Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxypropionate)

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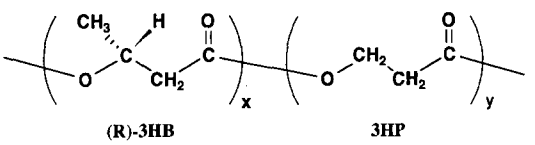
Copolymers of 3-hydroxybutyrate (3HB) and 3-hydroxypropionate (3HP) were synthesized in *Alcaligenes latus* from mixed carbon substrates of sucrose and 3-hydroxypropionic acid. The composition of copolyesters varied from 0 to 26 mol% 3HP, depending on the fraction of carbon substrates. The microbial copolyester was characterized by ^{1}H and ^{13}C nuclear magnetic resonance (n.m.r.) spectroscopy, gel permeation chromatography and differential scanning calorimetry. The copolymers were shown to have a random sequence distribution of 3HB and 3HP units by analysis of the 125 MHz ^{13}C n.m.r. spectra. The biosynthetic pathway of copolymers has been discussed.

(Keywords: hydroxybutyrate copolymer; microbial synthesis; nuclear magnetic resonance)

INTRODUCTION

Microbial poly(hydroxyalkanoate)s (PHA) are a biodegradable thermoplastic material with a wide range of physical properties[1-3]. A number of microorganisms synthesize isotactic homopolymers and copolymers of (*R*)-3-hydroxyalkanoic acids (3HA) with 4–14 carbon atoms as an intracellular storage material of carbon and energy[4,5]. Saturated[6-8], unsaturated[9,10], halogenated[11-13], branched[14] and aromatic[15] side chains in 3HA monomeric units have been found in the sequence of microbial PHA. In addition, some bacteria have been found to produce copolymers containing hydroxyalkanoate monomeric units without side chains such as 3-hydroxypropionate (3HP)[16], 4-hydroxybutyrate (4HB)[17,18] and 5-hydroxyvalerate (5HV)[19]. A random copolymer of (*R*)-3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) was produced by *Alcaligenes eutrophus* in nitrogen-free culture solutions containing 4-hydroxybutyric and butyric acids, and the copolymer compositions varied from 0 to 100 mol% 4HB, depending on the composition of carbon sources[17,18]. The physical properties and biodegradability of the copolyesters could be regulated by varying the copolymer composition[3,18]. As a result, the copolymers can be made in a wide variety of polymeric materials, from hard crystalline plastics to very elastic rubber.

In a previous paper[16], we reported that the copolymer of (*R*)-3-hydroxybutyrate and 3-hydroxypropionate, P(3HB-*co*-3HP), was produced by *Alcaligenes eutrophus* in nitrogen-free culture solutions containing 3-hydroxypropionic acid, 1,5-pentanediol, or 1,7-heptanediol. However, the mole fraction of 3HP unit in the copolymer was lower than 7 mol%.



(R)-3HB 3HP

In this paper we report that P(3HB-*co*-3HP) with a relatively wide range of compositions (0–26 mol% 3HP) is produced by *Alcaligenes latus* from mixed carbon substrates of 3-hydroxypropionic acid and sucrose. The sequence distributions of copolymers have been studied by analysis of ^{13}C n.m.r. spectra.

EXPERIMENTAL

Alcaligenes latus (ATCC 29713) was used in this study. Polyester synthesis was carried out by a single-stage fermentation of *A. latus*[20]. The strain was maintained in 10% (w/v) glycerin at −80°C. The bacterium was grown at 30°C under aerobic conditions on a reciprocal shaker in 500 ml Sakaguchi flasks with 100 ml of a mineral salts medium (pH 7.0) containing 0.8 g of sucrose and 3-hydroxypropionic acid as carbon sources. The mineral medium contained 8.6 g of $Na_2HPO_4 \cdot 12H_2O$, 1.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.06 g of ammonium iron(III) citrate and 0.01 g of $CaCl_2 \cdot 2H_2O$ per litre of distilled water. In addition, 1 ml of a microelement solution was added to the medium. The microelement solution contained the following (per

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Table 1 Production of poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) by *Alcaligenes latus*

