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Isolation of a *Pseudomonas* sp. Strain Exhibiting Unusual Behavior of Poly(3-hydroxyalkanoates) Biosynthesis and Characterization of Synthesized Polyesters

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Abstract A *Pseudomonas* sp. strain that is capable of utilizing dicarboxylic acids as a sole carbon source was isolated from activated sludge by using the enrichment culture technique. This organism accumulated polyhydroxyalkanoates (PHAs) with an unusual pattern of monomer units that depends on the carbon sources used. Polyhydroxybutyrate (PHB) homopolyester was synthesized from glucose or small C_{-even} alkanoic acids, such as butyric acid and hexanoic acid. Accumulation of PHB homopolyester was also observed in the cells grown on C_{-odd} dicarboxylic acids, such as heptanedioic acid and nonanedioic acid as the sole carbon sources. In contrast, a copolyester consisting of 6 mol% 3-hydroxybutyrate (3HB) and 94 mol% 3-hydroxyvalerate (3HV) was produced with a PHA content of as much as 36% of the cellular dry matter. This strain produced PHAs consisting both of the short-chain-length (SCL) and the medium-chain-length (MCL) 3-hydroxyacid units when heptanoic acid to undecanoic acid were fed as the sole carbon sources. Most interestingly, polyester consisting of significant amount of relevant fractions, 3HB, 3HV, and 3-hydroxyheptanoate (3HHp), was accumulated from heptanoic acid. According to solvent fractionation experiments, the polymer produced from heptanoic acid was a blend of poly(3HHp) and of a copolyester of 3HB, 3HV, and 3HHp units. The hexane soluble fractions contained only 3HHp units while the hexane-insoluble fractions contained 3HB and 3HV units with a small amount of 3HHp unit. The copolyester was an elastomer with unusual mechanical properties. The maximum elongation ratio of the copolyester was 460% with an ultimate strength of 10 MPa, which was very different from those of poly(3HB-*co*-3HV) copolyesters having similar compositions produced from other microorganisms.

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Polyhydroxyalkanoates (PHAs) are a class of naturally occurring polyesters that are synthesized by a wide range of bacteria as a carbon and energy reserve material usually under the unbalanced growth conditions. Approximately 120 different hydroxyalkanoic acids have been detected so far as constituents of these bacterial polyesters [31]. The monomeric compositions of PHAs vary as a function of the specificity of the PHA synthase system, the nature of the carbon source, and the metabolic pathways leading to PHA formation [8, 27].

PHA-synthesizing bacteria can be divided into two major groups on the basis of PHA synthase that they possess [2, 29]. *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) and many other bacteria possess a SCL-PHA synthase which preferably incorporate short-chain-length (SCL) monomer units having three to five carbon atoms. In contrast, *Pseudomonas oleovorans* and other pseudomonads belonging to rRNA homology group I contain a MCL-PHA synthase which preferably incorporates medium-chain-length (MCL) monomer units having six or more carbon atoms. The great majority of PHA-synthesizing bacteria are known to accumulate only either SCL-PHA or MCL-PHA. However, a few strains belonging to the genus *Pseudomonas* have been reported to accumulate polyesters containing both 3-hydroxybutyrate (3HB) and MCL 3-hydroxyalkanoic acids (3HA) as monomer units [15, 22, 32]. In addition, recombinant strains of *P. oleovorans* and *P. putida* harboring the PHA biosynthesis genes of *A. eutrophus* and *Thiocapsa pfennigii*, respectively, were shown to produce PHAs with

a content of 3HB along with MCL 3HA units [24, 32]. These polyesters containing both SCL and MCL 3HA monomer units are of a special interest since they are expected to exhibit a considerable range of thermomechanical properties that might be superior to those of PHAs containing only either SCL or MCL monomer units [5].

In this study, a *Pseudomonas* sp. strain that utilized dicarboxylic acids as the sole carbon source for growth and PHA production was isolated. This organism accumulated PHAs consisting of 3HA monomer units from 3HB to 3-hydroxydodecanoate depending on the carbon sources used. We describe the distinct PHA biosynthetic patterns of the isolate which are unusual when compared to those of other PHA-producing bacteria. In particular, biosynthesis of an interesting polyester consisting of both 3HB and 3-hydroxyvalerate (3HV) as well as 3-hydroxyheptanoate (3HHp) in significantly relevant fractions is emphasized.

