

**STRUCTURE AND PROPERTIES OF  
POLY(3-HYDROXYBUTYRATE-CO-4-HYDROXYBUTYRATE)  
PRODUCED BY *ALCALIGENES LATUS***

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**Summary**

Random copolymers of 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) with a wide range of compositions varying from 0 to 83 mol% 4HB were produced by *Alcaligenes latus* from the mixed carbon substrates of 3-hydroxybutyric and 4-hydroxybutyric acids. The structure and physical properties of P(3HB-co-4HB) were characterized by <sup>1</sup>H and <sup>13</sup>C NMR spectroscopy, gel-permeation chromatography, and differential scanning calorimetry. The isothermal radial growth rates of spherulites of P(3HB-co-4HB) were much slower than the rate of P(3HB) homopolymer. The enzymatic degradation rates of P(3HB-co-4HB) films by a PHB depolymerase were strongly influenced by the copolymer composition.

**Introduction**

A wide variety of microorganisms synthesize various kinds of poly(hydroxyalkanoic acids) (PHA) as an intracellular storage material of energy and carbon source (Anderson and Dawes, 1990; Doi, 1990). Since these microbial polyesters are biodegradable thermoplastics, they have attracted much attention as new environmentally compatible materials.

A copolymer of (R)-3-hydroxybutyrate and 4-hydroxybutyrate, P(3HB-co-4HB), was produced by *Alcaligenes eutrophus* in nitrogen-limited cultures containing 4-hydroxybutyric acid,  $\gamma$ -butyrolactone or 1,4-butanediol as carbon sources (Kunioka *et al.*, 1988; Kunioka *et al.*, 1989; Doi *et al.*, 1990). The copolymer composition was varied from 0 to 34 mol% 4HB, depending on the composition of carbon substrates supplied in feed. We also found that P(3HB-co-4HB) with high contents of 4HB unit (70-100 mol%) was accumulated in *A. eutrophus* cells from 4-hydroxybutyric acid in the presence of additives such as citrate and ammonium sulfate (Nakamura *et al.*, 1992). However, the yield of P(3HB-co-4HB) with compositions of 70-100 mol% 4HB was very low. Recently, we reported that P(4HB) homopolymer was produced from 1,4-butanediol by *Comamonas acidovorans* which was isolated from activated sludge (Saito and Doi, 1994).

Unlike many bacteria including *A. eutrophus*, which accumulate P(3HB) under conditions of the depletion of one or more essential nutrients, *Alcaligenes latus* produces efficiently P(3HB) from sucrose during the course of cell growth (Hänggi, 1990). In a previous communication (Hiramitsu *et al.*, 1993), we reported that *A. latus* produced P(3HB-co-4HB) (4HB = 0-45 mol%) with a high yield from a mixture of sucrose and  $\gamma$ -butyrolactone as carbon sources under growth conditions. In this communication, we produce P(3HB-co-4HB) from a mixture of 3-hydroxybutyric and 4-hydroxybutyric acids by *A. latus*, and study on the physical properties and enzymatic degradability of P(3HB-co-4HB) with a wide range of compositions.