Sample	Carbon source ($g l^{-1}$)		Cell dry weight ($g l^{-1}$)	Polyester content[a] (wt%)	Copolymer composition[b] (mol%)	
	Sucrose	$HOCH_2CH_2COOH$			3HB	3HP
1	8.0	0.0	4.0	60	100	0
2	7.5	0.5	3.5	49	90	10
3	7.0	1.0	2.6	40	89	11
4	6.0	2.0	3.0	43	81	19
5	4.0	4.0	2.0	29	74	26
6	0	2.0	0	–	–	–
7	0	3.0	0	–	–	–

[a] Polyester content in dry cells
[b] Determined by ^1H n.m.r. spectra

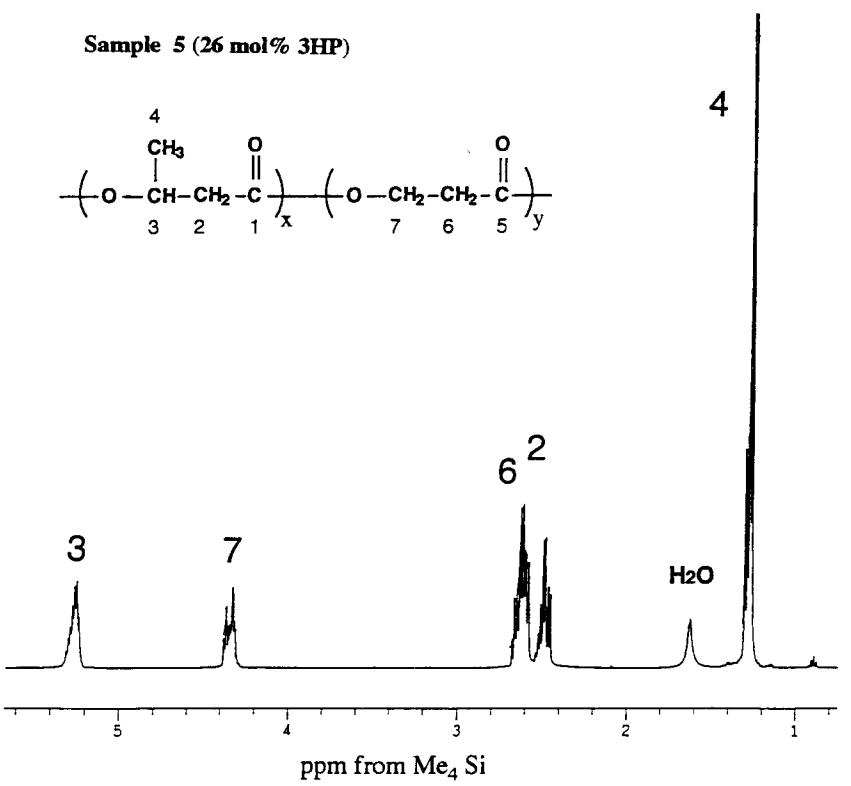


Figure 1 The 500 MHz ^1H n.m.r. spectrum of P(3HB-*co*-3HP) sample 5 (26 mol% 3HP) in $CDCl_3$

litre of 1.0 N HCl): 0.3 g of H_3BO_3, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.03 g of $MnCl_2 \cdot 4H_2O$, 0.03 g of $NaMoO_4 \cdot 2H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$ and 0.01 g of $CuSO_4 \cdot 6H_2O$. The cells were cultivated for 48 h at 30°C, harvested by centrifugation, washed with distilled 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 ^1H and ^{13}C n.m.r. analyses of polyester samples were carried out on a JEOL GX-500 spectrometer. The 500 MHz ^1H n.m.r. spectra were recorded at 27°C in a $CDCl_3$ solution of polyester (2 mg ml^{-1}) with a 4.5 μs pulse width (45° pulse angle), 5 s pulse repetition, 5000 Hz spectral width, 32K data points and 130 accumulations. The 125 MHz ^{13}C n.m.r. spectra were recorded at 27°C in a $CDCl_3$ solution of polyester (20 mg ml^{-1}) with 5.5 μs pulse width (45° pulse angle), 5 s pulse repetition, 2500 Hz spectral width, 64K data points and 7000 accumulations. Tetramethylsilane (Me_4Si) was used as an internal chemical shift standard.