MATERIALS AND METHODS

Isolation of Bacterial Strain

For the isolation of bacterial strains that utilized dicarboxylic acid for growth, the samples from activated sludge and soil were inoculated into a mineral salts medium [6] containing 20 mM of octanedioic acid as the sole carbon source, and then the medium was incubated at 30°C for 5 days under aerobic conditions. This procedure was repeated three times in order to enrich the microorganisms. The culture broth was successively diluted and streaked on octanedioic acid mineral agar plates. Several colonies that grew rather quickly were screened for their PHA production. Among these isolates, one strain designated as HJ-2 was selected and used in this study.

Identification of the Isolated Strain

Biochemical tests were performed using API 20NE (BioMerieux) and Biolog GN (Biolog) test kits, which were prepared according to the manufacturer's specifications. Additional tests for oxidase activity, catalase activity, nitrate reduction, and denitrification were carried out by the following standard methods [25]: Fatty acid composition of the isolate was analyzed by gas chromatography (Hewlett-Packard model 5890A) that was equipped with a flame ionization detector and a 5% phenyl methyl silicone fused silica capillary column (0.2 mm×25 m). Identification and quantification of the methyl esters of the fatty acids were performed using the standard MIS library generation software (Microbial ID). The resultant profiles were compared with those in a commercial bacterial library (MIDI Aerobe Library, version 3.8).

For analyzing the 16S rDNA sequence, an almost complete 16S rDNA of the isolate HJ-2 was enzymatically amplified by using universal primers, 8F and 1492R, on an MJ Research model PTC 1000 cycler [21]. The resultant PCR product was purified by using a DNA PrepMate (Bioneer Co., Korea). The purified PCR product was directly sequenced by using a SIVER SEQUENCE DNA Sequencing System (Promega Co., U.S.A.). The following sequencing primers were used: 5'-CCCAGACATTACT-CACCCG (129R: positions 129 to 110 [*Escherichia coli* numbering]), 5'-AGAGGGGGGTAGAATTCCAG (662F: positions 662 to 681), 5'-CTACCAGGGTATCTAATCC (803R: positions 803 to 785), 5'-GGGCCCGCACAAGCGG (927F: positions 927 to 942), 5'-ACCTCCCTCCGGTTTATC (1183R: positions 1183 to 1164), SRV3-1 and SRV3-2.

The 16S rDNA sequences of strain HJ-2 was aligned with those in the Ribosomal Database Project [28] and GenBank. Multiple alignment of sequences and calculations of levels of sequence similarity were carried out by using the CLUSTAL W [33]. The evolutionary distance matrix was calculated by the Jukes and Cantor method [14]. Phylogenetic trees were inferred by using three algorithms, the neighbor-joining [30], least squares [28], and maximum parsimony [19]. The confidence level of the resultant tree topology was evaluated by performing 1,000 bootstrap replications (heuristic search). The PHYLIP package [9] was used for all phylogenetic analyses. For determining the DNA base composition, *Escherichia coli* K12 was used as a standard. The G+C content of DNA was determined from the midpoint value (Tm) of the thermal denaturation profile by using an Ultrospec 2,000 spectrophotometer (Pharmacia Biotech Ltd., U.K.) equipped with a programmable peltier temperature control unit.

Culture Condition

The organism was grown on various carbon sources in 0.5×E2 medium, which is a mineral salts medium [20]. In 0.5×E2 medium, the concentrations of $NaNH_4HPO_4$, K_2HPO_4, and KH_2PO_4 are a half of the salt concentration in a E2 medium. With an exception for carbohydrates, the carbon sources were consistently added as the sodium salts, which were prepared by adding 10 M NaOH to carboxylic acids to produce a final pH of 7.0. Cells were cultivated in 1-l Erlenmeyer flasks containing 300 ml medium or in a 5-l jar fermentor (Korea Fermentor Co.) with a working volume of 3 l. Temperature and pH in a fermentor were automatically controlled at 30°C and 7.0, respectively. The cultures were inoculated with a 10% (v/v) inoculum of exponentially growing precultures. Cell growth was monitored spectrophotometrically by measuring optical density of the culture at 660 nm. After cultivation for a given time depending on the kinds of carbon sources used, cells were harvested by centrifugation, washed with distilled water, and then were lyophilized.

Purification of PHA

PHA was isolated from lyophilized cells by extracting with chloroform in a Soxhlet apparatus. The polymer was

dissolved in a small amount of chloroform and precipitated by dropwise addition into methanol while vigorously stirring for further purification of the polymers. This precipitation procedure was repeated twice.

