

## Polyesters Biosynthesis of *Alcaligenes eutrophus* H16 (ATCC 17699) from Various Mono- and Dicarboxylic Acids and Diols

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The polyesters (polyhydroxyalkanoates; PHAs) production capability in a two-step cultivation of *Alcaligenes eutrophus* H16(ATCC 17699) was investigated by using various organic carbon sources. The carbon sources used included linear C<sub>2</sub>~C<sub>10</sub> monocarboxylic acids, C<sub>3</sub>~C<sub>10</sub> dicarboxylic acids, crotonic acid, and several linear vicinal and ω-diols. The polyesters synthesized were characterized by 500 MHz <sup>1</sup>H-NMR spectroscopy, intrinsic viscosity[η] measurement in chloroform and differential scanning calorimetry (DSC). The PHAs synthesis data showed that the use of C-odd (C<sub>3</sub>, C<sub>5</sub>, and C<sub>7</sub>) monocarboxylic acids resulted in poly(3-hydroxybutyrate-co-3-hydroxyvalerate)(P(3HB-co-3HV)) (3HV content ranging 40 to 70 mol%) while the use of C<sub>9</sub> substrate gave the copolyester containing only 4 mol% of 3HV. All culture products obtained on C<sub>3</sub>~C<sub>10</sub> dicarboxylic acids gave exclusively P(3HB). 500 MHz <sup>1</sup>H-NMR analysis showed that all polyesters synthesized generally contained 1~2 mol% 3HV even for the unrelated substrates such as the carboxylic acids with even number of carbon. When α,ω-diols with even number of carbon were used as substrates, 4-hydroxybutyrate(4HB) was inserted into the polyester chain composed of P(3HB-co-4HB). Vicinal diols were generally not utilized by the bacterium for polyester production.

Various types of polyesters other than P(3HB) can be produced by changing the bacterial strain, the carbon source and the culture condition (1, 5, 6, 12). In recent years, studies on carbon source dependence have been extensively carried out by several research groups for the production of various types of polyesters from *A. eutrophus* H16 (5, 6), *Pseudomonas oleovorans* (2, 5, 6, 9, 10, 13) and *Pseudomonas citronellolis* (3, 4). Approximately ~40 different hydroxyalkanoic acid monomers have been detected among the bacterial polyhydroxyalkanoates(PHAs) found up to now (16).

*A. eutrophus* is known to produce P(3HB) homopolymer and various co- or terpolyesters from various organic carbon sources (5, 6). The six constituent comonomers found are 3-hydroxybutyrate(3HB), 3-hydroxyvalerate(3HV), 4-hydroxybutyrate(4HB), 5-hydroxyvalerate(5HV), 4-hydroxyvalerate(4HV), and 3-hydroxypropionate(3HP). The comonomer types found in the polyesters synthesized by *P. oleovorans* are far more diverse than

those found in *A. eutrophus*. The comonomers derived from *P. oleovorans* (2, 9, 10, 13) and *P. citronellolis* (3, 4) contain R-groups of various medium chain lengths, which thereby permits the incorporation of functional groups such as halogens, double-bonds and alkyl-branches into them.

The carbon sources used by several researchers so far, in the PHAs production of *A. eutrophus* H16 include C<sub>2</sub>~C<sub>6</sub> monocarboxylic acids, glucose, fructose, and linear C<sub>2</sub>~C<sub>10</sub> α,ω-diols (5, 6). However, there is no detailed study of carbon sources such as higher monocarboxylic acids (C<sub>7</sub>~C<sub>10</sub>); linear C<sub>3</sub>~C<sub>10</sub> ω-dicarboxylic acids; crotonic acid; several linear vicinal diols. In this study, we report the results of two-step PHAs synthesis of *A. eutrophus* H16 where the carbon sources mentioned above were used. Our systematic experimental results (15) revealed that the P(3HB) accumulation is unavoidable even under nutrient-rich condition. This means that the two-step culture product inevitably contains the P(3HB) produced under nutrient-rich condition. Therefore, we also reinvestigated the PHAs production from the carbon sources reported earlier.