Molecular-weight data of polyester samples were obtained by gel permeation chromatography (g.p.c.) using a Shimadzu LC-9A system equipped with an RID-6A refractive index detector and a Shodex K-80M column at 40°C. Chloroform was used as eluant at a flow rate of 0.5 ml min^{-1}, and a sample concentration of 1.0 mg ml^{-1} was used. Polystyrene standards with a low polydispersity were used to make a calibration curve.

The glass transition and melting temperatures of polyesters were recorded on a Shimadzu DSC-50 equipped with a cooling accessory under a nitrogen flow of 30 ml min^{-1}. Polyester samples of 2 mg were encapsulated in aluminium pans and heated at 10°C min^{-1} from 0 to 200°C. The melting temperature and enthalpy of fusion were determined from the d.s.c. endotherms. For the measurement of the glass transition temperature

Table 2 Analytical data of P(3HB-*co*-3HP) samples

Sample	Molecular weight[a]		Thermal properties[b]		
	$M_n \times 10^{-3}$	M_w/M_n	T_g (°C)	T_m (°C)	ΔH_m (cal g^{-1})
1	768	1.9	4.0	177	23.2
2	365	2.1	−1.0	155	10.3
3	343	2.2	−1.4	157	10.5
4	203	2.9	−2.3	150	7.2
5	293	2.2	−8.1	85	n.d.[c]

[a] Determined by g.p.c.
[b] T_g, T_m and ΔH_m were measured by d.s.c. at 10°C min^{-1}
[c] Not determined

(T_g), the samples were maintained at 200°C for 1 min and then rapidly quenched at −100°C. They were heated from −100 to +200°C at heating rate of 10°C min^{-1}. The T_g was taken as the midpoint of the heat-capacity change.

RESULTS AND DISCUSSION

Synthesis and properties of P(3HB-co-3HP)

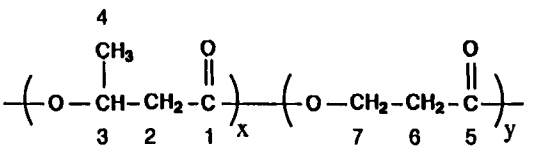
Table 1 lists the results of P(3HB-*co*-3HP) production by *Alcaligenes latus* from sucrose and 3-hydroxypropionic acid after 48 h cultivation at 30°C. *A. latus* accumulated P(3HB) homopolymer in the cells up to 60% of the dry weight during the course of growth, when sucrose was used as the sole carbon source[20]. In contrast, *A. latus* did not grow in the medium containing 3-hydroxypropionic acid as the sole carbon source. When 3-hydroxypropionic acid was fed with sucrose, P(3HB-*co*-3HP) copolymers were accumulated in *A. latus* cells. The copolymer content in dried cells decreased to 29 wt% with increasing proportion of 3-hydroxypropionic acid to sucrose in the culture solution, while the mole fraction of 3HP units in copolymers increased up to 26 mol%. The compositions of copolymers were determined by integration of the proton resonances of 3HB and 3HP monomeric units in the ^{1}H n.m.r. spectra (see *Figure 1*).

