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Isolation of *Pseudomonas putida* BM01 Accumulating High Amount of PHA_{MCL}

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A *Pseudomonas putida* strain able to accumulate high amount of polyesters of medium-chain-length 3-hydroxyalkanoic acids (PHA_{MCL}) was isolated from soil in a landfill site using an enrichment technique. Culture condition of the isolated strain for polyester production in a one-step culture was optimized in a mineral-salts medium against pH and concentrations of ammonium sulfate, carbon source(e.g., octanoate), and phosphate. The optimal values for maximal cell growth and PHA accumulation were: pH; 7~8, $(NH_4)_2SO_4$; 8 mM, octanoate; 40 mM. The optimum temperature was in the range of 20~30°C, which was rather broader than in other bacteria. Cell growth was strongly inhibited by the phosphate limitation to less than 1 mM. An increase of phosphate concentration above 1 mM showed little effect on cell growth and polyester accumulation. When the strain was grown on octanoate under this optimized condition it produced 3.4 g dry biomass per liter and yielded 1.7 g PHA per liter amounting to 53 wt% of dry cells. The monomer units composing the polyester synthesized from octanoate were 3-hydroxyoctanoate (3HO), 3-hydroxycaproate (3HC), and 3-hydroxybutyrate (3HB) (85 : 13 : 2, mole ratio). Other low linear C_3~C_{10} monocarboxylic acids were also tested for polyester production.

Many bacteria are able to accumulate polyhydroxyalkanoates (PHAs) as granular inclusions (1, 6, 7). The accumulation of PHAs is accelerated by nutrient limitation and carbon source excess. Approximately up to 40 different hydroxyalkanoic acid monomers have been reported. The bacterial monomers may be classified into two categories (16, 17); short-chain and medium-chain length monomers. The short-chain length (SCL) monomers include 3-hydroxypropionate (3HP), 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). The medium-chain length (MCL) monomers have 3 to 6 more carbon atoms than the SCL monomers have. *A. eutrophus* (7), *Rhodospirillum rubrum* (2) and *P. pseudoflava* (13) can accumulate copolyesters composed solely of SCL monomers, while *P. oleovorans* (3, 8, 10, 13), *P. putida* (9, 10, 17) and other fluorescent *Pseudomonas* strains (17) can biosynthesize copolyesters principally composed of MCL monomers. Steinbüchel and Wiese (16) recently isolated a *Pseudomonas* strain which can accumulate polyesters composed of both 3HB and MCL 3-hydroxyalkanoic acids. However, their experimental results showed that the polyester in cells was a blend rather than a copolyester.

According to many literatures (1-3, 6-10, 13, 14, 16, 17), the polyester production of MCL-polyester producing bacteria, e.g., *P. oleovorans, P. putida, P. citronellolis, P. aeruginosa*, and *etc.*, is rather low (ranging between 25 and 60 wt% of PHA in dry cells; less than 1 g/L biomass) in comparison with that of SCL-polyester producing bacteria. However, as long as we know no optimization study for the PHA production of the MCL-polyester producing bacteria have been reported. This may be partly due to the limited applications expected from the poor mechanical and thermal properties of MCL polyesters (1, 6, 7).

Despite such disadvantages, the MCL polyesters have chemically a great advantage over the SCL polyesters because of the easier incorporation of several functional groups such as halogens, olefins, branched alkyls, cycloalkyls and phenyl into the side-chain of MCL polyester by MCL polyester-producing bacteria (4-7, 11, 13). This gave us an impetus to screen out the bacteria accumulating high amount of MCL polyesters. In this study such a bacterium that accumulated high amount of MCL polyester from various monocarboxylic acids up to 53 wt% by solvent-extraction and weighing of the cell dry mass was isolated. The isolated bacterium was characterized with ID32GN and API20E kits in accordance with API identification program and was identified as a strain of

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Pseudomonas putida. We investigated optimum culture conditions for maximum accumulation of the PHA in this bacterium on shake flask-cultivation.

MATERIALS AND METHODS

Isolation and Characterization of Bacterial Strain.