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Key Words: Polyester biosynthesis, *A. eutrophus* H16, carboxylic acids, linear diols

## MATERIALS AND METHODS

### Carbon Sources and Other Materials

All chemicals used were reagent grade. Yeast extract and nutrient broth for growth media were purchased from Difco Laboratories. The following carbon sources were purchased from Sigma Chemical Co. and used as received; propionic, butyric, pentanoic, hexanoic, heptanoic, octanoic, nonanoic, decanoic, propanedioic, butanedioic, pentanedioic, hexanedioic, heptanedioic, octanedioic, and nonanedioic acids. Decanedioic acid, 1,5-pentanediol, 1,6-hexanediol, 1,2-hexanediol and other vicinal diols were from Aldrich Chemical Co.. 1,4-Butanediol, acetic acid,  $(\text{NH}_4)_2\text{SO}_4$ , and other inorganic salts were from Junsei Chemical Co..

### Bacterial Strain and Culture Media

The strain ATCC 17699 of *Alcaligenes eutrophus* H16 used in this study was purchased from the American Type Culture Collection (ATCC). Culture was maintained on nutrient broth (1% yeast extract, 1.5% nutrient broth) agar plates at 4°C and was subcultured every three weeks.

Two kinds of media were used in this study. Medium 1 used for exponential culture was a nutrient-rich medium containing 10 g of yeast extract, 15 g of nutrient broth, and 5 g of  $(\text{NH}_4)_2\text{SO}_4$  per liter of distilled water. The pH was adjusted to 7.0 with 5 M NaOH before the medium was autoclaved. Medium 2 used in the expedition of PHAs accumulation was a nitrogen-free medium containing a carbon source. The composition of the nitrogen-free medium was as follows (per liter of distilled water); 2.3 g of  $\text{KH}_2\text{PO}_4$ , 7.3 g of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 0.25 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g of  $\text{Fe}(\text{NH}_4)_2$ -citrate or  $\text{FeCl}_3 \cdot 12\text{H}_2\text{O}$ , 0.5 g of  $\text{NaHCO}_3$ , 0.2 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 1 ml of a microelement solution. The pH of the medium was adjusted to 7.0. The microelement solution contained 0.15 g of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g of  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.3 g of  $\text{H}_3\text{BO}_3$ , 0.8 g of  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.04 g of  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.02 g of  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$  and 0.03 g of  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  per liter of distilled water.

### Polyhydroxyalkanoates(PHAs) Synthesis

PHAs synthesis was performed by a two-step batch culture. First, 5 ml of inocula were transferred onto 500 ml of a nutrient-rich medium and cultivated at 30°C and 160 rpm. Cells were harvested by centrifugation (6,000 rpm, 10 min) after 22-hr cultivation time corresponding to the late log phase and washed with sterilized water. In the second step, the washed cells were transferred onto a nitrogen-free medium (500 ml) containing a carbon source to promote PHAs accumulation, and were cultivated for 48 hrs at 30°C and 160 rpm. The cultivated cells were harvested by centrifugation (6,000

rpm, 10 min), washed with acetone and finally dried under vacuum at 30~40°C.

### Polyesters Extraction and Purification

Dried cells were weighed and transferred onto an extraction thimble filter (28×100 mm, Advantec; Toyo Roshiki Kaisha, Ltd.). Polyesters were extracted from the dried cells with hot chloroform in a Pyrex Soxhlet apparatus for 6 hrs. The solvent extract was concentrated under reduced pressure on a rotary vacuum evaporator (EYELA; Tokyo Rikakikai Co.) and precipitated in a rapidly stirring cold methanol. The precipitate was separated by suction filtration and dried overnight under vacuum at 30~40°C. Dried polyesters were redissolved in chloroform and filtered. Polyesters were reprecipitated with cold methanol, washed with 80% methanol+20% chloroform solution to completely remove lipid components in the polyester and dried under vacuum at 30~40°C.