## Materials and Methods

*Alcaligenes latus* (ATCC 29173) was used as a strain in this study. P(3HB-co-4HB) synthesis was carried out through a one-stage cultivation in which cell growth and polymer synthesis occur simultaneously. *A. latus* was inoculated in a mineral medium (100 ml, pH 7.0) containing carbon sources of different composition of sodium salts of 3-hydroxybutyrate and 4-hydroxybutyrate, and ammonium sulfate as nitrogen source. The basic medium used for all cultures contained the following (per liter of distilled water); Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O of 8.6 g, KH<sub>2</sub>PO<sub>4</sub> of 1.0 g, MgSO<sub>4</sub>·7H<sub>2</sub>O of 0.2 g, CaCl<sub>2</sub>·2H<sub>2</sub>O of 0.01 g, ammonium iron (III) citrate of 0.06 g and trace element solution 1 ml. The trace element solution contained following minerals (per liter of 0.1N HCl); H<sub>3</sub>BO<sub>3</sub> of 0.3 g, CoCl<sub>2</sub>·6H<sub>2</sub>O of 0.2 g, ZnSO<sub>4</sub>·7H<sub>2</sub>O of 0.03 g, MnCl<sub>2</sub>·4H<sub>2</sub>O of 0.03 g, NaMoO<sub>4</sub>·2H<sub>2</sub>O of 0.03 g, NiCl<sub>2</sub>·6H<sub>2</sub>O of 0.02 g and CuSO<sub>4</sub>·6H<sub>2</sub>O of 0.01 g. The bacterium was cultivated under aeration at 30°C for 48 hours on a reciprocal shaker in a 500 ml flask. The cells were harvested by centrifugation, washed with water, and finally lyophilized. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with hexane.

The compositions and sequence distributions of copolymers were determined by analysis of <sup>1</sup>H and <sup>13</sup>C NMR spectra of P(3HB-co-4HB). The <sup>1</sup>H and <sup>13</sup>C NMR analyses were carried out on a JEOL ALPHA-400 spectrometer. The 400-MHz <sup>1</sup>H NMR spectra were recorded at 23°C in a CDCl<sub>3</sub> solution of polyester (4 mg ml<sup>-1</sup>) with a 5.0 μs pulse width, 16000 data points, and 16 accumulations. The 100 MHz <sup>13</sup>C NMR spectra were recorded at 25°C in a CDCl<sub>3</sub> solution of polyester (20 mg ml<sup>-1</sup>) with a 4.5 μs pulse width, 27000 Hz spectral width, 16000 data points, and 3000 accumulations. Tetramethylsilane (Me<sub>4</sub>Si, δ = 0) was used as internal chemical shift standard.

Molecular weight data were obtained at 40°C on a Shimadzu 6A GPC system and a 6A refractive index detector with serial columns of Shodex K-802 and K-806M using chloroform as effluent at a flow rate of 0.5 ml min<sup>-1</sup>. The sample concentration was 1.0 mg ml<sup>-1</sup> and polystyrene standards with a low polydispersity were used to make a calibration curve.

The differential scanning calorimetry (DSC) data of polyesters were recorded on a Shimadzu DSC-50 under a nitrogen flow of 30 ml min<sup>-1</sup>. The melting temperature (T<sub>m</sub>) and enthalpy of fusion (ΔH<sub>m</sub>) were determined from the DSC endotherms. In order to measure the glass-transition temperature (T<sub>g</sub>), the melt samples were rapidly cooled to -150°C and then heated again to 200°C at rate of 20°C min<sup>-1</sup>. The T<sub>g</sub> was taken as the midpoint of the heating capacity change.

Measurements of the isothermal radial growth rate of spherulites of P(3HB-co-4HB) samples were carried out on a Nikon optical microscope with crossed polarizers and a Linkam TH600 hot stage. Thin films of samples, as obtained by solvent casting, were inserted between two microscope cover glasses and heated to 200°C. After keeping at 200°C for 1 min, the temperature was lowered to a given crystallization temperature (T<sub>c</sub>) by means of N<sub>2</sub> gas flow and the samples allowed to crystallize isothermally. A video camera, attached to the microscope through the Linkam VTO232 interface, allowed real time measurements of spherulite dimensions on a TV monitor. The radial growth rate of P(3HB-co-4HB) spherulites was calculated as the slope of the line obtained by plotting the spherulite radius against time with more than ten data points.

For enzymatic degradation tests, an extracellular PHB depolymerase was purified from *Alcaligenes faecalis* (Shirakura *et al.*, 1983). The P(3HB-co-4HB) cast films (initial weight, 6 mg; initial film dimensions, 10 x 10 x 0.05 mm) were placed in small bottles containing 1.0 ml of 0.1 M phosphate buffer (pH 7.4). The reactions were started by the addition of an aqueous solution of PHB depolymerase (1.0 μg). After shaking the sample

bottles at 37°C for 2 h, the films were removed, washed with distilled water, and dried to constant weight *in vacuo* before analysis.

