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ACCUMULATION OF PHA AND ITS COPOLYESTERS BY *METHYLOBACTERIUM* SP. KCTC 0048

Choong K. Kang, Hyun S. Lee, Jung H. Kim*

Technology Research Institute, Sun Hill Glucose Co., Ltd.,
Gajwa-Dong, Seo-Ku, Incheon, 404-250, Korea
*Department of Biotechnology, KAIST, Kusung-Dong,
Yusung-Ku, Taejon, 305-701, Korea

Summary

Methylobacterium sp. KCTC 0048 isolated from soil, could synthesize a variety of copolyesters when secondary carbon substrates were added to nitrogen-limited cultures containing methanol as a major carbon and energy source. The copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate, P(3HB-co-3HV) accumulated when valeric acid, pentanol or heptanoic acid was added to the nitrogen-limited medium containing methanol. The copolyester of 3-hydroxybutyrate and 4-hydroxybutyrate, P(3HB-co-4HB) was synthesized from 4-hydroxybutyrate, 1,4-butanediol, or γ-butyrolactone, and the copolyester of 3-hydroxybutyrate and 3-hydroxypropionate (P(3HB-co-3HP)), from 3-hydroxypropionate as the secondary carbon substrates, respectively.

Introduction

Many different types of microorganisms are able to synthesize polyhydroxyalkanoate (PHA) as an intracellular carbon and energy reserve material, which is usually formed under nutrient-limited conditions (Haywood et al., 1989a). Recently, it became of industrial interest to evaluate the microbial polyesters as biodegradable plastics with several properties similar to conventional synthetic plasitcs. The copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate, P(3HB-co-3HV) has been commercially produced with *Alcaligenes eutrophus* from propionic acid and glucose by ICI under the trade name of Biopol (Holmes, 1985). Doi et al. (1988) showed that P(3HB-co-3HV) could be produced from butyrate and valeric acid with a wide range of 3HV fraction (0-90 mol%). The copolyester of 3-hydroxybutyrate and 4-hydroxybutyrate, P(3HB-co-4HB) could be accumulated from 4-hydroxybutyrate, 1,4-butanediol or γ-butyrolactone (Kunioka et al., 1989) and the copoyester of 3-hydroxybutyrate and 3-hydroxypropionate, P(3HB-co-3HP) from 3-hydroxypropionate, 1,5-pentanediol or 1,7-heptanediol by *A. eutrophus* (Nakamura et al., 1991).

For industrial use, the selection of particular production organism and carbon substrate will have a critical influcence on the cost of fermentation process. It has been argued that

methanol would appear to be an attractive substrate for P(3HB) production due to several advantages of low price, complete water miscibility, moderate requirement of oxygen, and so on (Byrom, 1987). Although a few methanol-utilizing bacteria were recently studied as PHA producers (Suzuki et al., 1986, Daniel et al., 1992, Ueda et al., 1992), little is known about methylotrophs having ability of producing the copolyesters other than P(3HB) or P(3HB-co-3HV). Recently, we observed the possibility of production of PHA and its copolyesters, P(3HB-co-3HV), P(3HB-co-4HB) and P(3HB-co-3HP) from various types of carbon substrates by a methylotroph isolated in our labaratory (Kang et al., 1992). In this article, we describe the experimental results on the accumulation of various types of copolyester by *Methylobacterium* sp. KCTC 0048. The development and optimization of a fermentation process for the copolyester production are being carried out.

Materials and Methods

PHA producer. Bacteria using methanol as a sole carbon and energy source were isolated from soil. Among forty six strains, one strain which was identified as *Methylobacterium* sp. KCTC 0048, was selected for its high ability to synthesize PHA and its copolyester. The strain was maintained in a frozen state at -80 °C.

Culture conditions. The synthetic medium (Choi et al., 1989) was used as a basal medium. The composition was as follows: Methanol, 0.5% (v/v), NH_4Cl 0.6g, KH_2PO_4 1.31g, Na_2HPO_4 2.13g, $MgSO_4 \cdot 7H_2O$ 0.45g, $CaCl_2 \cdot 2H_2O$ 3.3mg, $FeSO_4 \cdot 7H_2O$ 1.3mg, $MnSO_4 \cdot 4H_2O$ 130μg, $ZnSO_4 \cdot 4H_2O$ 130μg, $CuSO_4 \cdot 5H_2O$ 40μg, $Na_2MoO_4 \cdot 2H_2O$ 40μg, $CoCl_2 \cdot 6H_2O$ 40μg, H_3BO_3 30μg, and distilled water 1*l*. The pH was adjusted to 7.0. The batch cultures were performed in 2*l* fermentor (B. Braun) with working volume of 1.2*l* at 30 °C for PHA accumulation. For the accumulation of copolyester, the selected secondary carbon substrate was added at the end of exponential growth phase when the nitrogen source was exhausted with an excess presence of methanol (0.5%, v/v).

Analytical procedures. For determination of cell growth, 10ml of culture broth was centrifuged, washed with distilled water and freeze-dried in vacuum. The concentration of ammonium ion in the culture broth was determined by indole-phenol method (Weatherburn, 1967) in order to confirm the nitrogen-limited condition. P(3HB) and P(3HB-co-3HV) were determined by gas chromatographic method (Braunegg and Sonnleitner, 1978) with a Hewlett Packard 5890 GC equipped with a capillary column HP-FFAP (50m x 0.2mm). For determination of PHA and its copolyester other than P(3HB) and P(3HB-co-3HV), intracellular polyesters were extracted from lyophilized cells into chloroform by using a Soxhlet extractor for 6 hours, and purified by precipitation with hexane, washed with methanol. The spectra of ^1H and ^{13}C NMR of polyesters in $CDCl_3$ solution were recorded on a Bruker AM 500 spectrometer at 500 MHz and 125 MHz, respectively.

