ARTICLE

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Biosynthesis and Characterization of 3-Hydroxyalkanoate Terpolyesters With Adjustable Properties by *Aeromonas hydrophila*

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ABSTRACT: Polyhydroxyalkanoates (PHA) terployesters P(3HB-*co*-3HV-*co*-3HHx) consisting of 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) were produced by wild-type *Aeromonas hydrophila* 4AK4, its recombinant harboring PHA synthesis genes *phaPCJ* encoding PHA binding protein phasin, PHA synthase, and enoyl-CoA hydratase, and another its recombinant harboring *phaAB* encoding beta-ketothiolase and acetoacetyl-CoA reductase, respectively, when grown in lauric acid and/or valerate. The terpolyesters produced by *A. hydrophila* 4AK4 (*phaAB*) grown in velarate were found to produce copolymers P(3HB-*co*-3HV) containing high 3HV fractions with a maximum of 99 mol% 3HV. In terpolyesters, 3HV ranged from 9 to 32 mol% depending on the valerate concentration and strain used. A maximal terpolyester P(3HB-*co*-3HV-*co*-3HHx) content in dry cells was 71 wt%. Transmission electron microscopy study of *A. hydrophila* 4AK4 harboring *phaPCJ* revealed the full occupation of terpolyester P(3HB-*co*-3HV-*co*-HHx) in the cellular spaces. Terpolyesters with various monomer compositions showed changing thermal and mechanical properties. Those with higher 3HV fractions demonstrated an improved property over the lower HV containing ones.

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KEYWORDS: polyhydroxyalkanoates; 3-hydroxyvalerate; *Aeromonas hydrophila*; PHA synthase; mechanical property; biopolyester

Introduction

Polyhydroxyalkanoates (PHA) are produced by many bacteria as carbon and energy storage compounds, they can also serve to enhance microbial resistance to adverse environmental conditions (Hazer and Steinbüchel, 2007; Kadouri et al., 2005; Lee, 1996; Zhao et al., 2007). Due to their biodegradability, biocompatibility, thermoprocessibility, and sustainability, PHA have found applications as bioplastics and implant biomaterials (Chen and Wu, 2005; Doi and Steinbüchel, 2002; Tian et al., 2001).

Generally, PHA can be classified into three main types depending on the number of carbon atoms in the monomer units: short-chain-length PHA (SCL-PHA) containing 3–5 carbon atoms in the monomer, medium-chain-length PHA (MCL-PHA) consisting of 6–14 carbon atoms, and copolymer containing SCL- and MCL-PHA monomer (Madison and Huisman, 1999; Wu et al., 2000). SCL-PHAs are partially crystalline thermoplastic materials, while MCL-PHAs are sticky, elastic, and amorphous materials (Sudesh et al., 2000). A few bacteria were reported to produce PHA containing both SCL and MCL monomer units (Chen et al., 2001, 2004). These copolymers consisting of SCL and MCL 3HAs possess much better properties than SCL or MCL polymers (Doi et al., 1995). Recently, terpolyesters P(3HB-*co*-3HV-*co*-3HHx) were reported to be synthesized by recombinant *Escherichia coli* containing PHA synthesis genes cloned from *Aeromonas* spp. and recombinant *Aeromonas hydrophila* 4AK4 harboring genes *phaAB* (Lu et al., 2004; Park et al., 2001; Qiu et al., 2004; Zhao and Chen, 2007). *A. hydrophila* 4AK4 and its recombinant were found to be able to accumulate P(3HB-*co*-3HV-*co*-3HHx) when lauric acid and propionic acid or undecanoic acid were used as carbon sources (Lu et al., 2004; Zhao and Chen, 2007). However, 3HV fraction in P(3HB-*co*-3HV-*co*-3HHx) was well below 10%, this could not significantly change the

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terpolyester properties (Zhao and Chen, 2007). On the other hand, valerate was demonstrated to have the superior convertibility to form 3HV compared with that of propionic acid (Braunegg et al., 1998). It was expected that the variation of the 3HV in the terpolyesters would lead to a wide variety of thermo-mechanical properties that could meet various application requirements including tissue engineering, soft packaging, and strong and elastic fiber making, etc.

In this article, we demonstrated the possibility of broadly manipulating the 3HV content in the terpolyester P(3HB-*co*-3HV-*co*-3HHx) using recombinant *A. hydrophila* 4AK4 and wild-type *A. hydrophila* 4AK4 grown in lauric acid and/or valerate. Thermo-mechanical properties of the terpolyesters with high 3HV content were studied.

