C)	ΔH_m (J g^{-1})	T_g[a] (°C)	$T_{1/2}$[a] (min)	cryst temp[a] (°C)
F3	169	41.9	−1	0.48	75
F4	161	35.3	−3	0.46	80
F5	174	68.7	−3	2.09	70
F8	172	63.3	*c*	1.97	60
R[b]	158	72.2	*c*	0.90	60
P(3HB)	176	85.3	5	0.48	60
$F3_{H(a)}$	158	18.3	5	*c*	*c*
	173	34.0			
$F4_{H(a)}$	143	24.5	4	*c*	*c*
	168	55.4			
$F5_{H(a)}$	138	13.7	5	*c*	*c*
	173	74.5			

[a] T_g onset of the glass transition; $T_{1/2}$ and crystallization temperature refer to the minimum crystallization half-time and the temperature at which the minimum was observed. [b] Random copolymer with 6 mol % 3HV. [c] Not determined.

below 0.5 min (Figure 4c). The minimum crystallization half-times for these polymers were found to be close to that of P(3HB). PHA produced using more frequent feeding changes (PHA F5 and F8) displayed the crystallization behavior of random copolymers (Figure 4c). A single melting peak, which was very close to that of P(3HB) (173 °C), was observed for all PHA produced by alternate substrate feeding, indicating that the polymers crystallize in the P(3HB) lattice type with little or no disruption to the unit cell. This implies that the degree of cocrystallization is minimal and can account for the fast rate of crystallization of the PHA produced using less frequent feeding changes (PHA F3 and F4).

Compositional partition is likely to occur during the crystallization process in blends of P(3HB) and P(3HB-*co*-25% 3HV).[17] Such blends showed a single T_m, close to that of P(3HB), but the glass transition temperatures were not reported. As with PHA produced by alternate

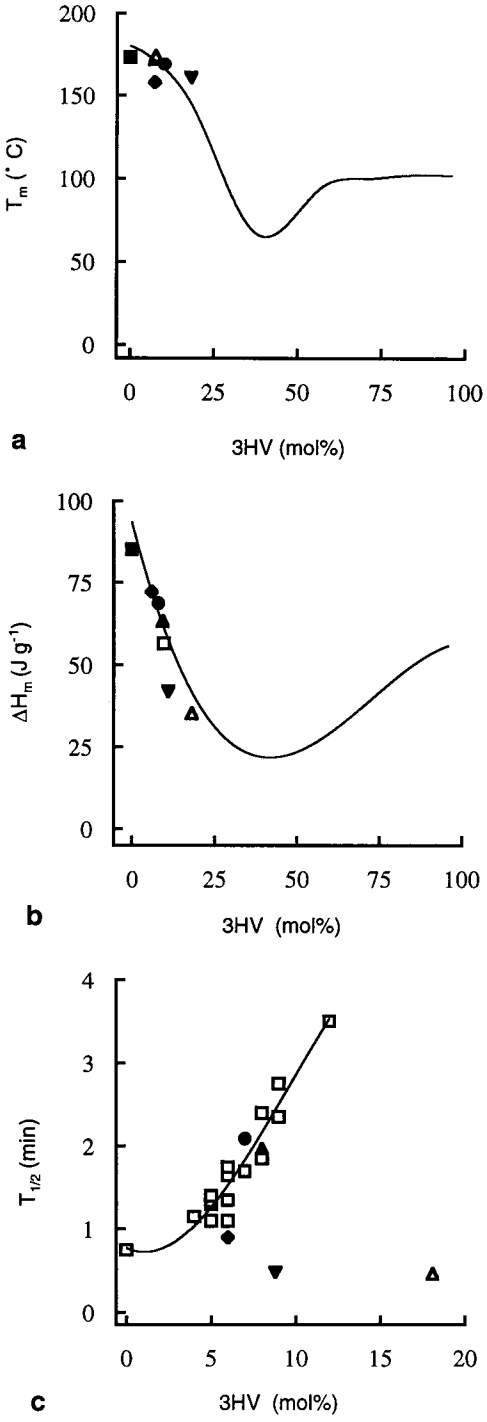

Figure 4. Thermal properties of PHA, showing variation in (a) melting temperature, (b) heat of fusion (solid lines represent random copolymers, data from ref 3), and (c) crystallization half-time, with increasing 3HV content. Other polymers (see Table 1) shown are P(3HB) (■), R (◆), PHA F3 (▼), F4 (△), F5 (●), F8 (▲), and Biopol samples (data supplied by Zeneca) (□).