The sample obtained from an activated sewage sludge was suspended with sterilized water and inoculated to mineral salts M1 medium containing octanoate as carbon source. The M1 medium consisted of 30 mM $KH_2PO_4/Na_2HPO_4 \cdot 12H_2O$, 10 ml of 100 mM $MgSO_4$, and 1 ml of microelement solution (15) in 1 liter of deionized water. After 2 days of incubation at 30°C, samples were withdrawn and inoculated onto octanoate mineral agar plates. After 1 day of incubation at 30°C, several single colonies were tooth-picked according to their opacity and purified by a series of spreading on octanoate agar plates. The colony with the highest degree of opacity and the largest size was picked out. The isolated bacterial strain was characterized in accordance with API identification program and identified as *Pseudomonas putida* BM01.

Optimization of PHA Accumulation

The optimization of polyester accumulation in cells was performed against concentrations of carbon-source (e.g., octanoate), ammonium sulfate and phosphate, initial pH and culture temperature in M1 mineral salts medium. Cultivation was allowed for 30~34 hrs at 200 rpm. The usual optimization experiment was carried out at 30°C. The volume of culture medium was 500 ml in 2 L flask. The PHA accumulation for the carbon-sources other than octanoate was performed using the optimization parameters for octanoate. The cultivation time of up to 40 hr was needed for PHA accumulation on various carbon sources. The PHA content in cells was measured after extracting the dry cells with chloroform (15).

Electron Microscopy

The washed cells were fixed doubly with 2.5% glutaraldehyde and 1% osmium tetraoxide (OsO_4) (15). Ultrathin sectioning was performed with a LKB-Ultratome using a diamond knife. These sections were then collected on a copper grid coated with a Formvar-carbon film and were poststained with lead citrate and uranyl acetate. Electron micrographs were taken with a HITACHI H-600 electron microscope under acceleration voltage of 75 kV. Cells for the scanning electron micrographic (SEM) study were stained with 2% phosphotungstic acid solution (pH 7.2).

PHA Characterization

The PHA accumulation during cultivation was traced by the gas chromatographic analysis of methyl esters recovered by methanolysis of PHA in dry cells. Ten milligrams of the cells were reacted in a mixed solution of 1 ml of chloroform, 0.85 ml of methanol and 0.15 ml of sulfuric acid for 2 h at 100°C (3). The methyl esters of 3-hydroxyacids in organic phase was analyzed by gas chromatography with a Hewlett and Packard HP5890A gas chromatograph equipped with a Carbowax 20M column and a flame ionization detector.

The monomer-units in polyesters were characterized by using a nuclear magnetic resonance spectroscopy. The ^1H-noise decoupled ^{13}C NMR spectra were recorded on a 100 MHz ^{13}C NMR (JEOL EX-400) spectrometer with 5.0-s pulse repetition, 10000 Hz spectral width, 64 K data points and 500~1000 accumulations. The chemical-shift assignment of carbon signals was made in comparison with the data reported earlier (8).

The melting transition temperature (Tm) of the PHAs was measured under a dry nitrogen purge by using a Setaram differential scanning calorimeter (Setaram micro-DSC) equipped with a data station. Approximately 20 mg of air-dried polymer for the DSC study was placed into a sample vessel. The specimen was subjected to annealing at room temperature for 3 weeks or more (5). The heating rate was 0.8°C/min. The scanning range was 20~80°C.

RESULTS AND DISCUSSION

Characterization of Isolated Strain

Table 1 shows the morphological, biochemical and nutritional characteristics of the isolated strain that accumulates high amount of medium-chain-length polyhydroxyalkanoate (MCL PHA). The strain has 4~6 polar flagella (Fig. 1), and it exhibited motility. Activites of arginine dihydrolase and cytochrome oxidase were present. Denitrification was not observed. Gelatine was not hydrolyzed. Glucose, L-arabinose, and D-mannose were utilized for growth. Thus, from the test results, by using the API identification program in accordance with standard methods (12), the isolate was identified as a strain of *Pseudomonas putida* and named *P. putida* BM01.

Little PHA accumulation occurred under the nutrient-rich condition (Nutrient-Broth 1.5 w/v%; Yeast extract 1 w/v%). However, lots of PHA granules appeared when grown on mineral salts medium with 20 mM octanoate+4 mM $(NH_4)_2SO_4$ (Fig. 2).