Materials and Methods

Bacterial Strains and Shake-Flash Culture Conditions

Wild-type *A. hydrophila* 4AK4 and its recombinants were used in this study. The recombinant strains were prepared using plasmid pTG01 harboring β-ketothiolase gene *phaA* and NADPH-dependent acetoacetyl-CoA reductase gene *phaB* cloned from plasmid pUC-AB (Gao et al., 2002) or plasmid pQHA08 containing PHA synthesis genes *phaPCJ* cloned from *Aeromonas caviae*. Construction of these plasmids was described previously (Han et al., 2004).

For the production of PHA terpolysters, the strains were grown at 30°C in a mineral salt medium (Xie and Chen, 2008) (pH 7.2) supplemented with lauric acid and sodium valerate. Kanamycin was added to the medium at a final concentration of 50 mg/L when needed. The cultures were incubated in 500 mL conical flasks containing 100 mL culture broth on a rotary shaker (FUMA QYC2112, Shanghai, China) at 200 rpm and 30°C. The strains were first cultivated in 50 mL conical flasks each containing 20 mL Luria–Bertani (LB) medium consisting of (g/L) 10 tryptone (Oxoid Ltd., Basingstoke, Hampshire, England, UK), 5 yeast extract (Oxoid), and 10 sodium chloride medium overnight (12 h), then 5% (v/v) seed cultures were transferred into mineral salt medium (pH 7.2) supplemented with 1 g/L yeast extract, 8 g/L lauric acid, and different amounts of valerate. Lauric acid (8 g/L) and 1 g/L valerate were added at the beginning of growth except 0.5 g/L valerate concentration, additional valerates were added in two equal pulse installments at an interval of 24 h (such as 1 g/L at 24th hour and another 1 g/L at 48th hour) when the concentrations of valerate were more than 1 g/L. Valerate means sodium valerate throughout the article. In addition, all of the other conditions were similar to previous description when *A. hydrophila* 4AK4 harboring *phbA* and *phbB* (*phaAB*) was cultured with valerate and sodium gluconate.

Fermentor Studies

Production of P(3HB-*co*- 3HV-*co*-3HHX) was conducted in a 7.5 L jar fermentor (Bioflo 110, NBS, New Jersey). Seed culture was prepared in a 500 mL flask containing 125 mL of medium and was incubating at 30°C for 12 h, then 5%(v/v) seed cultures were transferred into the fermentor. All fed-batch cultures were carried out in the 7.5 L jar fermentor with an initial volume of 2.5 L at 30°C. The initial culture medium in the fermentor was the same as described in the above shake-flask culture conditions. The pH value was set at 6.2 using a 25% (v/v) H_3PO_4 and a 10% NH_4OH aqueous solutions. Dissolved oxygen (DO) was provided by injecting filtered air and was maintained at 20% of air saturation by automatically adjusting the agitation rate from 200 to 800 rpm. The valerate was fed separately together with lauric acid.

PHA Extraction and Purification

Liquid cultures was collected by centrifugation (5,000*g* for 6 min) and washed twice with absolute ethanol and distilled water, respectively. After lyophilization overnight, intracellular PHA polymers were isolated from lyophilized cells using hot chloroform extraction at 100°C for 4 h in a high-pressure reactor under stirring conditions. After cooling to room temperature, the PHA was filtrated through a Whatman No. 1 paper filter to remove the debris, PHA dissolved in chloroform was then precipitated with 10-fold volume of ice-cold ethanol. The collected PHA was dried under vacuum at 40°C for 24 h to completely remove the solvent.

Gas Chromatography (GC) Analysis of Intracellular PHA

PHA accumulation and composition were analyzed by GC (Agilent Technologies Inc., Santa Clara, State of California, 6820, 30 m column length, 320 μm span) equipped with a DB1 column. Both lyophilized cells and extracted PHA were subjected to methanolysis in the presence of 3% (v/v) sulfuric acid and PHA was extracted and methyl esterified at 100°C for 4 h using benzoic acid as an internal standard (Braunegg et al., 1978; Chen et al., 2001). For peak identification, a PHA standard mixture (kindly donated by Tsinghua University, Beijing, China) was used. The average results of three parallel experiments were recorded.

Transmission Electron Microscopy (TEM)

Samples were prepared by washing harvested cells three times in phosphate buffer solution (PBS) at pH 7.0, followed by centrifugation at 3,000*g* in a Centrifuge (Eppendorf 5804 R, Hamburg, Germany). Samples were then fixed with 2.5% glutaraldehyde (in buffer) for 2 h then rinsed three times in PBS. Next, samples were treated with 2.5% OsO_4 (buffer) solution for 60 min and then washed three times in

PBS. A dehydration series of 50%, 80%, 90%, and 100% ethanol was performed. Finally the sample was infiltrated with embedding agent overnight and then cured at 63°C for 72 h. The blocks were then microtomed to create a smooth face. Sections were then placed on copper grids and dried. A Hitachi H-300 TEM was used for the microscopic studies.