### Nuclear Magnetic Resonance(NMR) Spectroscopy

The  $^1\text{H}$ -NMR analysis of the polyester samples was carried out on a Bruker AMX-500 spectrometer in the pulse Fourier transform (FT) mode. The 500 MHz  $^1\text{H}$ -NMR spectra were recorded at 25°C using  $\text{CDCl}_3$  solutions of the polyester(5 mg/ml) with 4.0-s pulse repetition, 5000-Hz spectral width, 32 K data points and 16 accumulations.

### Thermal Analysis of Polyesters

The melting transition temperature ( $T_m$ ) was measured by using a DuPont 9900 differential scanning calorimeter (DSC V2.2A) equipped with a data station. Samples for the DSC study were prepared by casting the polyester into aluminum pans from a ~5% solution in chloroform. The specimens were subjected to further drying under vacuum at room temperature for 1 day or more. Sample size was in the range of 10~15 mg. Experiments were carried out at a heating rate of 10°C/min under a dry nitrogen purge.

### Determination of Polyesters Molecular Weight

P(3HB) molecular weight was determined from the Mark-Howink equation which shows a relation between molecular weight and intrinsic viscosity. The intrinsic viscosities of various PHAs samples in chloroform at 30°C were measured in a capillary viscometer of the Cannon-Fanske type (capillary No. 50) which was immersed in a constant temperature bath. Molecular weights were calculated from the Mark-Howink equation (14):

$$[\eta] = k M^a$$

$$k; 7.7 \times 10^{-5} \text{ (cm}^3\text{g}^{-1}\text{) (in chloroform)}$$

$$a; 0.82$$

## RESULTS AND DISCUSSION

### Polyesters Accumulation on Monocarboxylic Acids

Table 1 shows the results of polyester production from C<sub>2</sub>~C<sub>10</sub> monocarboxylic acids by *A. eutrophus* H16. The contents of polyesters in dried cells were in the range of 1.7~55 wt%, depending on the carbon source used. A homopolymer of 3HB[P(3HB)] (actually, containing 1 mol% or less of 3HV) was produced from organic acids with even carbon number such as acetic, butyric, hexanoic and octanoic acids, but when decanoic acid was used as the carbon source, cell lysis occurred even at the concentration of 2 g/500 ml, probably because of its detergent-like behavior. Among aliphatic monocarboxylic acids, the accumulation on butyrate gave the highest P(3HB) production, 55 wt%, which is comparable to 62 wt% on crotonate. The highest yield for those C<sub>4</sub> substrates among the homologues may be ascribable to easier incorporation of them into the P(3HB) biosynthesis pathway via less steps of modification to prepare the metabolic intermediates ready for use in the P(3HB) synthesis. The PHA production yield strongly depends on the substrate concentration in the feed. The increase of crotonate concentration to 10 g/500 ml reduced the P(3HB) production yield down to 40 wt%. On the contrary, doubling acetate concentration from 5 to 10 g/500 ml doubled the yield from 16 to 35 wt%. Actually, the concentration of each substrate should be optimized for the maximum PHA production. The optimum concentration for the homologous aliphatic carboxylates is known to be in the range of 1~2% (wt/v) under our synthesis culture condition (5, 6).

