Appl Microbiol Biotechnol (1992) 37:698–701

Applied and Microbiology Biotechnology

Production of poly(3-hydroxyalkanoates) by a bacterium of the genus *Alcaligenes* utilizing long-chain fatty acids

Minoru Akiyama[1], **Yasuo Taima**[2], **and Yoshiharu Doi**[2]

[1] Asahi Chemical Industry, Ushio-dori 3-13, Kurashiki, Okayama 712, Japan
[2] Research Laboratory of Resources Utilization, Tokyo Institute of Technology, Nagatsuta 4259, Midori-ku, Yokohama 227, Japan

Received 10 February 1992/Accepted 18 May 1992

Summary. The synthesis of poly(3-hydroxyalkanoates) [P(3HA)] by a new *Alcaligenes* species was investigated. The new species was grown on various carbon sources such as *n*-alkanoic acids of carbon numbers ranging from C_2 to C_{22}, plant oils and animal fats, and accumulated P(3HA) within the cells. When the bacterium was cultured in mineral media containing sodium salts of *n*-alkanoic acids, the homopolymer of poly(3-hydroxybutyrate) [P(3HB)] was produced from *n*-alkanoates of even carbon numbers, whereas the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate units [P(3HB-co-3HV)] was produced from *n*-alkanoates of odd carbon numbers. Relatively high yields of both dry cells and P(3HA) were obtained by the use of *n*-alkanoates from C_{12} to C_{16} as the sole carbon source.

Introduction

Poly(3-hydroxybutyrate) [P(3HB)] is accumulated as a reserve material in granular form within cells of many bacteria and has been used as an index of microbial taxonomy. P(3HB) was the sole microbial polyester for about 50 years since its discovery (Lemoigne 1926). However, the situation has dramatically changed during the last two decades by the discovery of microbial copolyesters (Wallen and Rohwedder 1974). A number of copolyesters grouped into poly(hydroxyalkanoates) (PHA) have been found to be synthesized by approximately 60 different bacterial strains (Anderson and Dawes 1990; Brandl et al. 1990; Doi 1990). *Pseudomonas oleovorans* (Lageveen et al. 1988; Brandl et al. 1988) and other pseudomonads belonging to the rRNA homology group I (Huisman et al. 1989; Haywood et al. 1989; Timm and Steinbüchel 1990) produce copolyesters of medium-chain length 3-hydroxyalkanoates ranging from C_6 to C_{12}, when the cells are cultured on the corresponding alkanes and alkanoates. In contrast, *Alcaligenes eutrophus* (Holmes 1985; Doi et al. 1988) and other *Alcaligenes* species (Liebergesell et al. 1991) produce a copolyester of 3-hydroxybutyrate (C_4) and 3-hydroxyvalerate (C_5), P(3HB-co-3HV), from propionate or valerate.

There are few works on PHA synthesis by *Alcaligenes* species from medium- and long-chain fatty acids. In this paper, we report that a new *Alcaligenes* species grows on a wide range of *n*-alkanoates from C_2 to C_{22} and accumulates P(3HB) or P(3HB-co-3HV).

Materials and methods

Bacterial strain. A new strain of *Alcaligenes* species was used in this study. This strain was isolated by colonization on agar plates of a subculture of *P. oleovorans* (ATCC 29347), which exhibited an abnormal behaviour of P(3HA) biosynthesis, featured by relatively high percentages of 3HB units together with medium-chain-length 3HA units in polymer composition. A basal mineral medium containing 0.25 M sodium *n*-octanoate was used for the subculture and agar plates. Each colony was picked up from the plate and used as an inoculant for liquid culture, in which P(3HB) was accumulated in the cells. The stock cultures were submitted to NCIMB (Scotland) and also to the Japan Microbiological Clinic for bacterial characterization. The isolate was labelled AK 201 and identified as *Alcaligenes* species.