Optimization of PHA production by *P. putida* BM01

Cells were harvested 8 hrs after the O.D. of culture medium reached a steady-state value. Thus, the cultivation time was in the range of 30~34 hrs. Fig. 3 shows that the cell growth was strongly inhibited by the nitro-

Table 1. Characterization of the isolated PHA-producing bacterium

Test	Result	Test	Result	Test	Result
Cell shape	rod	Utilization of		Utilization of	
Cell size (μm)	0.6.~2.7	Glucose	+	D-Sorbitol	+
Gram staining	−	L-Arabinose	+	Propionate	+
Motility	+	D-Mannose	+	Valerate	+
Flagella number	4~6	L-Rhamnose	−	Caprate	+
Growth on nutrient agar	+	Maltose	−	Adipate	−
Growth on MacConkey agar	+	Sucrose	−	Malate	+
Biochemical test		D-Ribose	−	Acetate	+
Cytochrome oxidase	+	D-Saccharose	−	Malonate	+
Arginine dihydrolase	+	D-Melibiose	−	D-Lactate	+
Lysine decarboxylase	−	Fucose	−	Citrate	+
Tryptophan deaminase	−	Alanine	+	Itaconate	−
Urease	−	Salicine	−	Suberate	−
Denitrification	−	Histidine	+	Gluconate	+
Indole test	−	L-Serine	+	5-Ketogluconate	−
Voges-Proskauer test	−	L-Proline	+	2-Ketogluconate	+
Hydrolysis of		Phenylacetate	+	3-Hydroxybutyrate	+
Esculine	−	Glycine	−	3-Hydroxybenzoate	−
Gelatine	−	Inositol	−	4-Hydroxybenzoate	+
Ortho-Nitrophenyl-β-D-galactopyranoside	−	Mannitol	−	N-Acetyl-glucosamine	−
p-Nitrophenyl-glucosamine	−				

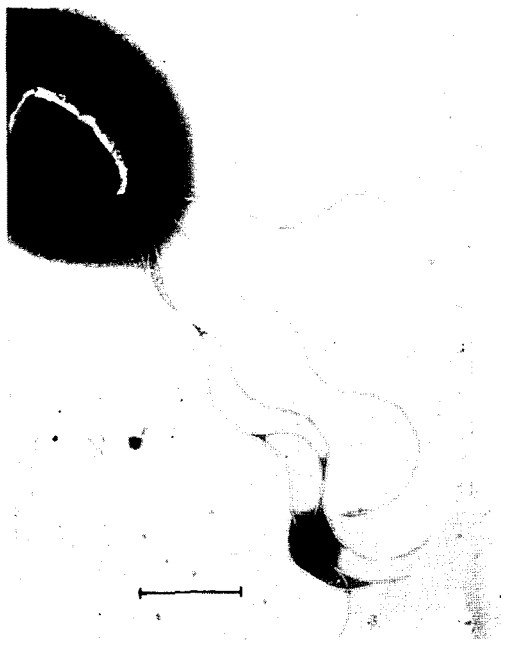

Fig. 1. Scanning electron micrograph of *Pseudomonas putida* BM01 negatively stained.(The marker represents 1 μm.)

gen concentration lower than 4 mM of $(NH_4)_2SO_4$. The PHA wt% data at these lower $(NH_4)_2SO_4$ concentrations were omitted due to insufficient amounts of harvested cells for solvent-extraction and weighing. A decrease in C/N ratio strongly supported the cell growth, reducing PHA accumulation (Fig. 3). At the C/N ratio of 5, cell growth and PHA accumulation occurred simultaneously.

In the optimization of octanoate concentration, the concentration of ammonium sulfate was increased to 8 mM. The dry cell weight, O.D. and PHA wt% were maxima at the octanoate concentration of 40 mM (Fig. 4). This point corresponds to the C/N ratio of 5 where the PHA wt% reached up to 50 and the biomass content was ~3 g. The growth curve data showed that the time to reach steady-state in O.D. was lengthened from 12 hr to 30 hr as the octanoate concentration increased from 10 mM to 40 mM (data not shown). The initial patterns of O.D. increase were similar to one another up to 40 mM. Further increase of octanoate concentration strongly inhibited the initial cell growth.

It was reported that the PHA accumulation in bacterial cells may be enhanced by phosphate limitation (1). However, in *P. putida* BM01 the decrease of phosphate concentration down to 2 mM strongly inhibited cell growth (Fig. 5). But, it had no effect on the PHA accumulation in cells.