PHA Molecular Weight Study Using Gel-Permeation Chromatography (GPC)

Average molecular weights were estimated by GPC using a Waters 1525 pump with a combination of four styragel columns series (Styragel HR, 5 μm) (Waters, Taunton, Massachusetts). A 2414 differential refractive index detector and UV detector were employed. Chloroform was used as eluent at a flow rate of 1.0 mL/min. The sample concentration was 2 mg/mL and the injection volume was 50 μL. The calibration curve was generated with polystyrene standards (Shodex, Japan) (Fernandez et al., 1986).

Physical Characterization of PHA Polymers

Differential scanning calorimetry (DSC) data were recorded in temperature range of −60 to 180°C at a heating rate of 10°C/min under nitrogen atmosphere (50 mL/min) using a TA Instruments Q100 (TA Instrument, New Castle, Delaware). Samples of cast films weighted approximately 1.7 mg were packed in aluminium pan and then heated from −60 to 180°C at a scanning rate of 10°C/min (first run). Glass transition temperature (T_g) was analyzed from the second heating round of the DSC curves. The melting temperature (T_m) was determined from the DSC maximum endothermal peaks during the first heating run.

Thermogravimetry analysis was performed using a TA-Q50 thermogravimetric analyzer (TGA) (TA Instrument). The temperature ranged from 10 to 500°C in a nitrogen atmosphere of 50 mL/min. Five to seven milligram samples were loaded with a heating rate 10°C min^{-1} each run.

Mechanical properties studied were conducted on the PHA solvent-casting films. The films were cut into dumbbell-shape specimen with a width of 6 mm and a thickness range from 0.14 to 0.26 mm. The stress–strain measurements of films were carried out using a Universal Testing Machine CMT 4204 (Sans Co., Ltd, Shenzhen, China) at room temperature. The speed of the cross-head was 5 mm/min. The values were the average value of three parallel measurements.

Results and Discussion

Effects of Valerate Concentration on Cell Growth and Production of Terpolyester P(3HB-*co*-3HV-*co*-3HHx) by Wild-Type *A. hydrophila* 4AK4 and Its Recombinants

Both wild-type *A. hydrophila* 4AK4 and its recombinant harboring *phaAB* were reported to produce P(3HB-*co*-3HV-*co*-3HHx) from mixed carbon sources of lauric acid and sodium propionate (Lu et al., 2004; Zhao and Chen, 2007). Due to the lower 3HV content, the terpolyesters did not show good application properties. When mixed carbon sources of lauric acid and valerate were used in this study, we demonstrated that P(3HB-*co*-3HV-*co*-3HHx) with high 3HV content could be accumulated by wild-type *A. hydrophila* 4AK4 and its recombinants. In addition, 3HV content was dependent on the amount of valerate added to the cells (Table I).

Table I. Effect of concentration of valerate on P(3HB-*co*-3HV-*co*-3HHx) produced by *Aeromonas hydrophila* 4AK4 (*phaAB*) grown in shake flasks.

Carbon source[a], valerate (g/L)	CDW (g/L)	PHA, content (wt%)[b]	PHA composition (mol%)[c]		
			3HB	3HV	3HHx
A					
0	3.61 ± 0.28	31.3 ± 2.55	81.2 ± 1.06	ND	18.8 ± 1.06
0.5	4.22 ± 0.47	40.8 ± 0.63	74.3 ± 2.33	8.6 ± 1.52	17.1 ± 0.91
1	5.37 ± 0.53	49.4 ± 0.19	64.8 ± 1.95	19.5 ± 2.65	15.7 ± 0.70
2	3.41 ± 0.55	56.2 ± 0.55	62.0 ± 0.64	18.8 ± 1.72	19.2 ± 1.46
3	0.60 ± 0.09	40.7 ± 3.54	69.1 ± 1.69	18.8 ± 0.92	12.1 ± 0.78
4	NG	—	—	—	—
5	NG	—	—	—	—
B					
1	4.85 ± 0.50	54.1 ± 2.02	56.3 ± 1.05	24.1 ± 0.59	19.6 ± 0.89
2	5.94 ± 0.71	58.5 ± 1.86	53.7 ± 0.75	27.4 ± 0.71	18.9 ± 1.24
3	3.19 ± 0.45	63.8 ± 2.72	55 ± 2.92	29.6 ± 2.04	15.4 ± 1.11
4	3.89 ± 0.0	67.6 ± 0.77	51.1 ± 2.58	32.0 ± 1.85	16.9 ± 0.73
5	3.8 ± 0.36	67.8 ± 2.36	52.6 ± 0.61	31.8 ± 1.23	15.6 ± 0.70

[a]Cells were grown in mixed carbon sources containing 8 g/L lauric acid and increasing concentration of valerate in a 500 mL shake flask with 100 mL culture at 30°C for 72 h (three parallel studies).