Media and growth conditions. In single-stage batch cultures, *Alcaligenes* sp. AK 201 was grown under aerobic conditions at 30° C and pH 7.0 for 48 h on a reciprocal shaker in a 500-ml Sakaguchi flask containing 100 ml of a defined mineral medium of different carbon substrates. The carbon substrates used were sodium salts of *n*-alkanoic acids of carbon numbers from C_2 to C_{22}, plant oils and animal fats. The mineral medium contained 1.1 g $(NH_4)_2HPO_4$, 5.8 g K_2HPO_4, 3.7 g KH_2PO_4, and 0.12 g $MgSO_4$ 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 litre of 1.0 M HCl): 2.78 g $FeSO_4 \cdot 7H_2O$, 1.98 g $MnCl_2 \cdot 4H_2O$, 2.81 g $CoSO_4 \cdot 7H_2O$, 1.67 g $CaCl_2 \cdot 2H_2O$, 0.17 g $CuCl_2 \cdot 2H_2O$ and 0.29 g $ZnSO_4 \cdot 7H_2O$. After cultivation, the cells were harvested by centrifugation and then lyophilized. The cells were completely dried to constant weight by lyophilization for 48 h. Polyesters were extracted from the lyophilized cells with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with *n*-hexane.

Present address: Polymer Chemistry Laboratory, RIKEN, Hirosawa, Wako-shi, Saitama 351-01, Japan

Correspondence to: Y. Doi

Analysis of polyesters. To determine the cellular polyester content and polymer composition, approximately 10 mg dry cells was subjected to methanolysis with a solution consisting of 1.7 ml methanol, 0.3 ml 98% sulphuric acid and 2.0 ml chloroform at 100° C for 4 h to convert the constituents to their methyl esters (Braunegg et al. 1978). Addition of 1 ml water to the reaction mixture induced phase separation. The lower chloroform layer was used for gas chromatography (GC) analysis which was performed on a Shimadzu GC-14A system equipped with a Neutra Bond-1 capillary column (25 m by 0.25 mm) and a flame ionisation detector.

The relative molecular weights of polyester samples were determined by gel permeation chromatography using a Shimadzu 6A GPC system. Approximately 1 mg/ml of sample was eluted by chloroform at a flow rate of 0.5 ml/min at 40° C. Polystyrene standards of low polydispersity were used to make a calibration curve.

Melting temperatures of the polyester samples were recorded on a Shimadzu DSC-50. The 3-mg portions of samples were encapsulated in aluminium pans and heated at 10° C/min from zero to 200° C.

The ^{13}C-nuclear magnetic resonance (NMR) spectra of copolyester samples were recorded on a JEOL GX-500 spectrometer to study the sequence distribution of monomeric units. The 125 MHz ^{13}C-NMR spectra were recorded at 27° C in a $CDCl_3$ solution of polyester (25 mg/ml) with a 10 μs pulse width (45° pulse angle), 5 s pulse repetition, 25000 Hz spectral width, 64000 data points, and 2000 accumulations.

Results

Characterization of Alcaligenes sp. AK 201

The isolated bacterial strain AK 201 was a Gram-negative, aerobic, and motile rod, which was catalase and oxidase positive and incapable of growing at 41° C. Further tests revealed that the strain was non-fluorescent, incapable of reducing nitrate, insensitive to Hugh and Leifson glucose OF medium, unable to form acid on glucose and unable to utilize glucose, arabinose, mannose, mannitol or maltose. On the basis of these phenotypic properties, strain AK 201 was identified as an *Alcaligenes* sp. Transmission electron microscopy clearly showed the presence of peritrichous flagella. The strain seems to be a new species because it is different from any other *Alcaligenes* species in several phenotypic properties examined (not shown).

Cultivation with various carbon sources

Various carbon substrates were used for single-stage batch culture of *Alcaligenes* sp. AK 201 as the sole carbon source. They include *n*-alkanes, *n*-alkanoic acids, natural oils and fats. As a result, *n*-octane or *n*-nonane, immiscible with the aqueous mineral medium, induced a small amount of bacterial growth at the initial supply of 1.8 g/l, but no polyester was accumulated within the cells. A greater supply of the carbon source failed to improve bacterial growth or polyester formation. A series of *n*-alkanoic acids with chain lengths ranging from C_2 to C_{22} induced not only bacterial growth but also polyester accumulation, which is detailed in the next section. The strain could not be grown on formate, the C_1 homologue. The strain was grown on gluconate, but with no formation of polyester. Natural oils and fats were good substrates for both cellular growth and polyester formation, described later in more detail.