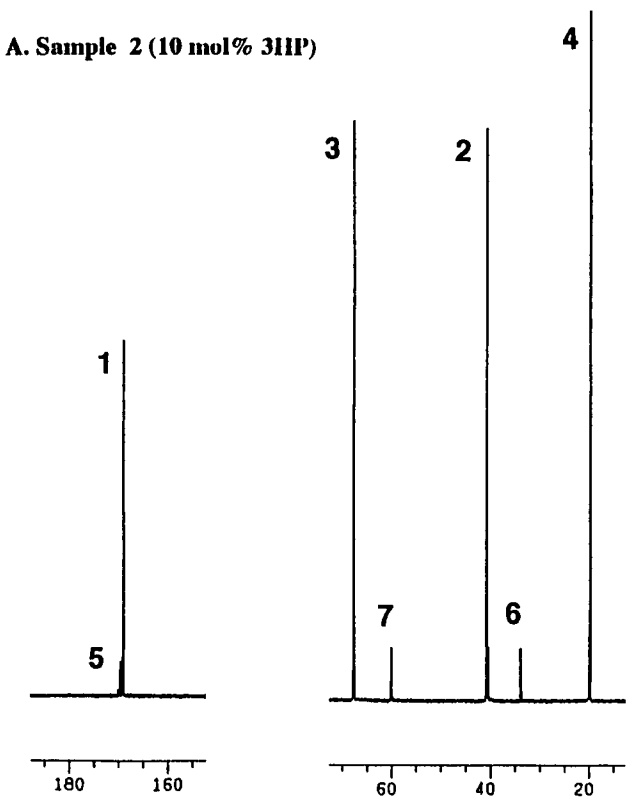
Table 2 shows the molecular weight and thermal properties of P(3HB-*co*-3HP) samples. The number-average molecular weights (M_n) of P(3HB-*co*-3HP) were in the range (2.0–3.7) × 10^5. The glass transition temperature (T_g) decreased from +4 to −8°C as the 3HP fraction increased from 0 to 26 mol%, and the melting temperature (T_m) decreased from 177 to 85°C. The enthalpies of fusion (ΔH_m) of P(3HB-*co*-3HP) decreased monotonically with an increase in the 3HP fraction, suggesting that the degree of crystallinity decreases with the 3HP fraction.

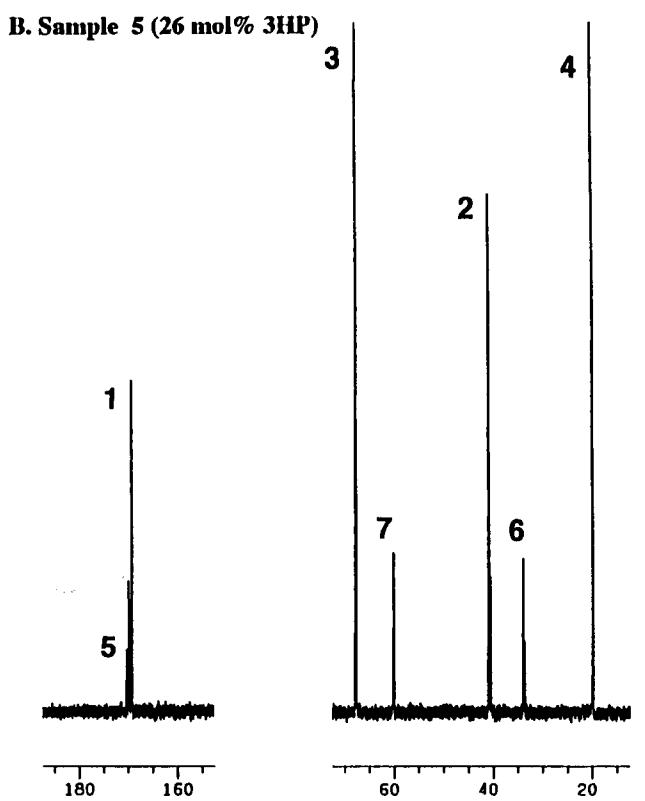
Sequence distribution of P(3HB-co-3HP)

Figure 2 shows the 125 MHz ^{13}C n.m.r. spectra of samples 2 and 5, together with the chemical shift assignment for each carbon resonance. All carbon resonances were split into several peaks, owing to different sequences of 3HB and 3HP units. The expanded spectra of seven carbon resonances for sample 5 (26 mol% 3HP) are shown in *Figure 3*. The ^{13}C chemical shifts and relative intensities of peaks a–f in seven carbon resonances are given in *Table 3*.