substrate feeding in the present study, no melting peak corresponding to the copolymer was observed. The spherulitic growth rate of blends of P(3HB) and P(3HB-*co*-25% 3HV) was also found to be close to that of P(3HB).[17] It was suggested that intermolecular interaction may take place between the copolymer and P(3HB), which could prevent a proportion of the P(3HB) molecules from occupying the crystalline phase. The authors concluded that some uncrystallized P(3HB) molecules form a homogeneous solution with the copolymer

Table 7. Solvent Fractionation of PHA Produced by Alternate Substrate Feeding[e]

fract	heptane (% v/v)	weight fract (%)	3HV (mol %)	diad intensities VV	VB + BV	BB	*D*
F3		**100**	**10.2**	**0.0326**	**0.1391**	**0.8283**	**5.6**
$F3_{H(a)}$[a]	55.6	86	7.0	0.0212	0.1028	0.8760	7.0
$F3_{H(b)}$	61.1	3	35.0	0.1312	0.4265	0.4423	1.3
$F3_{H(c)}$	65.6	8	41.0	0.1908	0.4370	0.3722	1.4
$F3_{H(d)}$[b]		3	41.0	0.1945	0.4338	0.3717	1.5
F4		**100**	**18.1**	**0.0622**	**0.2370**	**0.7008**	**3.1**
$F4_{H(a)}$	51.2	51	6.8	0.0264	0.0831	0.8905	17.2
$F4_{H(b)}$	60.8	25	29.4	0.0894	0.4093	0.5013	1.1
$F4_{H(c)}$	61.6	10	31.8	0.1139	0.4089	0.4772	1.3
$F4_{H(d)}$[b]		14	34.1	0.1330	0.4162	0.4508	1.4
F5		**100**	**7.5**	**0.0257**	**0.1093**	**0.8650**	**7.4**
$F5_{H(a)}$	50.0	90	4.4	0.0152	0.0587	0.9261	16.3
$F5_{H(b)}$	59.8	7	26.1	0.0553	0.4124	0.5323	0.7
$F5_{H(c)}$[b]		3	*d*	*d*	*d*	*d*	*d*
$F3_{AS}$[c]		95	6.7	0.0215	0.0905	0.8880	9.3
$F3_{AIS}$[c]		5	40.5	0.1953	0.4199	0.3848	1.7
$F4_{AS}$[c]		95	17.7	0.0442	0.2124	0.7434	2.9
$F4_{AIS}$[c]		5	43.0	0.1241	0.4045	0.4718	1.4

[a] $F3_{H(a)}$ refers to polymer fraction (a), obtained using heptane. [b] Polymer remaining in solution and not precipitated by further addition of heptane. [c] AS and AIS are acetone-soluble and -insoluble fractions. [d] Not determined. [e] Bold type used to distinguish PHA samples from their fractions.

fraction in the amorphous phase. The spherulitic growth rate of P(3HB) far exceeds that of the copolymer fraction and so, during the initial stages of crystallization, only P(3HB) crystals can grow. This could result in the copolymer fraction becoming trapped within the interlamellar regions, thus preventing its crystallization. This model can also account for the thermal properties of PHA produced by alternate substrate feeding. Another study[15] of PHA microstructure revealed that a blend of P(3HB) and P(3HB-*co*-25% 3HV) possessed a single T_m corresponding to that of P(3HB). The authors also concluded that the copolymer fraction remained in the amorphous phase.