In contrast with C-even substrates, the cultivation on C-odd substrates (propionate, pentanoate and heptanoate) produced the copolyester composed of 3HB and 3HV with 3HV content ranging from 40 to 66 mol%. The relative monomer compositions of the copolyester samples were determined from the areas of methyl proton resonances [HB(4) and HV(5)] in 3HB and 3HV units (Fig. 1). The composition of the copolyester synthesized from pentanoic acid is 3HB(58 mol%):3HV(42 mol%). The 3HV fraction in the copolyester from pentanoic acid is rather lower than that reported (75 mol%) by Doi *et al.*(7). They also reported that the cultivation on heptanoate produced no polyester (6), which is contrary to our result giving the copolyester with 66 mol% of 3HV (Fig. 1). When we consider the remaining P(3HB) of 8.5 wt% after the first-step cultivation (15), the copolyester production yield of 1.7 wt% suggests that most of the P(3HB) present in the cells must be degraded

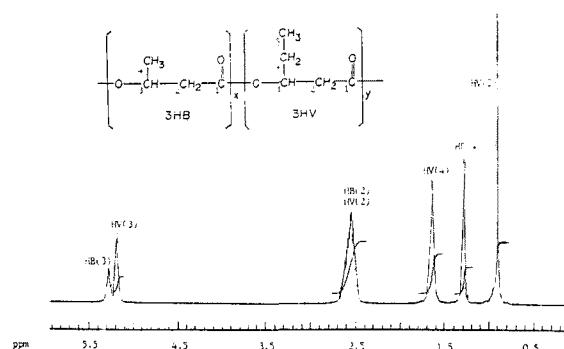


Fig. 1. 500 MHz <sup>1</sup>H-NMR spectrum of P(3HB-co-3HV) synthesized by *A. eutrophus* H16 from heptanoic acid.

Table 1. Biosynthesis of PHAs by *A. eutrophus* H16 from various monocarboxylic acids at 30°C.

| carbon source<br>(g/l culture medium) | wt. of dry<br>cells<br>(g/l) | wt. of<br>PHAs<br>(g/l) | wt% of<br>PHAs in<br>dry cells | PHAs composition<br>mol % <sup>a)</sup> |     | physical properties of PHAs |                   |                        |     |
|---------------------------------------|------------------------------|-------------------------|--------------------------------|---|-----|-----------------------------|-------------------|------------------------|-----|
|                                       |                              |                         |                                | 3HB                                     | 3HV | Tm (°C) <sup>b)</sup>       | [η] <sup>c)</sup> | Mv × 10 <sup>5d)</sup> |     |
| acetic acid                           | 10                           | 5.0                     | 0.82                           | 16.3                                    | 99  | 1                           |                   |                        |     |
| acetic acid                           | 20                           | 5.4                     | 1.88                           | 34.7                                    | 99  | 1                           |                   |                        |     |
| propionic acid                        | 10                           | 3.9                     | 0.72                           | 18.4                                    | 59  | 41                          |                   |                        |     |
| butyric acid                          | 10                           | 7.5                     | 4.14                           | 55.2                                    | 99  | 1                           |                   |                        |     |
| crotonic acid                         | 10                           | 8.2                     | 5.02                           | 61.6                                    | 99  | 1                           | 176               | 3.02                   | 4.0 |
| crotonic acid                         | 20                           | 5.8                     | 2.30                           | 40.0                                    | 99  | 1                           |                   |                        |     |
| pentanoic acid                        | 10                           | 3.9                     | 0.72                           | 18.4                                    | 58  | 42                          | 166               | 2.92                   | 3.8 |
| hexanoic acid                         | 10                           | 5.2                     | 2.18                           | 41.9                                    | 99  | 1                           |                   | 1.56                   | 1.8 |
| heptanoic acid                        | 10                           | 2.2                     | 0.04                           | 1.7                                     | 34  | 66                          |                   |                        |     |
| octanoic acid                         | 10                           | 2.0                     | 0.20                           | 10.0                                    | 99  | 1                           |                   |                        |     |
| nonanoic acid                         | 10                           | 2.7                     | 0.68                           | 24.6                                    | 96  | 4                           | 167               | 3.93                   | 5.5 |
| decanoic acid                         | 4                            | 0.0                     |                                |   |     |                             |                   |                        |     |
| 3-methylvalerate                      | 10                           | 3.0                     | 0.24                           | 8.0                                     | 97  | 3                           |                   |                        |     |