The carbonyl carbon resonances (1 and 5) at 169–170 ppm of sample 5 are resolved into four peaks,







ppm from Me_4Si

Figure 2 The 125 MHz ^{13}C n.m.r. spectra of P(3HB-*co*-3HP) samples 2 and 5 in $CDCl_3$

Table 3 Chemical shifts and relative intensities of ^{13}C resonances in P(3HB-*co*-3HP) samples 2 and 5

Carbon species	Peak[a]	Chemical shift (ppm)	Sequence	Relative intensities			
				Sample 2		Sample 5	
				Obs.	Calc.[b]	Obs.	Calc.[b]
$CH_3(4)$	a	19.78	BB*	0.90	0.90	0.67	0.74
	b	19.84	PB*	0.10	0.10	0.33	0.26
$CH_2(6)$	a	33.55	BP*P	0.14	0.10	0.21	0.19
	b	33.60	PP*P			0.10	0.07
	c	33.84	BP*B	0.86	0.90	0.48	0.55
	d	33.87	PP*B			0.21	0.23
$CH_2(2)$	a	40.43	BB*P	0.09	0.10	0.15	0.19
	b	40.46	PB*P			0.09	0.07
	c	40.81	BB*BB	0.91	0.90	0.76	0.74
	d	40.84	BB*BP				
	e	40.86	PB*BB				
	f	40.88	PB*BP				
$CH_2(7)$	a	59.93	BP*P	0.10	0.09	0.15	0.19
	b	60.01	BP*B	0.80	0.81	0.63	0.55
	c	60.07	PP*P	0.00	0.01	0.07	0.07
	d	60.14	PP*B	0.10	0.09	0.15	0.19
CH(3)	a	67.59	BB*P	0.10	0.09	0.14	0.19
	b	67.63	BB*B	0.80	0.81	0.63	0.55
	c	67.71	PB*P	0.02	0.01	0.06	0.07
	d	67.77	PB*B	0.08	0.09	0.17	0.19
CO(1)	a	169.15	B*B	0.81	0.81	0.56	0.55
	b	169.64	B*P	0.08	0.09	0.18	0.19
CO(5)	c	169.82	P*B	0.08	0.09	0.18	0.19
	d	170.25	P*P	0.03	0.01	0.08	0.07

[a] Peaks in *Figure 3*
[b] Calculated by Bernoullian statistics with the mole fraction of 3HP unit in *Table 1*

arising from different diad sequences of 3HB and 3HP units. The peak a at 169.15 ppm is assignable to the carbonyl resonance in the B*B (3HB*–3HB) sequence, since the chemical shift is consistent with that (169.16 ppm) of the carbonyl resonance in P(3HB) homopolymer. The peak d at 170.25 ppm is assigned to the carbonyl resonance in the P*P (3HP*–3HP) sequence, since the chemical shift is almost consistent with that (170.30 ppm) of P(3HP) homopolymer (polypropiolactone). The peaks b and c may be assigned to the carbonyl resonances in the B*P (3HB*–3HP) and P*B (3HP*–3HB) sequences, respectively. The other five carbon resonances are split into two, four, or six peaks as well as the carbonyl resonances. The chemical shift assignment of each peak has been made by a comparison of the chemical shifts and of the relative peak areas of all peaks in the ^{13}C n.m.r. spectra of P(3HB), P(3HP) and P(3HB-*co*-3HP) samples with different composition. The result is given in *Table 3*.

The diad and triad sequence distribution data for 3HB and 3HP monomeric units 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 in terms of the mole fractions F_i and F_j of i and j units as $F_{ij}=F_iF_j$, and the mole fraction F_{iji} of triad sequence iji as $F_{iji}=F_i^2F_j$. *Table 3* gives the diad and triad fractions of samples 2 and 5 calculated with the 3HP mole fractions, 0.10 and 0.26, respectively. The calculated diad and triad fractions are in good agreement with the observed values. Thus, it is concluded that the sequence distributions of 3HB and 3HP units in copolymer samples are statistically random.

Here, we propose a pathway of P(3HB-*co*-3HP) biosynthesis in *A. latus* from sucrose and 3-hydroxypropionic acid (*Figure 4*). Sucrose is transported into the cells and metabolized to acetyl-coenzyme A (CoA). A portion of acetyl-CoA enters the tricarboxylic acid (TCA) cycle for cell growth, while the other is metabolized into *R*-(−)-3-hydroxybutyryl-CoA. 3-Hydroxypropionic acid is converted to 3-hydroxypropionyl-CoA in the cells. Then, a random copolymer of 3HB and 3HP units may be produced by the copolymerization of *R*-(−)-3-hydroxybutyryl-CoA with 3-hydroxypropionyl-CoA under the action of PHA polymerase[21]. *A. eutrophus* grew on 3-hydroxypropionic acid, while *A. latus* did not. When 3-hydroxypropionic acid was used as the sole carbon source for *A. eutrophus*, P(3HB-*co*-3HP) was produced and the 3HP fraction was only 7 mol%[16]. These results suggest that 3-hydroxypropionyl-CoA is metabolized into acetyl-CoA in *A. eutrophus*. In contrast, *A. latus* may be incapable of metabolizing 3-hydroxypropionyl-CoA into acetyl-CoA. As a result, the P(3HB-*co*-3HP) with a relatively high content of 3HP units may be formed in *A. latus*.