Polyester synthesis

The isoalted strain was cultivated for 48 h at 30° C on sodium salts of *n*-alkanoic acids (3 g/l) with different chain lengths of C_1 to C_{22}. As can be seen from Fig. 1, *n*-alkanoates with chain lengths ranging from C_{11} to C_{19} yielded higher dry cell matter, over 2 g/l. It is notable that these alkanoates were sparingly soluble in the medium. No bacterial growth occured on formate. Acetate at 3 g/l induced moderate growth. The strain did not grow on propionate, butyrate or valerate at 3 g/l, but it grew at 1 g/l. It was rather strange that decanote was not effective for cell growth, although the other longer-chain alkanoates of more than seven carbons induced a great deal of cellular growth.

Cellular growth on *n*-alkanoates was accompanied by polyester formation in all cases tested. The contents of polyester in dry cells are shown in Fig. 2. Higher polyes-

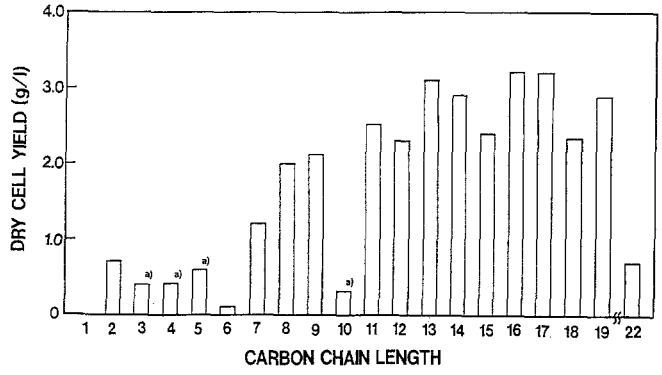

Fig. 1. Growth of *Alcaligenes* sp. AK 201 in basal mineral medium containing sodium salts of *n*-alkanoic acids of carbon chain length C_2–C_{22} as the sole carbon source. The bacterium was cultivated at 30° C for 48 h on 3 g/l of carbon source: *a),* 1 g/l of carbon source

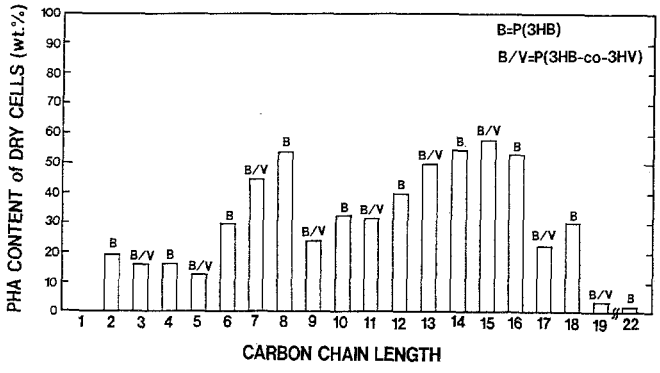

Fig. 2. The content of polyester (PHA) in dry cells from various *n*-alkanoates of C_2–C_{22}: *B* and *B/V* denote poly(3-hydroxybutyrate) [P(3HB)] and the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate [P(3HB-co-3HV)], respectively