The optimum pH for cell growth and PHA accumulation was found to be between 7 and 8 (Fig. 6). At higher pH ($\gg$10) and lower pH ($\ll$6), the cell growth and PHA accumulation were strongly inhibited. The effect of total phosphate concentration(buffer capacity) on maximal growth and PHA production of *P. putida* BM01 was also investigated (data not shown). In the study of buffer-capacity effect the strain BM01 was cul-

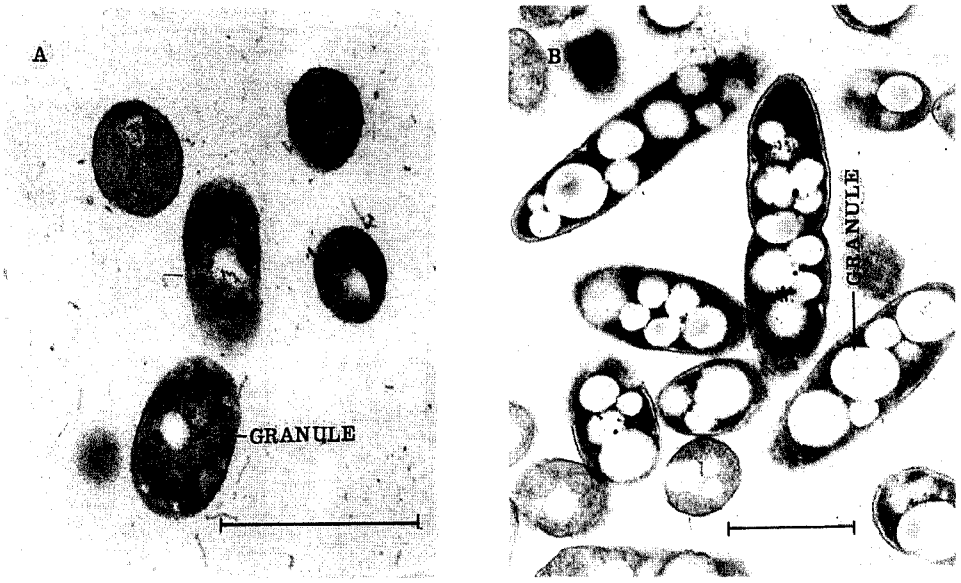

Fig. 2. Electron micrographs of *Pseudomonas putida* BM01 cultivated (A) in a nutrient rich medium and (B) on 20 mM octanoate plus 4 mM $(NH_4)_2SO_4$ in a mineral salts medium.(The marker represents 1 μm.)

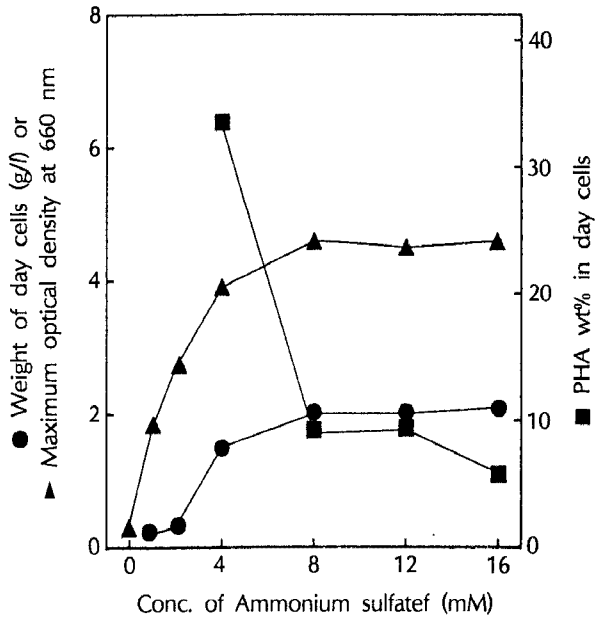

Fig. 3. Effect of ammonium sulfate concentration on biomass content, maximum cell growth, and PHA production of *Pseudomonas putida* BM01.
The strain was cultured in a modified mineral salts medium containing 20 mM of octanoate as carbon source.