## Results and Discussion

### *Microbial synthesis of P(3HB-co-4HB)*

Table 1 lists the result of P(3HB-co-4HB) production by *A. latus* from sodium salts of 3-hydroxybutyrate and 4-hydroxybutyrate for 48 h at 30°C. *A. latus* accumulated P(3HB) homopolymer in the cells up to 42 % of the dry weight, when 3-hydroxybutyrate was used as the sole carbon source. It is noted that 3-hydroxybutyrate was used for both cell growth and P(3HB) accumulation. In order to produce P(3HB-co-4HB), 4-hydroxybutyrate was added together with 3-hydroxybutyrate as carbon sources. The weight of dry cells decreased from 3.1 to 0.6 g/l as the fraction of 4-hydroxybutyrate in carbon sources was increased, but the mole fraction of 4HB unit in copolymers increased from 0 to 83 mol%. As shown in Figure 1, the 4HB fraction in copolymers increased proportionally with the fraction of 4-hydroxybutyrate in carbon sources.

Figure 2 shows a typical 100 MHz <sup>13</sup>C NMR spectrum of P(3HB-co-4HB) (54 mol% 4HB), together with <sup>13</sup>C chemical shift assignments (Doi *et al.*, 1988). The expanded carbonyl resonances ( $\delta=169-173$ ) were resolved into four groups of peaks, arising from different diad sequences of 3HB and 4HB units (3\*3 (3HB-3HB), 3\*4 (3HB-4HB), 4\*3 (4HB-3HB) and 4\*4 (4HB-4HB)). The diad sequence distributions of P(3HB-co-4HB) samples were determined from the relative peak areas of carbonyl resonances. The result is given in Table 2. The diad sequence distribution data were compared with the Bernoullian statistics applicable to a statistically random copolymerization. In the Bernoullian model, the mole fraction  $F_{ij}$  of diad sequence  $ij$  can be expressed with the mole

Table 1. Production of P(3HB-co-4HB) from the mixed carbon sources of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes latus* for 48 h at 30°C.

Sample	Carbon sources		N- <sup>c</sup> source (g/l)	Cell dry wt. (g/l)	Polymer content (%)	4HB fraction (mol%)	Molecular weight		Thermal properties		
	3HBA <sup>a</sup> (g/l)	4HBA <sup>b</sup> (g/l)					Mn x10 <sup>-3</sup>	Mw /Mn	Tg (°C)	Tm (°C)	ΔHm (cal/g)
1	8	0	1	3.1	42	0	166	2.6	4	175	22
2	7	1	1	3.2	40	13	153	2.8	-1	155	9
3	6	2	1	2.7	43	34	150	2.8	-3	57	4
4	4	4	1	2.4	46	54	165	3.1	-22	55	4
5	2	6	1	1.4	41	76	126	4.1	-37	46	3
6	1	7	1	0.6	63	83	126	2.8	-40	37	1
7	7.7	0.3	3	2.2	27	3	58	6.9	1	165	18
8	7.4	0.6	3	2.3	28	8	88	6.2	-1	161	13
9	7	1	3	2.2	30	13	74	5.0	-4	157	12
10	6	2	3	1.8	24	29	110	5.0	-3	144	5

a) Sodium (R)-3-hydroxybutyrate.

b) Sodium 4-hydroxybutyrate.

c) Ammonium sulfate was added as a nitrogen source.