[b]The PHA contents were determined by GC.

[c]Monomer composition of the terpolyesters was determined by GC; ND, not detected; NG, no growth; A: The addition of valerate was carried out at 0 h (to see the effect at the beginning); B: Initial valerate concentration was 1 g/L, rest of the valerate was added into the culture in equal amount every 24 h during the cell cultivation.

Valerate is a toxic substrate for the cells. A strong growth inhibition was observed when valerate was over 3 g/L in the culture (Table I A). At 2 g/L valerate, *A. hydrophila* 4AK4 (*phaAB*) grown in lauric acid accumulated over 56% PHA consisting of 62% 3HB, 19% 3HV, and 19% 3HHx. Increasing valerate concentration led to increased PHA accumulation and 3HV content in the terpolyesters up to 2 g/L. At 1 g/L valerate, cells grew to a maximal dry weight (CDW) of 5.4 g/L containing 49% PHA, which consists of 19% 3HV.

To avoid the valerate toxicity, only 1 g/L valerate was present in the initial culture, more valerate was added evenly after every 24 h. With such a valerate addition strategy, *A. hydrophila* 4AK4 (*phaAB*) grown in lauric acid accumulated 68% PHA consisting of 53% 3HB, 32% 3HV, and 16% 3HHx (Table IB). Although high valerate concentration still showed slightly inhibition to cell growth, PHA production, and 3HV content were positively affected by high valerate concentration added in the stepwise way. A maximum of 32% 3HV was observed in the terpolyester produced by the cells grown in the presence of 4 g/L valerate. Similar phenomenon was observed in the culture containing mixed substrates of propionic acid and glucose (Du et al., 2001). With propionic acid as a substrate, a lower 3HV yield was due to decarboxylation of propionyl-CoA to acetyl-CoA which can be used both for PHB synthesis and for precursors of other cellular metabolism (Lee et al., 1995). In contrast, valerate should generate acetyl-CoA, propionyl-CoA, and energy when beta-oxidized as described by Braunegg et al. (1998), leading to 3HB and more 3HV formation in the terpolyesters compared with propionate.

A. hydrophila 4AK4 harboring PHA synthesis genes *phaPCJ* was reported to produce PHBHHx and P(3HB-*co*-4HB-*co*-HHx) (Xie and Chen, 2008). The recombinant was able to accumulate P(3HB-*co*-3HV-*co*-3HHx) when grown on lauric acid and valerate (Table II). When fed with 2 g/L valerate, a maximum of 71% terpolyester consisting of 43% 3HB, 25% 3HV, and 32% 3HHx was produced, additional feeding on valerate reduced CDW and PHA formation. A maximal 3HV content of 30% was found in PHA produced from 3 g/L valerate. Cells of *A. hydrophila* 4AK4 (*phaPCJ*) were observed to be filled with many PHA granules (Fig. 1). Similar to the above observation (Table I), valerate can be used to change the terpolyester compositions.

Wild-type *A. hydrophila* 4AK4 also produced P(3HB-*co*-3HV-*co*-3HHx) with high 3HV content from mixture of lauric acid and velaric acid (Table III) although the wild-type produced less PHA and less CDW compared with the recombinants. With increasing valerate concentration, CDW showed a decreased trend. Interestingly, PHA content in CDW, 3HB, 3HV, and 3HHx contents in the terpolyesters remained relatively constant at valerate concentrations ranging from 2 to 5 g/L. In this case, the wild-type was not a good host for producing PHA with flexible PHA compositions.

In summary, wild-type *A. hydrophila*, its recombinants containing *phaAB* and *phaPCJ*, respectively, could be used to produce terpolyesters P(3HB-*co*-3HV-*co*-3HHx) containing 9% to 32% 3HV, with 3HHx also flexible from 12% to 34%, depending on the valerate concentration fed into the cultures. It was convenient to adjust appropriate composition in the materials via changing carbon source concentration. We were now able to produce the terpolyeters with more flexible compositions and thus, with flexible properties to meet various application requirements.