Analytical Methods

Dry cell weight (DCW) was measured by drying the harvested cells to a constant weight at 105°C. SCL-PHA content and its composition were determined by gas chromatography (Hewlett Packard 5890, U.S.A.) using PHA standards containing known proportions of 3HB and 3HV monomers after methanolysis of freeze-dried cells for 140 min at 100°C to yield the methyl esters of the constituent 3-hydroxyalkanoic acids [4]. The relative amounts of monomer units in MCL-PHA were determined as described in our previous paper [17]. Identification of repeating units was carried out by GC/MS analysis. A HP 5988 GC/MS system equipped with a SE30 nonpolar capillary column (0.32 mm×30 m×0.12 m) was used. The ionization energy was 70 eV for electron impact ionization, and methane was used for chemical ionization. Molecular weights of PHAs were determined by using a gel permeation chromatography system as described previously [18]. Differential scanning calorimeter (DSC) measurement was carried out by using a Thermal Analysis Model 2510. The temperature was scanned from -100°C to 210°C at a ramp of 20°C/min. NMR spectra were recorded using a Bruker AM 300 or a Bruker AMX 500. Tensile testing was performed under ambient conditions using an Automated Materials Testing System (Instron Corp., U.S.A.). The specimens were dumbbell-shaped according to ASTM-D 1708. A clamp separation of 24 mm and a crosshead speed of 50 mm/min were used.

RESULTS AND DISCUSSION

Isolation and Characterization of Isolated Strain

The isolated strain HJ-2 was a Gram-negative, aerobic, motile, and non-spore-forming rod (0.5 to 0.7 by 0.9 to 2.0 μm in size) that was catalase positive for oxidase reactions. On nutrient agar, the organism formed clear, translucent, smooth, round, and flat colonies. This strain did not produce pyocyanin, but it did produce diffusible yellowish nonfluorescent pigment on nutrient agar. The major fatty acids were 18:1 w7c (33.2%), 16:0 (24.6%), and 16:1 w7c/15:0 iso 2OH (21.5%). The G+C content of the DNA was 68.0 mol% by Tm. Agarose gel electrophoretic analysis of crude lysates did not reveal the presence of plasmid DNA. The phenotypic properties and the pattern of fatty acid suggested that the strain HJ-2 clearly belongs to the genus *Pseudomonas*. However, no clear reference to one particular species of the genus could be made at this time.

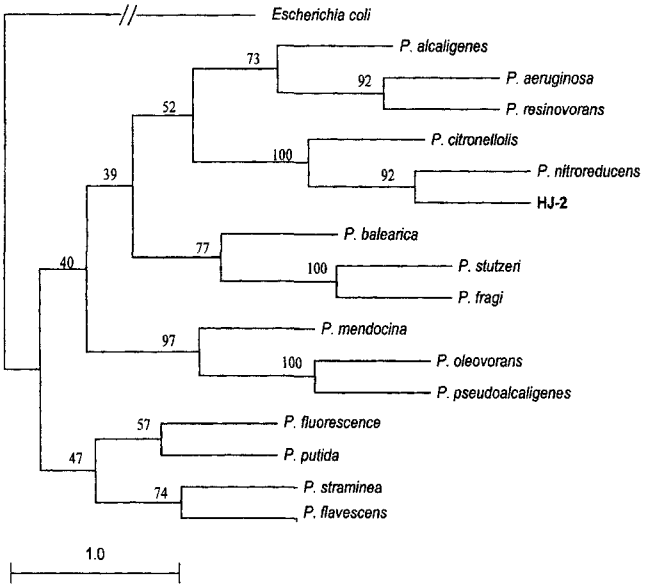

Fig. 1. Phylogenetic position of strain HJ-2.
The tree, constructed by using the neighbor-joining method, was based on a comparison of 16S rDNA sequences. Bootstrap values, expressed as percentages of 100 replications, are given at the branch points. 16S rDNA sequence of *E. coli* was used an outgroup sequence. Bar=1 nucleotide substitutions per 100 nucleotides.

A total of 1,375 nucleotides of the 16S rDNA of strain HJ-2 were determined and the primary structure was aligned with those of reference strains of RDP and GenBank. Cluster analysis of the 16S rDNA sequences of HJ-2 indicated that HJ-2 clearly grouped to species of *Pseudomonas* (Fig. 1). This genus belongs to the gamma subclass of the *Proteobacteria* and to rRNA group I. HJ-2 has shown to have the greatest sequence similarity (96.0%) to *Pseudomonas nitroreducens*. This sequence similarity is lower than those typically found between members of the same species [11]. Therefore, the phylogenetic analysis shows that HJ-2 probably represents a new species within *Pseudomonas*. *Pseudomonas* sp. HJ-2 has been deposited at the Korea Culture Type Collection as strain KCTC 0406BP.