<sup>a)</sup> Calculated from NMR data, <sup>b)</sup> Peak temperature from DSC data, <sup>c)</sup> Intrinsic viscosity, <sup>d)</sup> Viscosity molecular weight

and replaced with the copolyester during the accumulation step. When nonanoic acid was used as a carbon source, P(3HB-co-3HV) having only 4 mol% of 3HV was synthesized. It was expected that the copolyester from nonanoate would have contained at least as much of 3HV as in those produced from other lower carboxylates with odd carbon numbers. For its homologous structure, the unexpected low 3HV content of the copolyester from nonanoate may suggest its different degradation pathway from the lower carboxylate's.

#### Polyesters Accumulation on Dicarboxylic Acids

Table 2 lists the results for the biosynthesis of polyesters from dicarboxylic acids. The polyester contents in dried cells extracted with chloroform were in the range of 3.7–50.1 wt% depending on the dicarboxylic acid used as a carbon source. Nonanedioic acid was the best substrate among dicarboxylic acids for the high production of P(3HB), yielding 50.1 wt%. No general relation between monocarboxylic acids and the corresponding dicarboxylic acids of the same carbon number for polyester production was found. Compared to the average 8.5 wt% of residual P(3HB) in the first-step cultivation, the low P(3HB) production yield of 3.7 wt% from propanedioate means that most remaining P(3HB) degraded without any accumulation of polyesters during the second cultivation period. On the contrary to monocarboxylic acids, the use of dicarboxylic acids (C<sub>3</sub> to C<sub>10</sub>) produced exclusively P(3HB) homopolyester independent of the number of carbon in the substrate. The unexpected result may again imply that the polyester biosynthesis occurs via different pathway depending on the length of the linear chain and the number and kind of functional group of substrates.

#### Polyesters Accumulation on Linear ω- or Vicinal Diols

Doi group reported that the copolyesters containing 4HB (8, 11) and 3-hydroxypropionate(3HP) (5, 6) as comonomers were obtained when grown on 1,4-butanediol and 1,5-pentanediol, respectively. But we obtained ter-

polyesters instead of the copolyesters for the same carbon sources (Table 3). Fig. 2 shows the 400 MHz <sup>1</sup>H-NMR spectrum of the polyester synthesized from 1,6-hexanediol. In addition to the well-characterized proton resonances of 3HB, three resonances appear at 4.09, 2.34 and 1.92 ppm with identical intensities. These are due to 4HB(4), 4HB(2) and 4HB(3) protons, respectively. The chemical shift assignment for them was made according to the data published elsewhere (8, 11). The PHA accumulation on 1,6-hexanediol produced the copolyester consisting of 10 mol% 4HB and 90 mol% 3HB. The 4HB content in the polyester was lower than that in Doi *et al*'s experiment (5). The substrate concentration dependence on the polyester composition exhibited a reversed trend compared to their result. For the PHA synthesis on other α,ω-diols, a similar trend to Doi *et al*'s was observed. As shown in Table 3, the vicinal diols listed were likely not utilized for PHA production when compared to the P(3HB) content of 8.5 wt% accumulated under nutrient-rich condition.

#### Molecular Weights and Melting Temperature of Polyesters

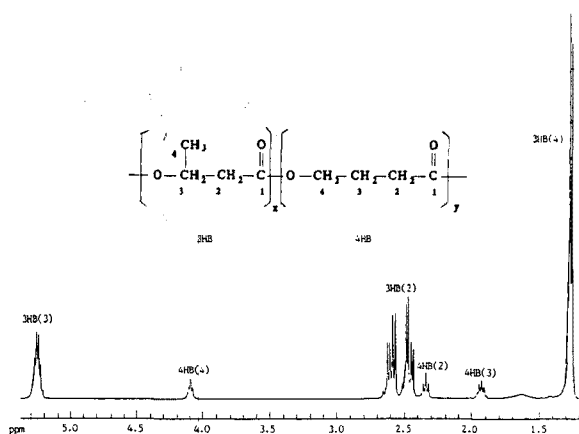


Fig. 2. 400 MHz <sup>1</sup>H-NMR spectrum of P(3HB-co-4HB) synthesized by *A. eutrophus* H16 from 1,6-hexanediol.