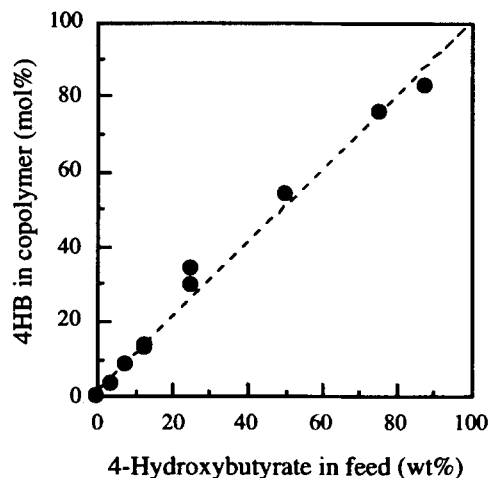


Figure 1. Relation between 4HB fraction in copolymer and 4-hydroxybutyrate in carbon sources.

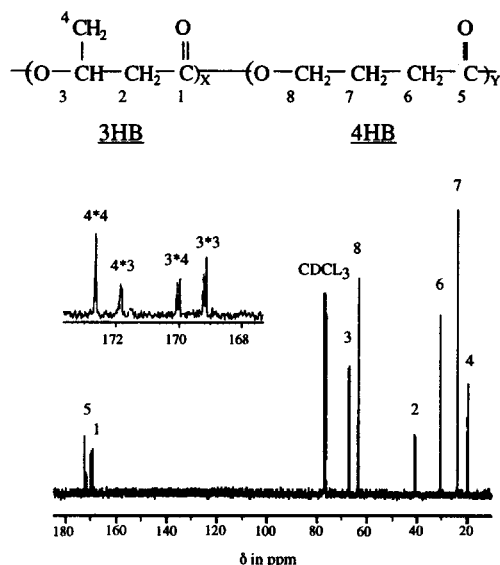


Figure 2. 100 MHz  $^{13}\text{C}$  NMR spectrum of P(3HB-co-54%4HB).

fraction  $F_i$  and  $F_j$  of  $i$  and  $j$  units as  $F_{ij} = F_i F_j$ . A parameter  $D$ , which is defined as  $(F_{33}F_{44})/(F_{34}F_{43})$ , was used to determine whether each sample was a random distribution or not. The value of  $D$  is equal to 1.0 for a statistically random copolymer. The  $D$  values of samples in Table 2 are in the range of 0.9 to 3.8, suggesting that the sequence distribution of 3HB and 4HB units in each samples is close to a random distribution.

Table 2. Diad sequence distributions of 3HB and 4HB units in P(3HB-co-4HB) samples.

Sample	Composition (mol%) <sup>a</sup>		Diad sequence distribution <sup>b</sup>				$D^c$
	3HB	4HB	$F_{33}$	$F_{34}$	$F_{43}$	$F_{44}$	
2	87	13	0.65	0.14	0.14	0.07	3.8
3	56	34	0.52	0.14	0.17	0.17	3.7
4	46	54	0.28	0.20	0.23	0.29	1.8
5	24	76	0.11	0.22	0.24	0.43	0.9
6	17	83	0.08	0.18	0.17	0.57	1.5
8	97	8	0.68	0.13	0.13	0.06	2.5
9	87	13	0.63	0.14	0.15	0.08	2.4
10	71	29	0.56	0.14	0.17	0.13	3.1

a) Determined by  $^1\text{H}$  NMR spectra.

b) The relative peak areas of carbonyl resonances in  $^{13}\text{C}$  NMR spectra.

c)  $D = F_{33}F_{44} / F_{34}F_{43}$

### Properties of P(3HB-co-4HB)

The number-average molecular weights ( $M_n$ ) of P(3HB-co-4HB) samples were in the range of  $0.6-1.7 \times 10^5$  (see Table 1). The thermal properties of P(3HB-co-4HB) samples are given in Table 1. The glass-transition temperature ( $T_g$ ) of P(3HB-co-4HB) samples decreased from 4 to  $-40^\circ\text{C}$  as the 4HB fraction increased from 0 to 83 mol%. The melting temperature ( $T_m$ ) also decreased from 175 to  $37^\circ\text{C}$  with an increase in the 4HB fraction. The heat of fusion ( $\Delta H_m$ ) decreased with an increase in the 4HB fraction, indicating that the degree of crystallinity of P(3HB-co-4HB) decreases with the 4HB fraction.