Fractionation of PHA Produced by Alternate Substrate Feeding. PHA produced by alternate substrate feeding were successfully fractionated both by precipitation from chloroform solution with heptane and by extraction into hot acetone (Table 7). It is clear that fractionation using heptane is by far the most efficient method of separating the individual polymers, yielding the most fractions. For each PHA, the first fraction to be precipitated using heptane accounted for the highest weight fraction and was composed primarily of 3HB monomer units. The *D* value was found to be high (7.0−17.2) for these fractions. All other fractions were found to be copolymers with a 3HV content of 26−41 mol %. A *D* value close to 1 was calculated, indicating that these polymers possessed a statistically random (Bernoullian) sequence distribution. One would assume that the random copolymers are synthesized during periods of propionic acid feeding, when no glucose was available to the bacteria. The 3HV content of the polymers yielded by heptane fractionation increased as the heptane concentration increased. This is due to the relative polarity of 3HV and 3HB monomers; the latter is more polar, and polymer rich in 3HB will be precipitated first on addition of heptane.[5]

In the case of heptane fractionation of PHA F3, four fractions were recovered (Table 7). The first fraction ($F3_{H(a)}$) was precipitated at a concentration of 55.6% (v/v) heptane. This fraction accounted for 86% of the total PHA and had a low 3HV content (7 mol %) and a high *D* value (7.0). This polymer is thought to be a mixture of 3HB homopolymer and P(3HB-*co*-3HV) that was not separated by the procedure. The remaining three fractions were found to be 3HV-rich (35−41 mol %) random copolymers (*D* value 1.3−1.5). Hot acetone fractionation of PHA F3 (Table 7) yielded a small amount of acetone-soluble polymer (5% w/w). Purification and characterization of this fraction established it as a random copolymer with 41 mol % 3HV. The similarity between the composition of this fraction and the last two fractions yielded by heptane fractionation may be because they are the same polymer. A small proportion (5% w/w) of PHA F4 polymer was also found to be soluble in acetone. This polymer was a random copolymer with 43 mol % 3HV. Four polymers were recovered by heptane fractionation of PHA F4 (Table 7). Three of these fractions were found to be random copolymers with *D* values close to 1. These polymers accounted for 49% of PHA F4, which correlates well with data in Tables 3−5. The average 3HV content of the random copolymers was 33.4 mol %, which agrees with previous calculations.

Synthesis of a polymer chain may commence during feeding of one substrate and be completed following the exchange of carbon source. Such chains will be of the type poly[P(3HB)-*block*-P(3HB-*co*-3HV)]. The average 3HV content of most of these chains will be low, relative to the copolymer chains, and so chains of this block nature would precipitate out of chloroform/heptane solution at about the same concentration of heptane as P(3HB) chains.

Due to the nature of PHA metabolism most bacteria accumulate small amounts of P(3HB) during unrestricted growth.[13] It has been noted that because of this, a mixture of PHA polymers with various 3HV contents would be accumulated during fed-batch production of P(3HB-*co*-3HV) in *R. eutropha*.[18] It has been reported[7] that during the production of P(3HB-*co*-4HB) in *R. eutropha*, the bacteria contained 9% (w/w) P(3HB) at the end of the initial growth phase. Fractionation of the final polymer revealed a substantial proportion of 3HB homopolymer. To produce a pure copolymer, it may be necessary to expose the cells to exogenous carbon starvation prior to the commencement of glucose and propionic acid feeding. This approach would force the cells to utilize their endogenous carbon source, i.e., P(3HB), although a potentially large decrease in viability is possible because cells may exhaust their P(3HB) reserves at different times and hence be deprived of this carbon and energy reserve.