Effects of Valerate Concentration on Cell Growth and Production of Copolymer P(3HB-*co*-3HV) by Wild-Type and Recombinant *A. hydrophila* 4AKA

P(3HB-*co*-3HV) with a high 3HV fraction could be produced by feeding *A. hydrophila 4AK4* (*phaAB*) with valerate only substrate (Table IV). 3HV fraction in the copolymer decreased from 99% to 64% with increasing valerate concentration from 1 to 5 g/L. Obviously, valerate was not a good carbon source for cell growth, indicated by low CDW ranging from 0.7 to 1.1 g/L after 72 h of growth. Increasing valerate concentration led to decreasing CDW, PHA, and 3HV contents. At a low valerate concentration of 0.5 g/L, no PHA was produced.

Table II. Effect of concentration of valerate on P(3HB-*co*-3HV-*co*-3HHx) produced by *Aeromonas hydrophila* 4AK4 (*phaPCJ*) grown in shake flasks.

Carbon source[a], valerate (g/L)	CDW (g/L)	PHA, content (wt%)[b]	PHA composition (mol%)[c]		
			3HB	3HV	3HHx
0.5	3.71 ± 0.23	45.4 ± 2.27	55.5 ± 1.08	10.5 ± 1.63	34 ± 0.93
1	4.72 ± 0.08	59.3 ± 0.11	47.4 ± 1.32	19.3 ± 3.52	33.3 ± 2.43
2	2.41 ± 0.13	71.1 ± 0.92	43.4 ± 0.11	24.7 ± 0.41	31.9 ± 0.41
3	2.33 ± 0.11	70.4 ± 0.92	40.8 ± 1.05	29.6 ± 1.99	29.6 ± 0.99
4	1.96 ± 0.25	64.7 ± 4.10	43.6 ± 2.04	25.7 ± 2.05	30.7 ± 0.1
5	2.15 ± 0.35	62.9 ± 3.46	45.1 ± 2.20	23.6 ± 1.07	31.3 ± 1.13

[a]Cells were grown in mixed carbon sources containing 8 g/L lauric acid and increasing concentration of valerate in a 500 mL shake flask with 100 mL culture at 30°C for 72 h (three parallel studies). Initial valerate concentration was 1 g/L, rest of the valerate was added into the culture in equal amount every 24 h during the cell cultivation.

[b]The PHA contents were determined by GC.

[c]Monomer composition of the terpolyesters was determined by GC.

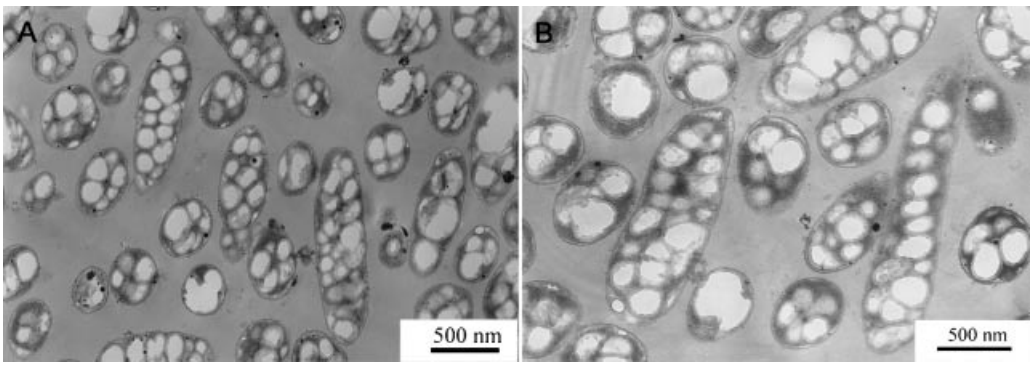

Figure 1. Transmission electron microphotographs of *Aeromonas hydrophila* 4AK4 (*phaAB*) cells containing 71.1 wt% P(3HB-*co*-3HV-*co*-3HHx).

When gluconate was served as co-substrate for *A. hydrophila* 4AK4 (*phaAB*), cell growth, and PHA content were improved significantly (Table IV). Copolymer content increased from around 20% in the absence of valerate to over 43% in the presence of gluconate and 4 g/L valerate. Interestingly, gluconate as carbon source did not contributed much to the 3HB formation, allowing the 3HV in the copolymers to be a dominant monomer with over 81% content. Similarly, increasing valerate concentration led to increasing 3HV in the copolymers (Table IV). In this case, gluconate was a carbon source for cell growth, while valerate was mainly utilized as precursors for both 3HB and 3HV, as described by Lu et al. (2004).