Table 2. Biosynthesis of PHAs by *A. eutrophus* H16 from various dicarboxylic acids at 30°C.

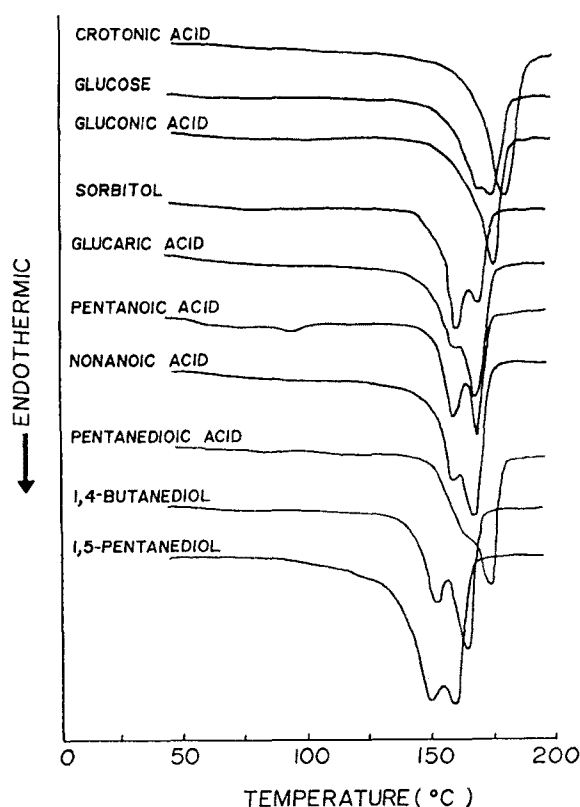
| carbon source<br>(g/l culture medium) | wt. of dry<br>(g/l) | wt. of<br>cells<br>(g/l) | wt% of<br>PHAs<br>dry cells | PHAs composition |                             | physical properties of PHAs |                    |                          |     |
|---------------------------------------|---------------------|--------------------------|-----------------------------|------------------|-----------------------------|-----------------------------|--------------------|--------------------------|-----|
|                                       |                     |                          |                             | PHAs in<br>3HB   | mol % <sup>(a)</sup><br>3HV | Tm (°C) <sup>(b)</sup>      | [η] <sup>(c)</sup> | Mv × 10 <sup>-5(d)</sup> |     |
| propanedioic acid                     | 10                  | 3.2                      | 0.12                        | 3.7              | 97                          | 3                           |                    |                          |     |
| butanedioic acid                      | 10                  | 5.6                      | 2.06                        | 37.0             | 99                          | 1                           |                    | 4.83                     | 7.1 |
| pentanedioic acid                     | 10                  | 5.7                      | 1.98                        | 34.6             | 99                          | 1                           | 173                | 4.42                     | 6.4 |
| hexanedioic acid                      | 10                  | 6.1                      | 2.68                        | 37.9             | 99                          | 1                           |                    | 4.64                     | 6.6 |
| heptanedioic acid                     | 10                  | 4.9                      | 0.46                        | 9.5              | 99                          | 1                           |                    | 4.47                     | 6.6 |
| octanedioic acid                      | 10                  | 5.2                      | 1.98                        | 38.0             | 99                          | 1                           |                    | 5.29                     | 7.9 |
| nonanedioic acid                      | 10                  | 7.2                      | 3.68                        | 50.1             | 99                          | 1                           |                    | 5.34                     | 8.0 |
| decanedioic acid                      | 10                  | 8.0                      | 3.18                        | 39.6             | 99                          | 1                           |                    | 5.05                     | 7.4 |