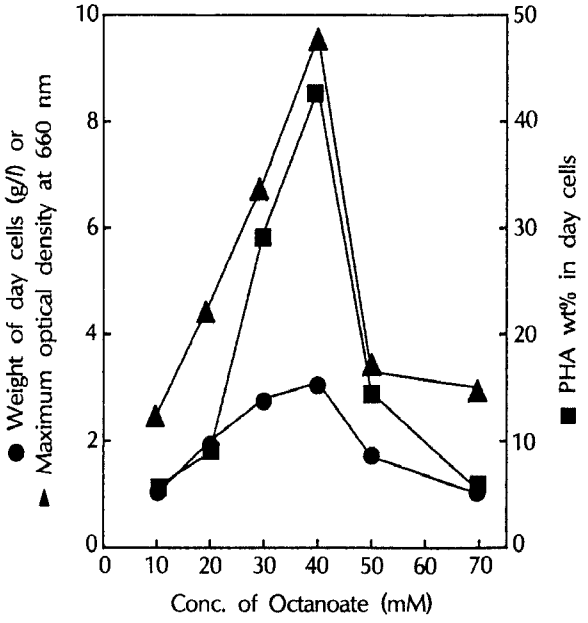

Fig. 4. Effect of octanoate concentration on biomass content, maximum cell growth, and PHA production of *Pseudomonas putida* BM01.
The strain was cultured in a modified mineral salts medium containing 8 mM of ammonium sulfate as nitrogen source.

tured in a modified mineral salts medium containing 40 mM of octanoate and 8 mM of ammonium sulfate whose initial pH was 7.2. In low-capacity buffers (lower than 10 mM of phosphate), the pH of the medium was initially lowered to 6 and no further growth was followed (O.D.=1.2). This is consistant with the pH optimization data. In higher buffer-capacity media (higher than 10 mM of phosphate) only a small change in pH (ΔpH=0.5) was observed with culture time. The biomass content and the PHA wt% were also constant with increasing phosphate concentration up to 50 mM.

The temperature optimization revealed a rather broad range of optimum temperature between 20~30°C for both the cell growth and PHA accumulation (Fig. 7).

The PHA accumulation kinetics data of *P. putida* BM 01 under these optimization conditions showed that both the cell growth and PHA accumulation reached a steady-state after 22~24 hours of cultivation without a time-lag between them (Fig. 8). In *P. oleovorans* (13) and *P. aeruginosa* (17), the PHA accumulation was accelerated after the cell growth reached a stationary phase in which nitrogen source was almost completely deple-

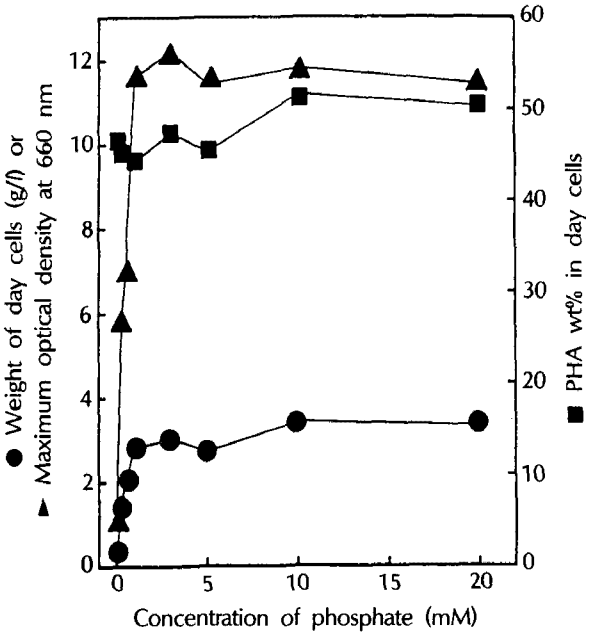

Fig. 5. Effect of phosphate concentration on biomass content, maximum cell growth, and PHA production of *Pseudomonas putida* BM01.
The culture medium contained 40 mM octanoate and 8 mM ammonium sulfate. 30 mM Sodium bicarbonate was used as a buffer whose initial pH was 7.0.