Physical Characterization of P(3HB-*co*-3HV-*co*-3HHx) With Various Monomer Contents

GPC showed that number-average molecular weight (M_n) and polydispersity (M_w/M_n) of the terpolyesters varied from 336,000 to 551,000 and 1.71 to 2.81, respectively, independent of their monomer compositions (Table V). *A. hydrophila* 4AK4 harboring *phaAB* or *phaPCJ* produced copolymers with a broader molecular weight distribution compared with that of the wild-type. This is not uncommon in some of the copolymers produced by microorganisms.

Thermal stabilities of PHA including PHBV, PHBHHx, and terpolyesters with different compositions were studied using TGA (Fig. 2). The temperature at 5% weight loss termed (T_d) was employed to evaluate polymer thermal stability. Results showed that the terpolyesters exhibited an obviously higher thermo-degradation temperature than those of copolymers PHBV and PHBHHx. Temperature at 5% weight loss of terpolyesters ranged from 246.7 to 258.1°C (Table V).

DSC showed that T_g of terpolyesters were significantly lower than those of the copolymers P(3HB-*co*-5% 3HV) and P(3HB-*co*-12% 3HHx) (Fig. 3). T_g of terpolyesters changed from −1.89 and −12.48°C with variations of monomer compositions (Table V). The decrease of glass transition temperature (T_g) will lead to increasing elastomeric behavior at room temperature (Doi et al., 1990; Spyros and Marchessault, 1996). It is therefore possible to regulate the terpolyester compositions to change their glass transition temperature (T_g). Terpolyesters with different compositions did not exhibit a cold crystallization during the cycle

Table III. Effect of concentration of valerate on P(3HB-*co*-3HV-*co*-3HHx) produced by wild-type *Aeromonas hydrophila* 4AK4 grown in shake flasks.

Carbon source[a], valerate (g/L)	CDW (g/L)	PHA, content (wt%)[b]	PHA composition (mol%)[c]		
			3HB	3HV	3HHx
0.5	3.56 ± 0.25	19.6 ± 1.28	80.2 ± 0.82	8.6 ± 1.02	11.2 ± 0.14
1	3.33 ± 0.31	23.2 ± 1.78	72.1 ± 1.86	15.2 ± 2.01	12.7 ± 0.31
2	1.22 ± 0.29	36.3 ± 3.15	66.7 ± 0.15	21.3 ± 0.83	12.0 ± 0.95
3	1.11 ± 0.11	35.4 ± 1.84	67.4 ± 0.35	19.6 ± 0.64	13.0 ± 0.61
4	1.18 ± 0.13	41.4 ± 0.31	66.0 ± 0.28	20.6 ± 2.32	13.4 ± 2.53
5	1.15 ± 0.06	35.1 ± 3.14	67.4 ± 1.66	21.1 ± 0.88	11.5 ± 0.8

[a]Cells were grown in mixed carbon sources containing 8 g/L lauric acid and increasing concentration of valerate in a 500 mL shake flask with 100 mL culture at 30°C for 72 h (three parallel studies). Initial valerate concentration was 1 g/L, rest of the valerate was added into the culture in equal amount every 24 h during the cell cultivation.
[b]The PHA contents were determined by GC.
[c]Monomer composition of the terpolyesters was determined by GC.

Table IV. Effect of concentration of valerate and sodium gluconate on P(3HB-*co*-3HV) produced by *Aeromonas hydrophila* 4AK4 (*phaAB*) grown in shake flasks.

Carbon source[a], valerate (g/L)	CDW (g/L)	PHA, content (wt%)[b]	PHA composition (mol%)[c]	
			3HB	3HV
A				
0.5	0.83 ± 0.07	ND	—	—
1	1.11 ± 0.03	23.1 ± 1.08	1.2 ± 0.02	98.8 ± 0.02
2	1.02 ± 0.03	24.8 ± 0.75	5.6 ± 0.06	94.4 ± 0.06
3	0.80 ± 0.12	17.1 ± 1.04	17.9 ± 3.57	82.1 ± 3.57
4	0.95 ± 0.15	18.2 ± 1.86	17.9 ± 4.26	82.1 ± 4.26
5	0.67 ± 0.05	17.2 ± 0.58	35.8 ± 2.37	64.2 ± 2.37
B				
0	1.55 ± 0.53	ND	—	—
0.5	2.18 ± 0.15	ND	—	—
1	2.37 ± 0.12	ND	—	—
2	2.89 ± 0.17	14.5 ± 0.75	19.3 ± 1.51	80.7 ± 1.51
3	3.28 ± 0.39	45.5 ± 4.44	15.2 ± 0.31	84.8 ± 0.31
4	4.17 ± 0.24	43.3 ± 1.24	12.7 ± 2.63	87.3 ± 2.63
5	1.72 ± 0.24	26.2 ± 1.36	10.8 ± 0.27	89.2 ± 0.27

[a]Cells were grown in a 500 mL shake flask with 100 mL culture at 30°C for 72 h (three parallel studies).
[b]The PHA contents were determined by GC.
[c]Monomer composition of the terpolyesters was determined by GC; ND, not detected; A: Increasing concentration of valerate were used as the sole carbon source; B: 8 g/L sodium gluconate and increasing concentration of valerate were used as carbon source.