Thermal Analysis of Polymer Fractions. Thermal analysis of the first polymer fraction recovered by heptane fractionation of PHA F3, F4, and F5 revealed that each possessed two distinct melting peaks (Figure 5). The major melting peak was close to that of P(3HB) (173 °C). The first fraction ($F5_{H(a)}$) of the F5 polymer had a major melting peak at 173 °C, with a corresponding ΔH_m of 74.5 J g^{-1}. Comparing this with pure P(3HB) (T_m 173 °C, ΔH_m 83.2 J g^{-1}) leads to the conclusion that this peak is attributable to 3HB homopolymer (Table 6). The minor melting peak (138 °C, ΔH_m 13.7 J g^{-1}) is probably a copolymer, which was not separated from the mixture by the fractionation procedure. Thus fraction $F5_{H(a)}$ appears to be a mixture of P(3HB) and copolymer.

NMR analysis of polymer fractions of PHA produced by alternate substrate feeding indicated that the poly-

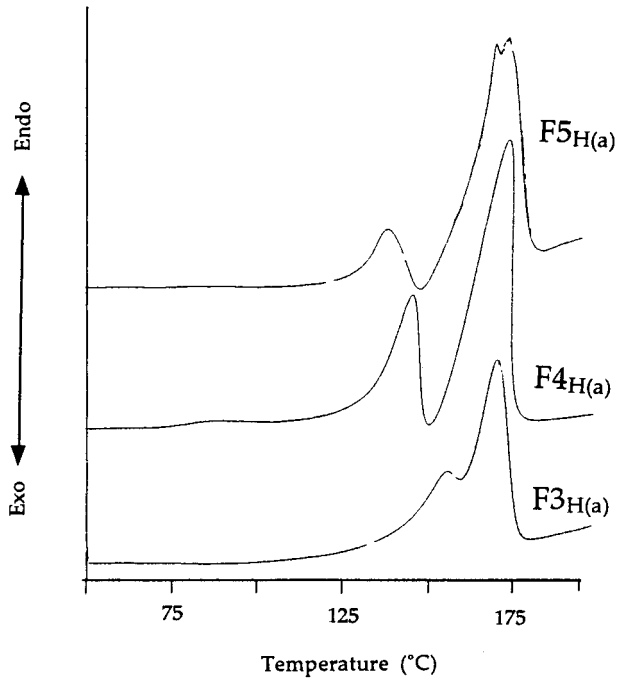

Figure 5. DSC thermograms of polymers obtained by heptane fractionation of PHA produced by alternate substrate feeding.

mer is a mixture of 3HV-rich random copolymer and P(3HB). Thermal analysis of the fractionated polymer supports the hypothesis that PHA produced by alternate substrate feeding are a mixture of P(3HB) and a random copolymer.

Changes in Mechanical Properties upon Storage. The closest approximation to PHA produced by alternate substrate feeding is of in vitro blends of P(3HB) and a 3HB/3HV copolymer. One study has been published on the mechanical properties of such blends.[19] The blends consisted of P(3HB) and P(3HB-*co*-18% 3HV), over a wide range of blend compositions. These blends also displayed the signs of aging characteristic of P(3HB)[20] and P(3HB-*co*-3HV).[21] Tests carried out on aged (5 weeks) samples revealed that their extension to break was in the region of 10−50%, depending on actual blend composition. Typically, the value was 10% when the overall 3HV content of the blend was below 15 mol %.

The mechanical properties of P(3HB) and P(3HB-*co*-3HV) are known to deteriorate upon storage at ambient temperatures, just above their T_g.[20,21] To ascertain whether the mechanical properties of PHA produced by alternate substrate feeding deteriorate with time, films were prepared by compression molding and tested immediately after preparation (0 days), after 7 days, and again after 28 days (Table 8).

The tensile strength and modulus of PHA produced by alternate substrate feeding were similar to those of in vitro blends.[19] Thus PHA produced by alternate substrate feeding offer no improvement in mechanical properties over blends of P(3HB) and P(3HB-*co*-3HV) or random copolymers. They also display the same detrimental aging effect, which appears to be an intrinsic property of P(3HB) and P(3HB-*co*-3HV).