In conclusion, the use of *A. latus* is of practical

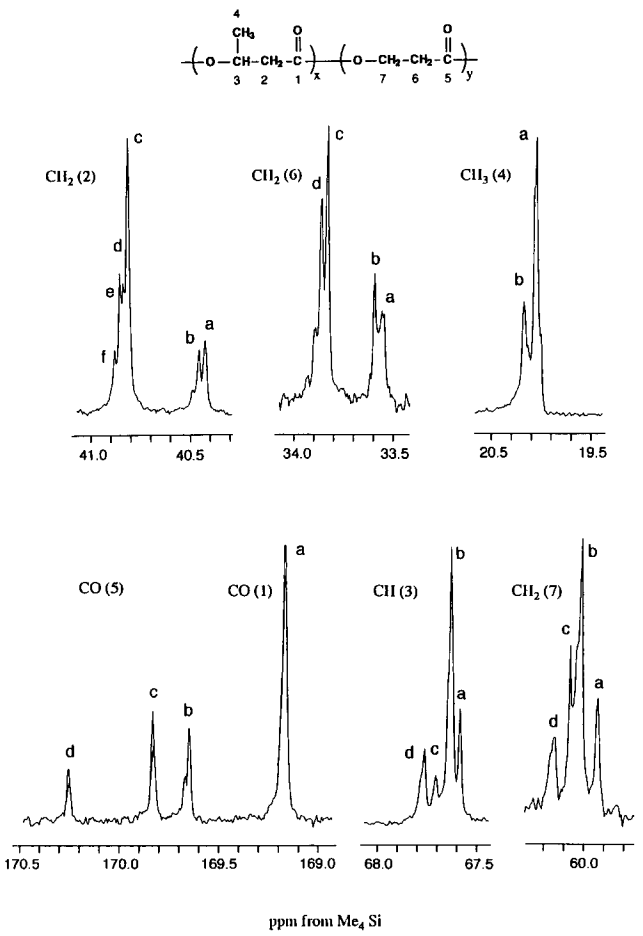


Figure 3 Expanded ^{13}C n.m.r. spectra at 125 MHz of sample 5 (26 mol% 3HP). The ^{13}C chemical shift assignments are given in *Table 3*

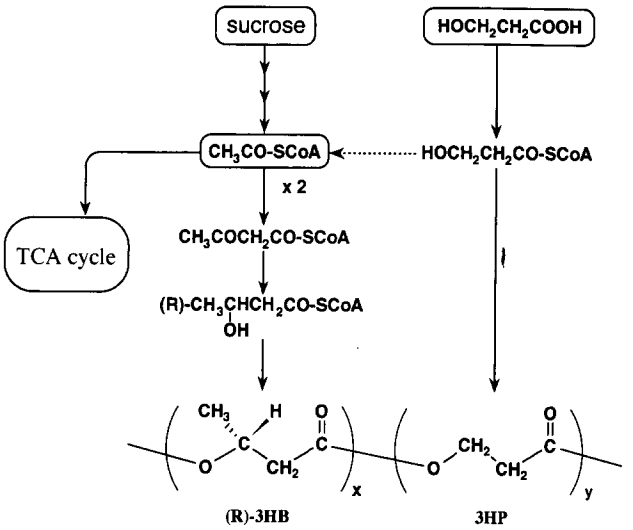


Figure 4 Schematic pathway of P(3HB-*co*-3HP) biosynthesis

importance in the production of a new copolymer, P(3HB-*co*-3HP). We are now studying the mechanical properties and biodegradability of P(3HB-*co*-3HP).

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