Accumulation of Polyesters from Various Carbon Sources

Pseudomonas sp. HJ-2 was examined to elucidate whether this organism synthesized PHAs when it was grown in a mineral salts medium containing different carbon sources. This organism accumulated PHAs with unusual pattern of monomer units depending on the carbon sources used (Table 1). Only a small amount (less than 10% DCW) of PHB homopolyester was accumulated when this organism was grown with glucose. The accumulation of PHB from simple carbohydrates is a well known characteristic of SCL-PHA producing bacteria. However, HJ-2 cells grown on gluconate biosynthesized a PHA composed of 3HB as the major constituent and three other monomer units, 3-

Table 1. Biosynthesis of PHAs by *Pseudomonas* sp. HJ-2 from various carbon substrates.

Carbon source (g/l)	Culture time (h)	DCW (g/l)	PHA content (%DCW)	PHA composition (%)[a]								
				3HB (C4)	3HV (C5)	3HHx (C6)	3HHp (C7)	3HO (C8)	3HN (C9)	3HD (C10)	3HUD (C11)	3HDD (C12)
Glucose (10)	48	1.21	5.8	100								
Gluconate (10)	110	0.79	11.4	76.7				5.3		15.3		2.7
Fructose (10)	120	0.98	18.4					29.2		60.7		10.1
Butyric acid (5)	74	1.54	12.3	100								
Valeric acid (5)	125	1.16	36.2	6.3	93.7							
Hexanoic acid (5)	50	2.11	25.6	100								
Heptanoic acid (5)	125	0.64	17.2	21.6	43.1		35.3					
Octanoic acid (5)	50	2.38	23.1	89.7		1.7		8.6				
Nonanoic acid (5)	75	0.73	8.22	1.2			15.7		83.1			
Decanoic acid (5)	125	1.12	42.8	72.5				27.5				
Undecanoic acid (5)	75	0.98	3.1	1.5	1.7		20.3		61.6		14.9	
Heptanedioic acid (5)	54	1.26	8.7	100								
Octanedioic acid (5)	110	0.88	3.4					14.8		69.9		15.3
Nonanedioic acid (5)	75	0.95	24.2	100								
Decanedioic acid (5)	110	0.73	5.5	13.6		58.3				28.1		

[a]GC area %.

hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD), and 3-hydroxydodecanoate (3HDD). These results suggested that there is a possible relationship between *de novo* fatty acid biosynthesis and PHA synthesis pathways [8]. Similar results were recently reported for *Pseudomonas* sp. 61-3 grown with gluconate [1]. In this case, the polyester was reported to contain 3HB and MCL 3HA of all even carbon numbers (C6, C8, C10, and C12). However, *P. putida* and many other fluorescent pseudomonads accumulate MCL-PHAs containing primarily 3HD when gluconate or glucose is fed as the sole carbon source [12, 13, 34].

N-Alkanoic acids from butyric acid to undecanoic acid supported *Pseudomonas* sp. HJ-2 for producing PHAs. Butyric acid and hexanoic acid supported the production of PHB homopolyester while valeric acid supported the production of a random copolyester consisting of 6 mol% 3HB and 94 mol% 3HV with PHA content as much as 36% of the cellular dry matter. In contrast, this strain produced PHAs containing both SCL and MCL 3HA units when grown with alkanoic acids from heptanoic acid to undecanoic acid. The polyesters from octanoic acid and decanoic acid were composed of 3HB and 3HO, while those from heptanoic acid, nonanoic acid, and undecanoic acid contained 3HB and 3HA monomer units with an odd number of carbons ranging from 3HV to 3HUD. The capability of accumulating PHAs with both SCL- and MCL 3HA monomer units revealed that *Pseudomonas* sp. HJ-2 biosynthesized PHAs via pathways both for SCL-PHA and MCL-PHA production. Moreover, these results in this study clearly demonstrated that the PHA synthase system of HJ-2 was not very specific for substrate, therefore, allowing for the incorporation of a variety of different repeating units into the polymer.