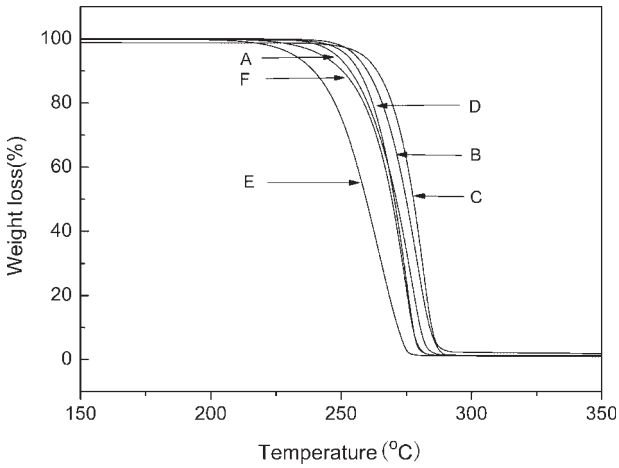

Figure 2. Thermogravimetry analysis (TGA) of P(3HB-*co*-3HV), P(3HB-*co*-3HHx), and P(3HB-*co*-3HV-*co*-3HHx). **A**: P(3HB-*co*-13.1 mol% 3HV-*co*-11.7 mol% 3HHx), (**B**) P(3HB-*co*-20.8 mol% 3HV-*co*-13.1 mol% 3HHx), (**C**) P(3HB-*co*-19.9 mol% 3HV-*co*-22.9 mol% 3HHx), (**D**) P(3HB-*co*-23.3 mol% 3HV-*co*-15.7 mol% 3HHX), (**E**) P(3HB-*co*-5 mol% 3HV), and (**F**) P(3HB-*co*-12 mol% 3HHx).

process employed in the DSC study (Fig. 3 and Table V), revealing that the terpolyesters were random copolymers. Melting temperatures (T_m) of P(3HB-*co*-3HV-*co*-3HHx) changed depending on monomer contents of 3HV and 3HHx.

The mechanical properties of PHB, PHBV, PHBHHx, and P(3HB-*co*-3HV-*co*-3HHx) with different monomer contents were studied using stress–strain measurement (Table VI). Among the polyesters tested, terpolyesters P(3HB-*co*-11% 3HV-*co*-10% 3HHx) and P(3HB-*co*-17% 3HV-*co*-10% 3HHx) were found to have the suitable combination of Young's modulus, elongation at break and tensile strength were great, ranging from 97 to 235 Mpa, 341% to 833%, and 8.4 to 14.3 Mpa, respectively. Other homo-, co-, and terpolymers suffered from at least one setback. Therefore, to meet a particular application, one can find a suitable monomer composition and then tailor-make the properties. Due to the high cost of these series of materials, one of the applications could be the tissue engineering joints that require high elongation to break, biocompatibility, and thermo-processibility. If cost could be reduced, such materials could be exploited for applications in environmentally friendly packaging areas. Their soft and more flexible properties could be exploited by material experts for developing more applications in various fields.

Table V. Thermal properties and molecular weights of P(3HB-*co*-3HV-*co*-3HHx) with different monomer compositions.

PHA monomer (mol%)[a]						Molecular weight[c]		
3HB	3HV	3HHx	$T_{d(5\%)}$ (°C)[b]	T_g (°C)	T_m (°C)	M_w (×10⁴ Da)	M_n (×10⁴ Da)	M_w/M_n
95	5	0	231.8	2.25	170.1	—	—	—
88	0	12	242.39	−1.22	96.23	44.0	21.0	2.03
75.2	13.1	11.7	248.1	−1.89	101.3	94.4	33.6	2.81
66.1	20.8	13.1	255.3	−3.59	68.5	126.7	55.1	2.30
67.2	12.6	20.2	246.7	−6.35	58.2	80.9	36.6	2.21
61.0	23.3	15.7	252.1	−9.65	ND	74.0	43.2	1.84
57.2	19.9	22.9	258.1	−12.48	ND	75.0	40.9	2.09
47.9	23.8	28.3	250.48	−5.14	54.2	94.2	45.1	1.71

T_g, glass-transition temperature (second run); T_m, melting temperature (first run); M_w, weight-average molecular weight; M_n, number-average molecular weight; M_w/M_n, polydispersity; ND, not detected.
[a]Monomer composition in PHA was determined by gas chromatography.
[b]Temperature at 5% weight loss was determined by TGA.
[c]Molecular weights were measured by GPC.