Results

In order to examine the possibility of producing PHA and its copolyesters other than P(3HB), 24 kinds of C_3-C_{11} alkanoic acids, alkanes, alcohols and some of their derivatives as secondary carbon substrates were tested with nitrogen-limited cultures with an excess presence of methanol (0.5%, v/v). As a result, it was found that P(3HB-co-3HV) could only be accumulated when valeric acid, pentanol, or heptanoic acid was added to the 24 hours culture. Cell growth and polymer synthesis were significantly suppressed by increasing the secondary carbon substrates in the presence of methanol (Table 1). Total copolyesters content of the cells varied from 5.1% to 29.1% with a wide range of 3HV fraction (13.5-99.9

mol%) by changing the secondary carbon substrate and its concentration. It was noted that a homopolymer of 3-hydroxyvalerate, P(3HV) could be obtained from valeric acid only in the absence of methanol. On the other hand, some amounts of 3HB unit were synthesized when heptanoic acid or pentanol was used as a sole secondary carbon substrate in the absence of methanol. These results suggest that heptanoic acid might be partially cleaved to acetyl-CoA, going to 3HB synthesis, the remainder being incorporated into the copolyester as 3HV units. In the case of pentanol, part of this substrate might be oxidized by alcohol dehydrogenase which is commonly presented in methanol-utilizing bacteria and metabolized subsequently to acetyl-CoA. Table 2 shows that the copolyester of P(3HB-co-4HB) could be synthesized when 4-hydroxybutyrate, 1,4-butanediol or γ-butyrolactone were added as the secondary carbon substrate in nitrogen-limited culture. The copolyester contents of the cells varied from 2.0% to 13.1% with a range of 4HB fraction from 1.9 mol% to 13.2 mol%. *Methylobacterium* sp. KCTC 0048 also produced the copolyester of 3-hydroxybutyrate and 3-hydroxypropionate, P(3HB-co-3HP) from 3-hydroxypropionate (Table 3). However, carbon substrates over C_8 could not be incorporated for PHA production. It seems that the substrate specificity of PHA synthase of this methylotroph might be restricted to short-length alkanoates (Haywood et al., 1989b).

From the above results, it is noted that *Methylobacterium* sp. KCTC 0048 can accumulate various types of copolyesters from different carbon substrates in the presence of methanol and would have a great potential for the copolyester production through the process development and optimization to improve the productivity.

Table 1. Accumulation of P(3HB-co-3HV) by *Methylobacterium* sp. KCTC 0048 with the deficiency of ammonium ion.

Secondary Carbon Substrate* (mmoles)		Methanol* (%,v/v)	Cell Conc.† (g/*l*)	Polyester Contents† (% of dry cell)	Fraction of 3HV (mol%)
Valeric acid	10	0	1.32	8.7	99.9
	5	0.5	2.34	24.5	31.8
	10	0.5	2.18	18.7	40.6
	20	0.5	0.86	5.1	70.6
Pentanol	10	0	1.32	7.1	46.5
	5	0.5	2.01	24.9	36.5
	10	0.5	1.34	19.4	40.0
	20	0.5	1.30	13.1	13.5
Heptanoic acid	10	0	1.32	13.3	30.9
	5	0.5	2.21	29.1	27.1
	10	0.5	2.00	16.2	13.4
	20	0.5	2.30	15.7	15.0

* Each carbon substrate was added to 24 hrs culture.
† Cell concentration and polyester contents were determined at 48 hrs after the addition of the secondary carbon substrate.

Table 2. Accumulation of P(3HB-co-4HB) by *Methylobacterium* sp. KCTC 0048 with the deficiency of ammonium ion.*

Secondary Carbon Substrate (mmoles)		Methanol (%,v/v)	Cell Conc. (g/l)	Polyester Contents (% of dry cell)	Fraction of 4HB (mol%)
4-hydroxy-butyrate	10	0	1.43	3.5	8.5
	5	0.5	2.13	13.1	5.3
	10	0.5	1.96	8.4	7.6
	20	0.5	1.79	3.0	13.2
1,4-butanediol	10	0	1.00	2.0	4.2
	5	0.5	2.06	9.8	8.1
	10	0.5	1.18	3.9	8.2
	20	0.5	1.17	2.9	3.6
γ-butyro-lactone	10	0	1.07	2.0	5.5
	5	0.5	1.88	12.4	1.9
	10	0.5	1.97	10.5	4.2
	20	0.5	1.80	4.9	3.5

*All experimental conditions are the same as Table 1.

Table 3. Accumulation of P(3HB-co-3HP) by *Methylobacterium* sp. KCTC 0048.*

3-hydroxy-propionic acid (mmoles)	Methanol (%,v/v)	Cell Conc. (g/l)	Polyester Contents (% of dry cell)	Fraction of 3HP (mol%)
10	0	1.13	3.9	4.3
5	0.5	1.86	9.8	9.0
10	0.5	1.66	11.8	7.2
20	0.5	1.86	8.2	10.5

*All experimental conditions are the same as Table 1.

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