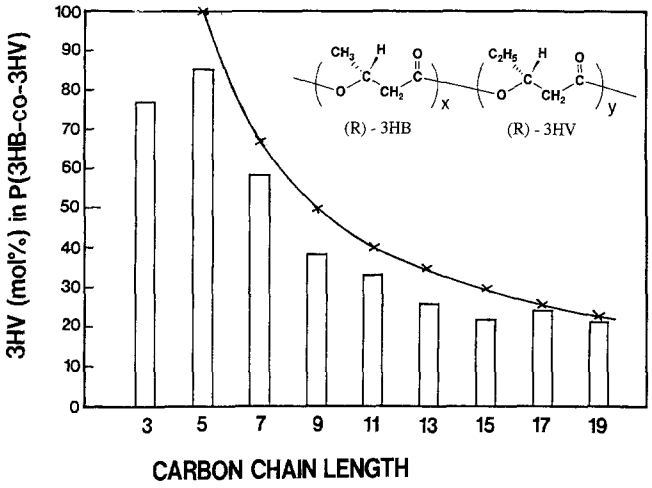

Fig. 3. The mole fraction of the 3HV unit in P(3HB-co-3HV) copolymers produced by *Alcaligenes* sp. AK 201 from *n*-alkanoates of odd carbon numbers

Table 1. Production of poly(3-hydroxybutyrate) [P(3HB)] by *Alcaligenes* sp. AK 201 from various oils and fats at 30° C

Carbon source (g/l)	Cultivation time (h)	Cell dry wt (g/l)	P(3HB) content (wt.%)
Rapeseed oil (2.75)	92	2.48	44
Olive oil (3.00)	61	3.11	47
Lard (3.00)	61	2.41	31
Soybean oil (3.00)	48	2.41	33
Corn oil (3.00)	48	2.79	39
Palm oil (3.00)	48	2.13	40

ter contents over 50 wt.% were obtained on longer-chain alkanoates of C_7–C_{16}. More notably, P(3HB) was produced from *n*-alkanoates of even carbon numbers, whereas P(3HB-co-3HV) was produced from *n*-alkanoates of odd carbon numbers. Interestingly, the mole fraction of the 3-HV unit in the copolymer was strongly dependent on the carbon number of odd alkanoates, and it decreased almost smoothly with increasing carbon number of *n*-alkanoates (Fig. 3).

Several plant oils and animal fats were used as the sole carbon source for single-stage batch culture of the isolate. Lard is a semi-solid, palm oil is a mixture of oil and solid and the others are oils. The results are given in Table 1. Since these carbon substrates are hydrophobic liquid or semi-solid, they were immiscible with the mineral medium, resulting in a two-phase medium system. All the hydrophobic oils and fats were good substrates for both cellular growth and polyester production as well as long-chain alkanoates. It should be noted that the polyester produced was P(3HB) in all cases.

Properties of polyesters

Polyesters produced by the isolate from a series of *n*-alkanoates were characterized by composition, melting temperature, and relative molecular weight. These basic properties are listed in Table 2. The melting temperatures of P(3HB) drifted a little from sample to sample, but were in the range 170–180° C. Melting temperatures of P(3HB-co-3HV) copolymers were dependent on their composition and were in a good agreement with previously reported values (Holmes 1988; Kunioka et al. 1989).

Table 2. Composition, relative molecular weight and melting temperatures (T_m) of polyesters produced from *n*-alkanoates of C_2–C_{22}

Alkanoates	Composition (mol%)		Relative molecular weights[a]			T_m (°C)
Carbon number	3HB	3HV	$M_n \times 10^{-4}$	$M_w \times 10^{-4}$	M_w/M_n	
2	100	0	95.1	154.9	1.6	179
3	23	77	16.3	31.4	1.9	176
4	100	0	16.2	35.7	2.2	102
5	15	85	18.5	37.3	2.0	—
6	100	0	—	—	—	69
7	42	58	164.1	289.7	1.8	172
8	100	0	98.4	138.6	1.4	68
9	62	38	85.2	155.5	1.8	171
10	100	0	9.3	23.2	2.5	115
11	67	33	10.0	20.7	2.1	173
12	100	0	16.1	30.4	1.9	102
13	74	26	70.8	173.8	2.5	175
14	100	0	45.7	141.6	3.1	106
15	78	22	57.5	109.5	1.9	174
16	100	0	58.5	144.2	2.5	108
17	76	24	22.6	54.2	2.4	171
18	100	0	52.0	98.6	1.9	116
19	79	21	2.1	3.6	1.7	—
22	100	0	—	—	—	

[a] M_n and M_w denote the number- and weight-average molecular weights, respectively

P(3HB-co-3HV) synthesized by *A. eutrophus* is known to have a random sequence distribution of repeating units (Kunioka et al. 1989). This was confirmed in the present study by analysis of the carbonyl carbon resonances in the 125-MHz ^{13}C-NMR of P(3HB-co-3HV) produced from tridecanoate.