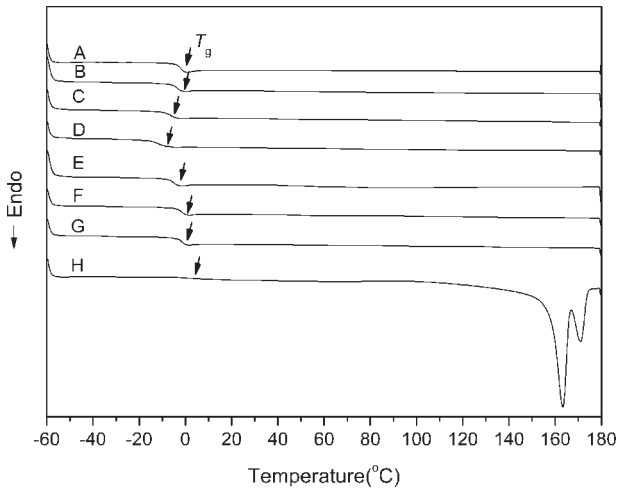

Figure 3. DSC thermograms (second run) of PHB, P(3HB-*co*-12 mol% 3HHx), P(3HB-*co*-5 mol% 3HV), and P(3HB-*co*-3HV-*co*-3HHx), T_g: glass transition temperature. **A**: P(3HB-*co*-13.1 mol% 3HV-*co*-11.7 mol% 3HHx), (**B**) P(3HB-*co*-20.8 mol% 3HV-*co*-13.1 mol% 3HHx), (**C**) P(3HB-*co*-12.6 mol% 3HV-*co*-20.2 mol% 3HHx), (**D**) P(3HB-*co*-19.9 mol% 3HV-*co*-22.9 mol% 3HHx), (**E**) P(3HB-*co*-23.8 mol% 3HV-*co*-28.3 mol% 3HHx), (**F**) P(3HB-*co*-23.3 mol% 3HV-*co*-15.7 mol% 3HHx), (**G**) P(3HB-*co*-12 mol% 3HHx), (**H**) P(3HB-*co*-5 mol% 3HV).

Conclusions

A series of P(3HB-*co*-3HV-*co*-3HHx) consisting of high 3HV monomer content were synthesized by wild-type *A. hydrophila* 4AK4, its recombinant harboring *phaAB* encoding beta-ketothiolase and acetoacetyl-CoA reductase, its another recombinant harboring *phaPCJ* encoding PHA binding protein phasin, PHA synthase, and enoyl-CoA hydratase when grown on lauric acid and/or valerate. 3HV and 3HHx contents in P(3HB-*co*-3HV-*co*-3HHx) were adjusted conveniently via changing lauric acid and/or valerate concentration. *A. hydrophila* 4AK4 harboring *phaAB* produced P(3HB-*co*-3HV) with a very high 3HV fraction depending on valerate concentration. A P(3HB-co-98.8% 3HV) was the maximal 3HV content achieved in this study. *A. hydrophila* 4AK4 harboring *phaPCJ* cultured in mixed carbon sources of lauric acid and valerate accumulated up to 71% terpolyester P(3HB-*co*-3HV-*co*-3HHx). All terpolyesters produced in this study showed a lower T_g ranging from $-2 \sim -13^{\circ}C$, higher thermal stability and more flexibility compared with copolymers of P(3HB-*co*-5% 3HV) and P(3HB-*co*-12% 3HHx). P(3HB-*co*-3HV-*co*-3HHx) consisting of different compositions provide materials with higher flexibility and adjustable tensile strength for various applications.

Table VI. Mechanical properties of P(3HB-*co*-3HV-*co*-3HHx) of different monomer compositions.

PHA (mol%)[a]			Young's modulus (Mpa)	Elongation at break (%)	Tensile strength (Mpa)
3HB	3HV	3HHx			
100	0	0	1510	4.45	18.5
95	5	0	1465	2.31	18.09
88	0	12	497.8	112.9	9.36
78.8	10.9	10.3	234.9	340.9	8.38
72.8	16.8	10.4	97.0	739.7	14.3
72.1	13.3	14.6	66.4	833.3	12.8
55.2	25.7	19.1	2.08	133.3	0.28

[a]Monomer composition in PHA was determined by gas chromatography as described in Materials and Methods Section.

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