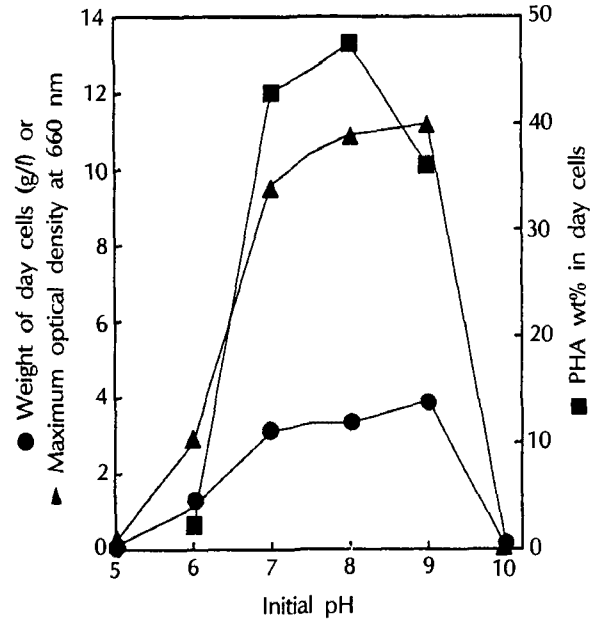

Fig. 6. Effect of initial pH on biomass content, maximum cell growth, and PHA production of *Pseudomonas putida* BM01.
The culture medium contained 40 mM octanoate and 8 mM ammonium sulfate. KH_2PO_4/Na_2HPO_4 buffer was used in the pH range of 5~8 and at the higher pH 9~10 a glycine buffer was used.

ted. A *Pseudomonas* strain GP4BH1 did not exhibit such time-lag when grown on octanoate, while it displayed a little time-lag when grown on gluconate (16).

It is interesting to note the invariability of the monomer-unit composition of polyester during cultivation (Table 2). It means that every polymer chain has a similar

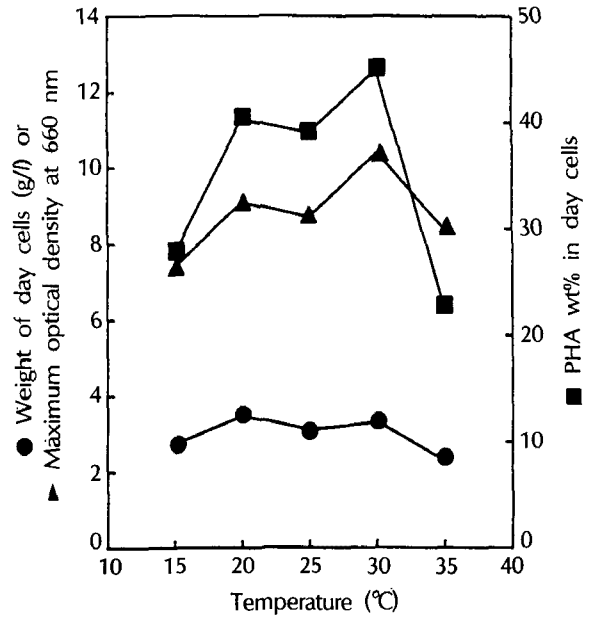

Fig. 7. Effect of temperature on biomass content, maximum cell growth, and PHA production of *Pseudomonas putida* BM 01.
The culture medium contained 40 mM octanoate and 8 mM ammonium sulfate, whose initial pH was 7.2.

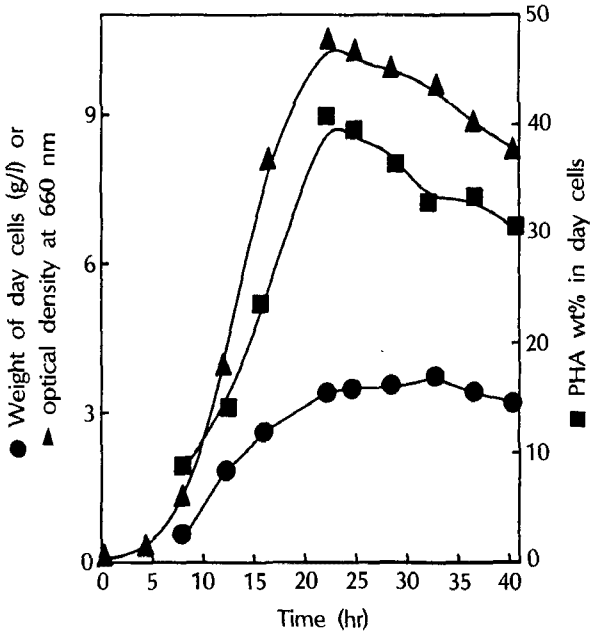

Fig. 8. Accumulation of polyhydroxyalkanoate by *Pseudomonas putida* BM01 with culture time.
Cells were cultivated with octanoate as a sole carbon source.

monomer composition which could never be obtained in chemical copolymerization if we would use the comonomers with different reactivities. Thus, this type of microbial copolymer may exhibit unique physico-chemical properties compared to the petrochemical polymers with uneven monomer distribution.

