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Production and characterization of terpolyester poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate-*co*-3-hydroxyhexanoate) by recombinant *Aeromonas hydrophila* 4AK4 harboring genes *phaPCJ*

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Abstract

Terpolyester consisting of 3-hydroxybutyrate (3HB), 4-hydroxybutyrate (4HB) and 3-hydroxyhexanoate (3HHx) abbreviated as P(3HB-*co*-4HB-*co*-3HHx), was produced by recombinant *Aeromonas hydrophila* 4AK4 harboring polyhydroxyalkanoate (PHA) synthesis genes *phaPCJ*. The monomer content of 4HB in the terpolyesters was dependent on the concentration of 1,4-butanediol, ranging from 4.3 to 8.5 mol% in the presence of 5–25 g/l 1,4-butanediol, respectively, accompanying by a decreasing 3HHx content from 22 to 17.6 mol% and a constant 3HB content of 74 mol% during growth in lauric acid containing mineral medium. Number average molecular weights of the terpolyesters were 440,000 to 550,000, much higher than 220,000 of the copolyester consisting of 3HB and 12 mol%3HHx commonly produced by wild type *A. hydrophila* 4AK4. Thermal and mechanical properties of the above terpolyesters were better than those of homopolyester poly(3-hydroxybutyrate) (PHB) and its copolymers P(3HB-*co*-7 mol%4HB), P(3HB-*co*-12 mol%3HHx). Moreover, physical properties of the terpolyesters changed with variations of monomer composition. The terpolyesters were characterized using nuclear magnetic resonance (NMR), gas chromatography (GC), gel-permeation chromatography (GPC), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) and stress–strain measurements. The introduction of 4HB and 3HHx monomers into PHB enhanced the thermal stability, changed its crystallinity and flexibility compared with homopolyester PHB and their two monomer containing copolyesters.

Keywords: PHB; *Aeromonas hydrophila*; 1,4-Butanediol; Terpolyester; Polyhydroxyalkanoates; PHA

1. Introduction

Polyhydroxyalkanoates (PHA) are accumulated by a wide variety of microorganisms as carbon and energy storage materials under unbalanced growth conditions in the presence of excess carbon source [1–3]. They have promised various industrial applications based on their biocompatibility, biodegradability, strong mechanical properties and piezoelectricity [4–7]. PHA seems to become one of the favorable candidate materials to replace synthetic petroleum-based polymers [8].

Generally, PHA can be classified into three main types based on their monomer structures: short-chain-length PHA (SCL-PHA) which contains 3–5 carbon atoms in the monomer, medium-chain-length PHA (MCL-PHA) which contains 6–14 carbon atoms, and copolymer containing SCL-PHA monomer and MCL-PHA monomer such as 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx) containing polymers P(3HB-*co*-3HHx) or PHBHHx which was biosynthesized by *Aeromonas vaviae, Aeromonas hydrophila* and recombinant *Escherichia coli* [8–11,15,26]. Terpolyesters consisting of 3-hydroxybutyrate, 3-hydroxyvalerate (3HV) and 3-hydroxyhexanoate termed P(3HB-*co*-3HV-*co*-3HHx) or PHBVHHx were seldom reported [12]. A number of studies demonstrated PHA containing 3-hydroxyalkanoates with longer chain lengths than 3-hydroxyvalerate (3HV) has low melting temperature, low glass transition temperature and low degree of crystallinity [13,14]. Study also revealed copolymer PHBHHx possessed similar mechanical properties to low density polyethylene, PHBHHx became softer with increasing 3HHx monomer content [15–17]. Also, poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate) or P(3HB-*co*-4HB) have been studied in details with similar discovery that 4HB reduces PHB crys-

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tallinity [18–21]. The elongation to break of P(3HB-*co*-4HB) increased from 5 to 1320% as the fraction of 4HB units increased from 0 to 82 mol% [22].

Terpolyesters poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*–4-hydroxybutyrate) [P(3HB-*co*-3HV-*co*-4HB)] and P(3HB-*co*-3HV-*co*-3HHx) were reported to be synthesized by *Alcaligens* spp., recombinant *E. coli* or *Ralstonia eutropha* [12,23,24]. P(3HB-*co*-3HV-*co*-4HB) showed better thermal and mechanical properties compared with that of PHB, PHBV and PHBHHx [23]. Tajima et al. reported the synthesis of P(3HB-*co*-4HB-*co*-3HHx) by *Bacillus* sp. INT005 isolated from soil sample in which 4HB and 3HHx molar fractions in the terpolyester were 1.8 and 1.7%, respectively [25]. No detailed mechanical property study of P(3HB-*co*-4HB-*co*-3HHx) was conducted. In this study, for the first time, P(3HB-*co*-4HB-*co*-3HHx) was synthesized with higher molar fractions of 3HB and 4HBx by recombinant *A. hydrophila* 4AK4 harboring PHA synthesis genes *phaPCJ*, which are *phaP*, *phaC* and *phaJ* genes encoding phasin, PHA synthase (*PhaC*) and (*R*)-specific enoyl-CoA hydratase (*PhaJ*) cloned from *Aeromonas caviae*. In addition, thermo-mechanical properties of the terpolyesters were studied.

2. Materials and methods

2.1. Bacterial strain and plasmid

The strain and plasmid used in this study was recombinant *A. hydrophila* 4AK4 harboring plasmid pQHA08 containing PHA synthesis genes *phaPCJ* cloned from *A. caviae*. Construction of this plasmid was described previously [26].