<sup>(a)</sup> Calculated from NMR data, <sup>(b)</sup> Peak temperature from DSC data, <sup>(c)</sup> Intrinsic viscosity, <sup>(d)</sup> Viscosity molecular weight

**Table 3. Biosynthesis of PHAs by *A. eutrophus* H16 from various linear  $\omega$ - or vicinal diols at 30°C.**

| carbon source<br>(g/l culture medium) | wt. of dry<br>cells<br>(g/l) | wt. of<br>PHAs<br>(g/l) | wt% of<br>PHAs in<br>dry cells | PHAs composition (mol%) |                   |                   |     |    |
|---------------------------------------|------------------------------|-------------------------|--------------------------------|-------------------------|-------------------|-------------------|-----|----|
|                                       |                              |                         |                                | 3HB                     | 4HB <sup>a)</sup> | 3HP <sup>a)</sup> | 3HV |    |
| 1,4-butanediol                        | 10                           | 3.7                     | 0.50                           | 13.8                    | 96                | 2                 |     | 2  |
| 1,5-pentanediol                       | 10                           | 4.7                     | 1.72                           | 36.4                    | 98                |                   | 1   | 1  |
| 1,2-pentanediol                       | 10                           | 2.8                     | 0.22                           | 7.5                     | 97                |                   |     | 3  |
| 1,6-hexanediol                        | 10                           | 4.7                     | 1.70                           | 35.6                    | 90                | 10                |     | <1 |
|                                       | 15                           | 3.5                     | 0.50                           | 15.2                    | 92                | 8                 |     |    |
|                                       | 20                           | 3.0                     | 0.06                           | 2.0                     | 96                | 4                 |     |    |
| 1,2-hexanediol                        | 5                            | 2.7                     | 0.20                           | 7.4                     | 98                |                   |     | 2  |
| 1,2-hexanediol<br>&                   | 7.6                          | 2.6                     | 0.14                           | 5.4                     | 96                | 2                 |     | 2  |
| 1,6-hexanediol                        | 2.4                          |                         |                                |                         |                   |                   |     |    |
| 1,2-hexanediol<br>&                   | 2.4                          | 2.6                     | 0.28                           | 10.8                    | 98                |                   |     | 2  |
| 1,6-hexanediol                        | 7.6                          |                         |                                |                         |                   |                   |     |    |
| 2,5-hexanediol                        | 10                           | 2.7                     | 0.28                           | 10.6                    | 98                |                   |     | 2  |

<sup>a)</sup> HB; 4-hydroxybutyrate, 3HP; 3-hydroxypropionate



**Fig. 3. DSC thermograms of PHAs produced by *A. eutrophus* H16 from various carbon sources.**

The viscosity molecular weight data examined for various samples are shown in Tables 1 and 2. The viscosity molecular weights of the polyesters synthesized from monocarboxylic acids were in the range of  $1.8$  to  $5.3 \times$

$10^5$ , whereas, those of the polyesters synthesized from dicarboxylic acids were in the range of  $6.6$  to  $8.0 \times 10^5$ . This means that the molecular weight of polyester could be controlled by choosing the appropriate carbon source.

Fig. 3 shows the DSC (Differential Scanning Calorimetry) profiles of polyesters synthesized from various carbon sources. The  $T_m$  values of the polyesters were around  $160^\circ\text{C}$ , similar to the values reported earlier (6). The thermogram of the polyester synthesized from pentanoic acid shows another endothermic peak at  $95^\circ\text{C}$ . This lowered endotherm is ascribable to the melting of P(3HB-co-3HV) copolyester domain in the sample because the incorporation of 3HV lowers the melting point. The existence of two melting transitions means that the polyester sample is a mixture of P(3HB) and 3HV enriched P(3HB-co-3HV). No general systematic trend toward the melting transition depending on carbon sources was found.

### Acknowledgement

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