Kinetics of Polymer Accumulation. Changes in the M_w, polydispersity, and total number of polymer chains per liter of culture ($[N]_t$) during the production of PHA F3 are shown in Figure 6. From these data the rates of chain propagation (R_p) and chain termination

Table 8. Mechanical Properties of PHA Produced by Alternate Substrate Feeding

polymer	age (days)	tensile strength (MPa)	elongation to break (%)	Young's modulus (MPa)
F3	0	29.0	17.4	472
	7	31.8	13.2	546
	28	34.4	9.5	788
F4	0	28.7	121.1	286
	7	26.6	23.5	518
	28	23.0	26.6	404
F5	0	19.0	13.6	272
	7	9.3	18.2	750
	28	8.2	11.2	476

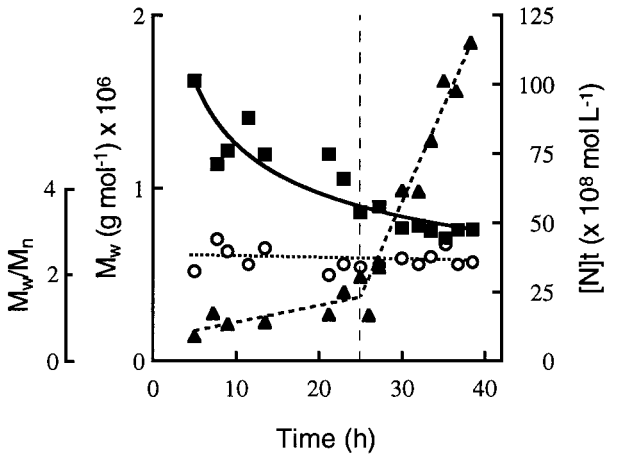

Figure 6. Changes in M_w (■), polydispersity (○), and number of PHA chains (▲) during the production of PHA F3.

Table 9. Rates of PHA Synthesis during Production of PHA F3

feed time (h)	PHA substrate	rate of PHA synthesis[a]	$10^2 R_p$[b]	$10^9 R_t$[b]	$10^{-5} R_p/R_t$
5−25	propionic acid	0.015	0.242	6.03	4.01
25−40	glucose	0.041	2.850	66.67	4.27

[a] g of PHA (g of non-PHA biomass)$^{-1}$ h^{-1}. [b] mol L^{-1} h^{-1}.

(R_t) can be calculated.[22] The ratio R_p/R_t is known as the kinetic chain length and ultimately determines the M_w of the polymer chain.[22] The kinetic chain length of polymer was 4.0×10^5 and 4.3×10^5 during the propionic acid and glucose feeding periods, respectively, indicating that the carbon source has little effect on the M_w of the polymer (Table 9). The kinetics of P(3HB) accumulation from butyric acid, by *R. eutropha* ATCC 17699, have been described,[22] and it has been calculated[23] that the PHA synthase catalyzes two propagation reactions per second. Our own data for P(3HB) production from glucose by *R. eutropha* NCIMB 40529 corresponds to a somewhat faster rate of 6.7 propagations per second.[24] From these data, a polymer chain of M_w 700 000 g/mol (e.g., PHA F5: Table 1) would be completed within approximately 0.3 h. Thus to produce poly[P(3HB)-*block*-P(3HB-*co*-3HV)] from propionic acid and glucose, it may be necessary to exchange carbon sources as frequently as every 5 min. Under these conditions, it is likely that a substantial proportion of random copolymer would be produced because both glucose and propionic acid will be present immediately after exchange of carbon sources.

Conclusions

Polymers produced by alternate substrate feeding are composed of P(3HB) and a smaller amount of 3HV-rich

random copolymer, P(3HB-*co*-3HV). These polymers are produced as mixtures. Block copolymers are not formed by alternate substrate feeding. The process of chain termination results in the polymer mixtures being formed rather than block polymers. The thermal properties of the polymer mixtures resemble those of P(3HB), and their mechanical properties were similar to those reported for random 3HB/3HV copolymers.

Acknowledgment. We thank Monsanto for financial support, for providing analytical services, and for useful discussions; in particular we express gratitude to Pierre Berger, Gerald Hook, and Ed Remsen.

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MA980606W