Dicarboxylic acids are extremely toxic substrates for the cell growth of *Pseudomonas* spp. [23]. Exceptionally, *P. cirtonellolis* was reported to utilize the dicarboxylic acids (C4 to C10) for the production of MCL-PHA copolyesters in which 3HD was the principal monomer unit regardless of the size of the carbon substrate [6]. HJ-2 grown with dicarboxylic acids containing odd numbered carbons accumulated only PHB homopolyester, while the same organism grown with octanedioic acid produced MCL-PHA containing 3HD as the main constituent. This polyester contained almost equimolar amounts of 3HO and 3HDD as minor constituents. These results suggested that dicarboxylic acids were metabolized in *Pseudomonas* sp. HJ-2 via different pathways for the synthesis of polyesters which were mostly determined by the carbon chain length.

Biosynthesis and Properties of PHA Consisting of 3HB, 3HV, and 3HHp

Among the PHAs produced by *Pseudomonas* sp. HJ-2, the most attractive ones were those containing 3HB, 3HV, and 3HHp units. The PHA isolated from cells grown with heptanoic acid was found to be the most interesting one. In contrast to the known characteristics of SCL- and MCL-PHAs, the polyester films were soft, tough, and could be easily fabricated into elastic bands or fibrous forms.

When the initial concentration of heptanoic acid was higher than 7.5 g/l, cell growth and PHA production was severly inhibited. The contents of PHA and the proportions of monomer units varied depending on the culture

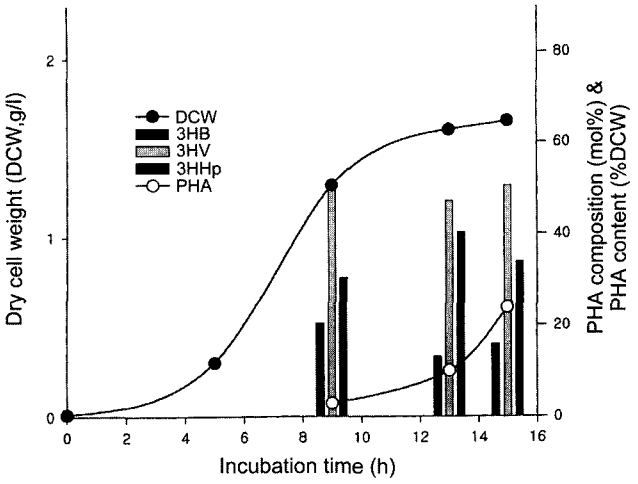

Fig. 2. Time courses of cell growth and PHA accumulation during the batch cultivation of *Pseudomonas* sp. HJ-2 in a mineral medium containing 0.5% heptanoic acid as the sole carbon source.

conditions (manuscript in preparation). Figure 2 shows typical time courses of cell growth and PHA accumulation during the batch cultivation of *Pseudomonas* sp. HJ-2 in a mineral salts medium containing 0.5% of heptanoic acid as the sole carbon source. PHA accumulation started during the exponential phase and reached its peak at the stationary phase. The maximum value of PHA content was lower than 30% of DCW and the monomeric composition of polyester remained almost unchanged during the batch fermentation process. The sizes of the granules in the cells varied but most frequently were 0.5-1.0 μm in length. Only a few bacteria, including *Rhodocyclus gelatinosus* strain 2150 [24] and recombinant *A. eutrophus* mutants harboring the heterologous PHA synthase gene from *Aeromonas caviae* [10] have been found to produce polyesters consisting of 3HB, 3HV, and 3HHp. However, in these cases, the molar fractions of 3HHp in the polyesters were lower than 10 mol%.

Solvent fractionation using chloroform and hexane as good and poor solvents, respectively, was carried out to elucidate whether SCL and MCL 3HA units in these polyesters were copolymerized or not. The results are given in Table 2. The polyester from heptanoic acid was fractionated into two fractions, hexane-soluble fraction and hexane-insoluble fraction. The hexane-insoluble fraction was the most likely copolyester of 3HB and 3HV [poly(3HB-*co*-3HV)] plus small amounts of 3HHp. It was not clear if the small amount of 3HHp units were covalently bound to 3HB and 3HV units. This hexane-insoluble fraction showed one glass transition (-4°C) and one melting transition (80°C). The hexane-soluble fraction contained only the 3HHp unit indicating that this polymer was a homopolyester of 3HHp. The glass transition temperature of this polymer was approximately -35°C and

Table 2. Hexane fractionation result of the PHA produced by *Pseudomonas* sp. HJ-2 from heptanoic acid.