Discussion

Alkanoates with straight carbon chains are well-known carbon sources that result in the production of P(3HA) during cultivation of various bacteria (Huisman et al. 1989; Haywood et al. 1989; Timm and Steinbüchel 1990; Liebergesell et al. 1991). The isolated strain, *Alcaligenes* sp. AK 201 characteristically utilizes a wide range of *n*-alkanoates from C_2 to C_{22} to produce P(3HB) or P(3HB-co-3HV). The P(3HB) homopolymer was produced from *n*-alkanoates of even carbon numbers, while the P(3HB-co-3HV) copolymer was produced from *n*-alkanoates of odd carbon numbers (see Fig. 2). The use of plant oils and animal fats as the carbon source resulted in the accumulation of the P(3HB)

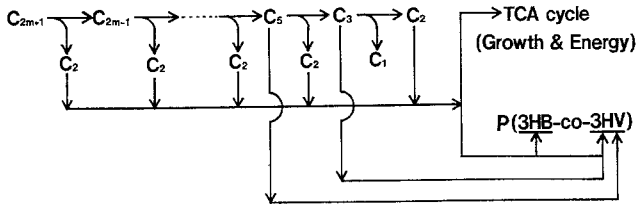

Fig. 4. Biosynthetic pathway of P(3HB-co-3HV) from *n*-alkanoates of odd carbon numbers: C_{2m+1} ($m \geq 1$) and C_2 denote acyl-coenzyme A: TCA cycle, tricarboxylic acid cycle

homopolymer in fairly high amounts on a dry cell basis. Production of P(3HB) reflects the fact that all the natural oils and fats used in this study are a mixture of triglycerides of saturated and/or unsaturated fatty acids of even carbon numbers.

Here, we propose a schematic pathway (Fig. 4) of P(3HB-co-3HV) synthesis from *n*-alkanoates of odd carbon numbers. As shown in Fig. 3, the mole fraction of 3HV units in copolymers decreased from 85 to 21 mol% as the carbon number of *n*-alkanoates increased from 5 to 19. The solid line in Fig. 3 is the curve calculated from Eq. 1.

$$C_{2m+1}H_{4m+2}O_2 \rightarrow (m-2)/2 \cdot (C_4H_6O_2) + (C_5H_8O_2) \quad (1)$$

where $C_{2m+1}H_{4m+2}O_2$ ($m \geq 2$), ($C_4H_8O_2$) and ($C_5H_8O_2$) denote *n*-alkanoates of an odd carbon number ($\geq C_5$), 3HB and 3HV repeating units, respectively. From Eq. 1, the theoretical value of 3HV mol% is given by 200/m. The experimental values of 3HV mol% are smaller by 2–15% than the theoretical values (see Fig. 3). The difference between the experimental and theoretical values of 3HV mol% may be attributed to the formation of acetyl-CoA from propionyl-CoA via the elimination of CO_2, as shown in Fig. 4. The 3HB unit is formed from two molecules of acetyl-CoA, which leads to a lower value of 3HV mol% than the theoretical value. When propionate was used as the sole carbon source, a copolymer of 23 mol% 3HB and 77 mol% 3HV units was produced. The 3HV unit may be formed by the reaction of propionyl-CoA with acetyl-CoA (Doi et al. 1987).

Acknowledgements. The authors thank Dr. Y. Nakamura for ^{13}C-NMR spectral analysis and Dr. Y. Kawaguchi for experimental guidance.

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