PHA Production from Linear Monocarboxylic Acids

Other linear monocarboxylic acids (C_3~C_{10}) were tes-

ted for their utilization for PHAs synthesis by *P. putida* BM01 (Table 3). The content of monomer-units was determined by the gas chromatographic analysis of methyl esters of the constituent monomer-units. The C_3~C_5 acids supported principally cell growth but no or little PHA accumulation.

Fig. 9 shows the 100 MHz ^{13}C-NMR spectrum of a PHA sample synthesized by *P. putida* BM01 grown on octanoate. The chemical-shifts of carbon atoms in PHAs were assigned in comparison to those reported earlier (8). The absorption band at 170 ppm indicates the presence of carbonyl carbons in polymers. Two distinctly resolved peaks appeared at 71.49 and 71.24 ppm ascribable to methine carbons singly bonded to ester oxygen. The two signals were from 3-hydroxyoctanoate (3 HO) and 3-hydroxycaproate (3HC), respectively. A small absorption occurred at 68.28 ppm, also due to the methine carbon in 3-hydroxybutyrate (3HB). Other absorptions at the up-field region were assigned (8) as in Fig. 9. This indicated that the isolated polymer was a copolyester which consisted of 1.6 mol% 3HB, 13.2 mol% 3HC and 85.2 mol% 3HO. We analyzed 22 PHA samples obtained from the optimization experiments of PHAs production by *P. putida* BM01 grown on octanoate. The result showed that the content of 3HO varied between 74.1~85.4 mole% with average 80.5± 2.4 mole%. The contents of the other two monomer units 3HC and 3HD varied in the range of 14.2~21.9 and 0.0~7.5 mole%, respectively. Thus, the composition of monomer-units slightly depends on the cultivation condition.

The cells grown on valerate produced a wide range of monomer-unit compositions (6 monomers of C_5 to C_{10} 3-hydroxyacids). The monomer-unit compositions of PHAs synthesized by *P. putida* BM01 from the C_6~C_{10} acids were similar to those by *P. oleovorans* (3, 8, 10, 13), *P. citronellolis* (5), *P. aeruginosa* (17) and *P. putida* KT2442 (9). The principal monomer-units of PHAs synthesized from the C_5~C_9 acids have the same number of carbon atoms as the substrates fed. Thus, the PHA monomer-units may be derived from the intermediates of β-oxidation (9).

Fig. 10 shows the DSC thermogram for the polyester mainly consisted of 3HO, which was synthesized from octanoate. The sample for DSC experiment was aged at room temperature for 3 weeks or more to enhance its crystallinity. The T_m of the PHA sample was 51°C. The endothermic heat content was 14.9 J/g. The low T_m of MCL PHAs may be due to inefficient crystalline-packing of side chains. It has been suggested that the MCL PHA with the n-alkyl pendant group equal to or greater than n-pentyl may crystallize in a similar manner as described for other comb-type polymers with lengthy (C_{15}~C_{18}) side chains such as poly(r-n-octadecyl L-glutamate), while the poly(3-hydroxybutyrate) (PHB) may cry-

Table 2. Compositional invariability with culture time of monomer units in polyesters synthesized by *Pseudomonas putida* BM01 grown on octanoate

Time (hrs)	PHA composition (mol%)[a]		
	HC[b]	HO	HD
8	18.9	74.5	6.2
12	15.8	80.6	3.6
16	14.2	80.9	4.9
22	14.2	81.5	4.3
24.5	14.5	81.1	4.4
28.5	14.0	80.4	5.6
32.5	14.1	81.1	4.8
36.5	13.7	81.6	4.6
40.5	14.1	81.1	4.8

[a] Calculated from G.C. data.
[b] For abbreviations, see the footnote in Table 3.
[c] The minor monomer-unit such as 3HB (less than 1 mol%) was neglected in the calculation of monomer-unit composition.

Table 3. PHA production of *Pseudomonas putida* BM01 grown on various monocarboxylic acids

Carbon source (mM)		Dry cell weight (g/L)	PHA wt% in dry cells	PHA composition (mol%)						
				HB	HV	HC	HH	HO	HN	HD[a]
Propionate	60	1.3	NA[b]							
Butyrate	40	1.4	<1				nd[c]			
Butyrate	60	1.7	<1				nd			
Valerate	40	1.7	<1				nd			
Valerate	60	1.9	3		33.3	2.1	15.7	12.7	10.9	25.3
Hexanoate	50	1.7	11			86.1		10.0		3.9
Heptanoate	40	2.6	37		3.8		92.0		4.2	
Octanoate	40	3.4	53	1.6		13.2		85.2		
Nonanoate	40	3.3	45		6.2		35.6		58.2	
Decanoate	40	3.7	47			14.6		54.4		30.9

[a] HB; 3-hydroxybutyrate, HV; 3-hydroxyvalerate, HC; 3-hydroxycaproate, HH; 3-hydroxyheptanoate, HO; 3-hydroxyoctanoate, HN; 3-hydroxynonanoate, HD; 3-hydroxydecanoate, [b] No PHA accumulation, [c] Not determined.