Fraction	PHA (g/l)	PHA composition (mol%)[a]		
		3HB	3HV	3HHp
Whole PHA	1.0	20	62	18
Hexane-insoluble	0.868	23	73	4
Hexane-soluble	0.132			100

[a]Determined by gas chromatography.

no melting endotherm was observed in the DSC thermogram. These results showed that *Pseudomonas* sp. HJ-2 produced a physical mixture of poly(3HHp) and poly(3HB-*co*-3HV) copolyester that could contain a small amount of 3HHp units. Similarly, fractionation studies conducted of other PHAs containing 3HB and MCL 3HA units from either octanoic acid or decanoic acid revealed that these polymers were also composed of PHB homopolyester and copolyester of MCL 3HA monomer units.

Even though poly(3HB-*co*-3HV) samples become more flexible compared to PHB as the 3HV mol% in the copolyester increase, it has been reported that poly(3HB-*co*-3HV) copolyesters containing 3HV units of more than 70 mol% are as stiff and brittle as PHB [7, 27]. However, the hexane-insoluble poly(3HB-*co*-3HV) fraction containing 73 mol% 3HV and 4 mol% 3HHp was an elastomer with good mechanical properties. The maximum elongation ratio and the ultimate strength of the copolyester strip at room temperature were approximately 460% and 10 MPa, respectively. These properties were very different from those of poly(3HB-*co*-3HV) copolyesters having similar compositions produced from other microorganisms.

As most physical properties of poly(3HB-*co*-3HV) have been characterized for the copolyesters produced by *R. eutropha* [3, 7], a poly(3HB-*co*-3HV) with 82 mol% 3HB and 18 mol% 3HV was prepared by a batch culture of *R. eutropha* NCIMB11599 using glucose and propionic acid as the main carbon substrate and cosubstrate, respectively, as described in a previous report [35]. The DSC thermogram of this copolyester was compared with that of poly(3HB-*co*-3HV) having a similar composition produced by HJ-2 grown with a mixture of 40 mM valeric acid and 10 mM hexanoic acid. The DSC thermograms of the copolyesters isolated from *R. eutropha* and HJ-2 are shown in Fig. 3. Figure 3 shows that the poly(3HB-*co*-3HV) biosynthesized by *R. eutropha* had two melting transitions at 78 and 174°C while the poly(3HB-*co*-3HV) biosynthesized by HJ-2 had one melting temperature at 142°C. These results indicated that the polymer obtained from *R. eutropha* was a mixture of PHB and poly(3HB-*co*-3HV), while the polymer produced by HJ-2 was a homogeneous copolyester. The molecular weight distribution of these polymers were very similar. The average molecular weights and polydispersity

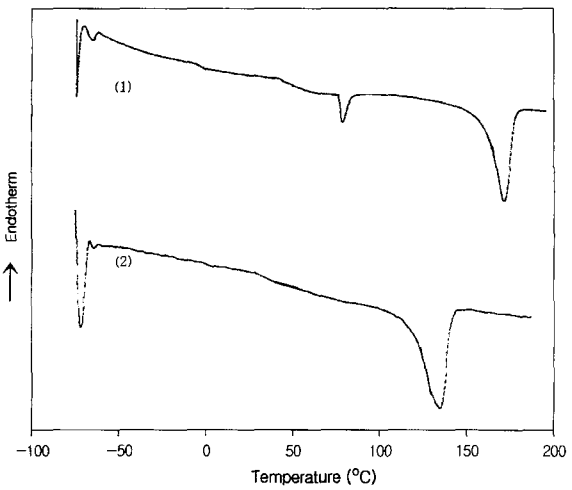

Fig. 3. DSC heating curves of poly(3HB-*co*-18 mol% 3HV) biosynthesized by *R. eutropha* (1) and *Pseudomonas* sp. HJ-2 (2).

indices were approximately 200,000-220,000 and 1.9-2.2, respectively. These results suggested that the difference in physical properties between poly(3HB-*co*-3HV) copolyesters biosynthesized by HJ-2 and those reported in literatures for poly(3HB-*co*-3HV) biosynthesized by *R. eutropha* are due to the different microstructures (composition distributions) of the polymers. Recently, some investigators have observed that bacterial poly(3HB-*co*-3HV) does not have a narrow composition distribution but is composed of polymer chains containing differing proportions of 3HB and 3HV monomers [16, 27]. Since the composition distribution may influence on various properties of polymers [36], it is necessary to know its effects on the physical properties of poly(3HB-*co*-3HV). Further investigation is in progress to understand the composition-property relationship of the poly(3HB-*co*-3HV) biosynthesized by HJ-2.

Acknowledgments

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