The spherulites of P(3HB-co-4HB) samples were observed with a polarized optical microscope. The samples were isothermally crystallized at a given temperature after melting at  $200^\circ\text{C}$  for 1 min. After crystallization, uniform spherulites were well-developed throughout the film. The spherulite radius increased linearly with time. Figure 3 shows the rate of spherulite ( $G$ ) of P(3HB-co-4HB) samples at different crystallization temperatures. The rates of spherulite growth were dependent on both copolymer composition and the crystallization temperature. The spherulite growth rate decreased with an increase in the 4HB fraction, and the crystallization curves were shifted toward lower temperatures. These results suggest that the incorporation of 4HB units into a P(3HB) sequence leads to a remarkable decrease in the rate of deposition of 3HB segments at the growing front of crystalline lamella.

Enzymatic degradation of P(3HB-co-4HB) films were carried out at  $30^\circ\text{C}$  in 0.1 M phosphate buffer (pH 7.4) of PHB depolymerase from *A. faecalis*. The weight loss (erosion) data of P(3HB-co-4HB) films after enzymatic degradation of 2 h are shown in Figure 4. The erosion rate of P(3HB-co-4HB) films increased sharply with an increase in the 4HB fraction up to 13 mol%, followed by a gradual decrease of the rate with the 4HB fraction up to 83 mol%. The highest rate of enzymatic degradation was observed on the film of P(3HB-co-13%4HB), and the rate was about 10 times faster than the rate of P(3HB) homopolymer.

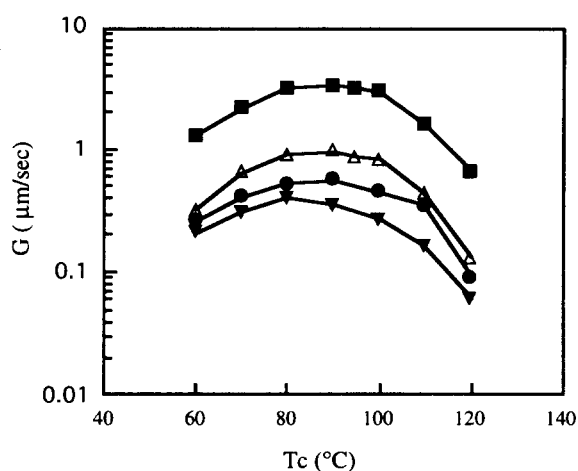


Figure 3. Radial growth rate ( $G$ ) of spherulites at various crystallization temperatures ( $T_c$ ) for P(3HB-co-4HB) samples with different 4HB fractions of 3 mol% ( $\Delta$ ), 8 mol% ( $\bullet$ ) and 13 mol% ( $\blacktriangledown$ ), and for P(3HB) homopolymer ( $\blacksquare$ ).

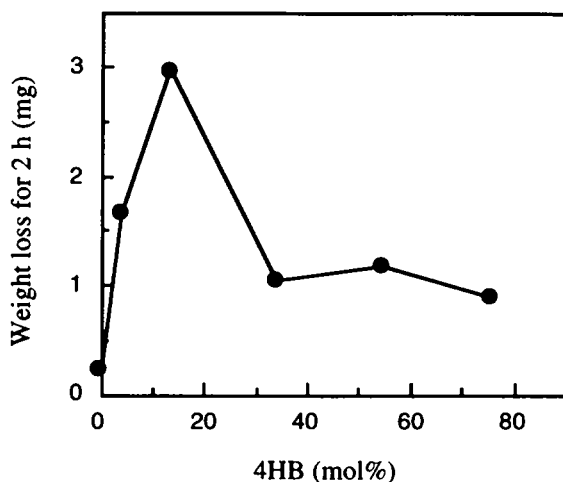


Figure 4. Enzymatic erosion data for 2 h at 37°C of P(3HB-co-4HB) films in aqueous solution of PHB depolymerase (1µg/ml) from *A. faecalis*.

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