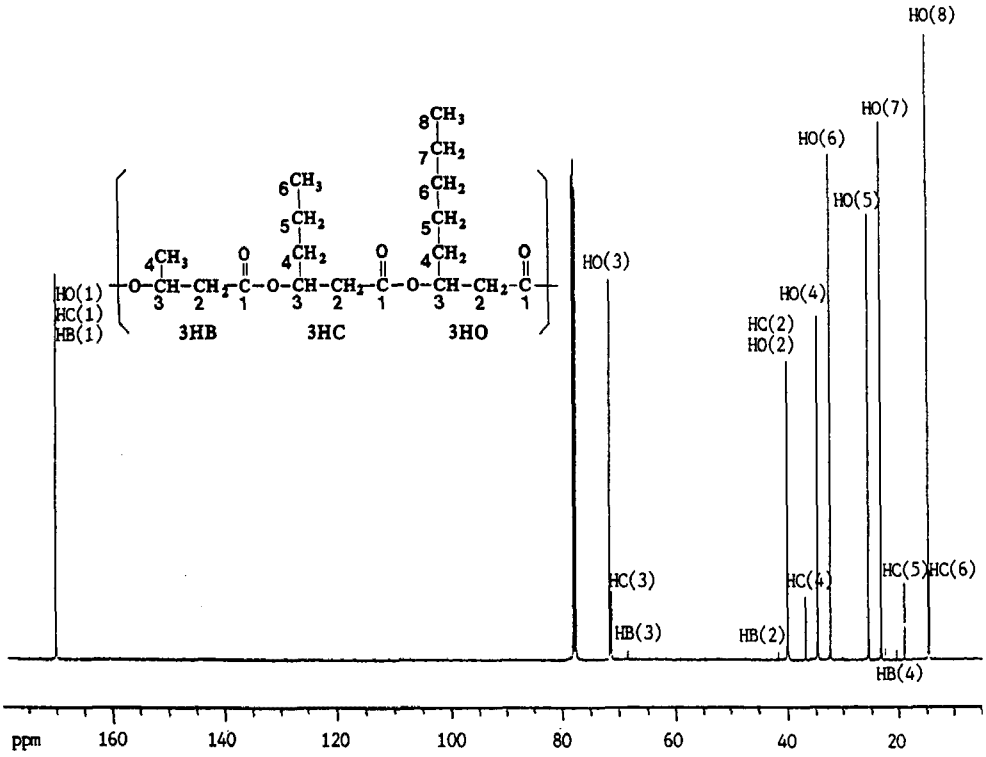

Fig. 9. 100 MHz ^{13}C-NMR spectrum of polyester synthesized by *Pseudomonas putida* BM01 grown on octanoate.

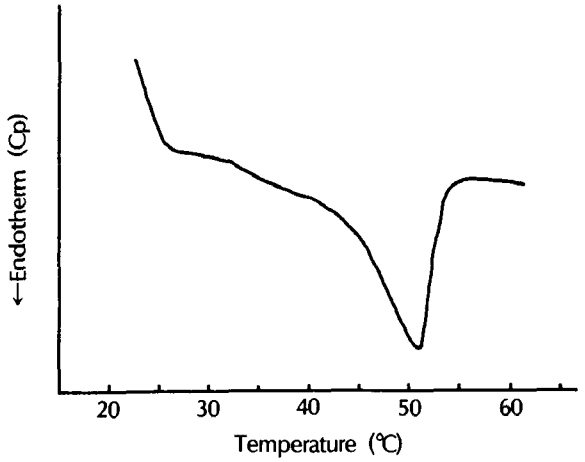

Fig. 10. DSC thermogram of PHA synthesized by *Pseudomonas putida* BM01 grown on octanoate.

stallize in the layered form of helical back-bone chains (5, 7, 8).

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