2.2. Culture conditions

For the production of PHA terpolyesters, the fed medium contained lauric acid as precursor for 3HB and 3HHx, 1,4-butanediol as 4HB precursor. The recombinant *A. hydrophila* 4AK4 (*phaPCJ*) were first cultivated overnight (12 h) in 50 ml conical flask containing 20 ml Luria-Bertani (LB) medium consisting of ($g l^{-1}$) 10 Tryptone (Oxoid, UK), 5 Yeast Extract (Oxoid, UK) and 10 sodium chloride medium. Then, 5% (v/v^{-1}) seed cultures were transferred into mineral salt (MS) [3] medium (pH 7.2) supplemented with 1 $g l^{-1}$ yeast extract, 8 $g l^{-1}$ lauric acid, and different amounts of 1,4-butanediol ranging from 0 to 25 $g l^{-1}$ under pH 6.5. When the pH was adjusted to 6.5, the lauric acid did not lead to foaming problems (Table 2). Lauric acid was added to MS medium and sterilized at 121 °C for 20 min. 1,4-Butanediol was fed at different times and different amounts after UV sterilization (Tables 1 and 2). The mineral salt medium consists of ($g l^{-1}$) $9Na_2HPO_4{\cdot}12H_2O$, $1.5KH_2PO_4$, $1(NH_4)_2SO_4$, $0.41MgSO_4{\cdot}7H_2O$, 0.05Fe(III)-NH_4-citrate, $0.02CaCl_2{\cdot}2H_2O$. In addition, 1 ml trace element solution containing (per liter of 1 mol l^{-1} HCl) 100 mg $ZnSO_4{\cdot}7H_2O$, 30 mg $MnCl_2{\cdot}4H_2O$, 300 mg H_3BO_3, 200 mg $CoCl_2{\cdot}6H_2O$, 10 mg $CuSO_4{\cdot}5H_2O$, 20 mg $NiCl_2{\cdot}6H_2O$ and 30 mg $NaMoO_4{\cdot}2H_2O$, was added to the medium. Kanamycin (50 μg ml^{-1}) was added to the medium when needed. The cultures were incubated in 500 ml conical flasks containing 100 ml culture broth on a rotary shaker set at 200 rpm and pH 6.5 (FUMA QYC2112, Shanghai, China) and 30 °C for 72 h.

Table 1
Effects of feeding time of 1,4-butanediol (10 $g l^{-1}$) on the cell growth (CDW), PHA contents and monomer compositions of the terpolyesters

Feeding time (h)	CDW ($g l^{-1}$)	PHA content (wt%)[a]	PHA composition (mol%)[b]		
			3HB	4HB	3HHx
0	3.26 ± 0.05	23.6 ± 1.2	73.9	5.1	21
4	3.69 ± 0.09	22.7 ± 1.0	73.9	5.2	20.9
8	4.01 ± 0.04	24.2 ± 1.3	73.8	5.0	21.2
12	3.71 ± 0.12	19.8 ± 1.7	73.7	4.9	21.4
16	3.82 ± 0.16	18.4 ± 1.2	73.9	4.8	21.3
20	3.79 ± 0.20	17.1 ± 1.4	73.8	4.9	21.3
24	3.64 ± 0.08	16.3 ± 1.6	73.7	4.7	21.6

The results were analyzed from cells grown after 72 h of cultivation. 3HB, 3-hydroxybutyrate; 4HB, 4-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate.
[a] The PHA contents were determined by GC.
[b] Monomer compositions of the terpolyesters were determined by GC.

2.3. The feeding of 1,4-butanediol for P(3HB-co-4HB-co-3HHx) production

Shake-flask experiments were carried out to investigate how 1,4-butanediol addition affected terpolyesters production by recombinant *A. hydrophila* 4AK4/pQHA08 (*phaPCJ*). 10 $g l^{-1}$ 1,4-butanediol were added each time at different times to the cultures, cell growth and PHA production were studied at 4 h interval addition time ranging from 0 to 24 h in cultures containing a lauric acid concentration of 8 $g l^{-1}$ in MS medium, respectively. Final concentrations from 5 to 25 $g l^{-1}$ of 1,4-butanediol were also used to study how precursor concentration affected cell growth and PHA production in the presence of 8 $g l^{-1}$ lauric acid in MS medium after 8 h cultivation.

Table 2
Effects of concentration of 1,4-butanediol on the cell growth (CDW), PHA contents and monomer compositions of the terpolyesters

Carbon source[a]	CDW ($g l^{-1}$)	PHA content (wt%)[b]	PHA composition (mol%)[c]		
1,4-butanediol ($g l^{-1}$)			3HB	4HB	3HHx
0	4.77 ± 0.15	58.6 ± 0.8	74	–	26
5	3.84 ± 0.1	26.2 ± 0.9	73.7	4.3	22
10	4.01 ± 0.04	24.2 ± 1.3	73.8	5.1	21.2
15	3.86 ± 0.3	22.7 ± 1.1	73.9	6.6	19.5
20	3.65 ± 0.25	20.1 ± 0.8	73.8	7.6	18.6
25	3.68 ± 0.2	19.7 ± 0.6	73.9	8.5	17.6

The results were analyzed from cells grown after 72 h of cultivation.
[a] The concentration of lauric acid was maintained at 8 $g l^{-1}$ during the cultivation process with the concentration of 1,4-butanediol increased at a 5 $g l^{-1}$ interval.
[b] The PHA contents were determined by GC.
[c] Monomer composition of the terpolyesters was determined by GC.