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Effect of C/N Ratio on the Production of Poly(3-hydroxyalkanoates) by the Methylotroph *Paracoccus denitrificans*

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Two series of carbon sources, linear primary C_1~C_9 alcohols and linear C_2~C_{10} monocarboxylic acids were tested for PHA synthesis in *Paracoccus denitrificans*. The results showed that the growth-associated synthesis of PHA could be referred only to the carbon sources with odd number of carbon except methanol. For all carbon sources with even number of carbon, nitrogen limitation was required to induce PHA synthesis in *P. denitrificans*. Poly(3-hydroxyvalerate)[P(3HV)] homopolymer was synthesized from C_5, C_7, and C_9 while growing in the presence of nitrogen, but the nitrogen depletion in the later growth period incorporated 3-hydroxybutyrate(3HB) unit into the polymer chain. The optimum C/N ratio for P (3HV) homopolymer production was found to be 10 when the strain was grown on 10 ml/l of valeric acid for 96 h. *P. denitrificans* synthesized P(3HB-co-3HV) copolymers from *n*-hexanoic and *n*-octanoic acid. The microstructural characterics of the P(3HB-co-3HV) copolymer from *n*-propanol was investigated using ^{13}C-nuclear magnetic resonance spectroscopy, showing a structural heterogeneity.

Many bacteria are known to synthesize polyhydroxyalkanoates (PHAs) as carbon and energy reserve materials under nitrigen- or oxygen-limited condition (1, 4, 5). The PHAs are usually produced as copolymers except few homopolymers such as poly(3-hydroxybutyrate) [P(3HB)], poly(4-hydroxybutyrate)[P(4HB)] (11), poly(3-hydroxyvalerate)[P(3HV)] (15, 17) and poly(3-hydroxy-5-phenylvalerate) (6, 14).

Most short-chain-length(SCL) PHA-producing bacteria are able to synthesize P(3HB) homopolymer from a variety of carbon sources. However, the other homopolymers P(4HB), P(3HV) and poly(3-hydroxy-5-phenylvalerate) are synthesized by a limited species of bacteria from a specific type of carbon precursors. The preparation of such homopolymers is necessary because of their usefulness as reference compounds in the characterization of their copolymers or homopolymer blends (10).

Recently, P(3HV) homopolymer has been synthesized in substantial amounts in cells by *Paracoccus denitrificans* (17) and *Chromobacterium violaceum* (15) from valeric acid or *n*-pentanol. *P. denitrificans* is a facultative methylotroph having the ability to grow and produce useful metabolites from a cheap compound, methanol. It is also a useful bacterium for the production of P(3HB-co-3HV) copolymers containing high level of 3HV content and their comonomer ratios can be easily controlled by changing the feed ratio of the two monomer precursors (16). Yamane *et al.* prepared P(3HV) homopolymer by using a fed-batch cultivation of the bacterium (17). According to their results, mol % of 3HB monomer increased during the initial 5 h and, then, began to decrease around 5 h of cultivation, eventually disappeared. Instead, 100 mol % 3HV homopolymer was produced. However, on futher cultivation 3HB-monomer started to reappear when the nitrogen was used up around 20 h. This suggests that C/N ratio during cultivation is an important parameter in the preparation of 3HB-free P(3HV) homopolymer.

Therefore, we decided to determine the optimum parameter for an efficient production of P(3HV) homopolymer in a batch cultivation. In addition, two series of carbon sources primary alcohols (C_1~C_9) and carboxylic acids (C_2~C_{10}) were tested for PHA synthesis in *P. denitrificans*.

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MATERIALS AND METHODS

Organism and Culture Conditions

Carbon sources and most other reagent-grade chemi-

cals were purchased from Aldrich Chemical Co. and Sigma Chemical Co. The strain KCTC 2530 of *P. denitrificans* used in this study was purchased from the Korean Culture Type Collection. Inocula were grown in 5-ml test tubes containing nutrient-rich media consisted of 1 %(w/v) yeast extract, 1.5 %(w/v) nutrient broth and 0.2 %(w/v) $(NH_4)_2SO_4$. Yeast extract and nutrient broth were purchased from Diagnostic Pasteur Co. and Difco Co., respectively.

A one-step or two-step cultivation technique was employed for polyester accumulation in cells. Cells were precultured in the nutrient-rich medium at 30°C for 24 h. The precultured cells were cultured at 30°C under aerobic condition with 190 rpm of shaking speed for an appropriate time depending on the carbon source used. The medium used in the first step of the two-step cultivation was composed of (per liter) 0.2 g of yeast extract, 3 g of $(NH_4)_2SO_4$, 1.4 g of KH_2PO_4, 7.6 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 31.5 mg of Fe^{3+} Citrate, 0.3 g of $NaHCO_3$, 31.5 mg of $CaCl_2 \cdot 2H_2O$, 5.25 mg of $ZnSO_4 \cdot 7H_2O$, 5.25 mg of $MnCl_2 \cdot 4H_2O$, 0.53 mg of $CuSO_4 \cdot 5H_2O$ and an appropriate amount of the selected carbon source (16). In the medium used in the PHA accumulation stage yeast extract, $NaHCO_3$ and $(NH_4)_2SO_4$ were omitted.

For the carbon source on which no PHA was produced in the first-step, the cells grown on the carbon source were transfered to the nitrogen-free PHA synthesis medium containing the carbon source to induce PHA accumulation in the second-step.

Polymer Isolation and Characterization

Polyesters were isolated and characterized using a procedure published elsewhere (2, 3, 12, 13). Quantitative determination of the monomer units in polyesters was performed by gas chromatography (Hewlett-Packard HP 5890A gas chromatograph) and ^{1}H-NMR (Bruker DPX-300 spectrometer) experiments. Glass transition temperature, melting temperature and fusion of enthalpy for the isolated polymers were measured by a TA Thermal Analysis System (DSC V4.0B DuPont 2000). Viscosity molecular weight of the isolated polyesters was determined by measuring the intrinsic viscosities with a Cannon-Fenske viscometer (capillary No. 50) and calculating with the equation (8) relating the molecular weight to the determined intrinsic viscosity.

Monitoring of Cell Growth

Growth culture was sampled at appropriate time intervals to analyze the medium and the cells. Residual carbon source in the medium was measured by gas chromatography. Remaining NH_4^+ was measured with Nessler's reagent (2). The monomer unit composition of polyesters in cells was determined by gas chromatography.

^{13}C-NMR Analysis for the Determination of Dyad Sequences of Polyesters

^{1}H-noise decoupled 75 MHz ^{13}C NMR spectra were recorded at 25°C in $CDCl_3$ on a Bruker DPX 300 spectrometer with 5-s pulse repitition, 20000 Hz spectral width and 1024 accumulation. Splittings of the individual resonances were assigned as previously reported (5). The overlapped splittings were deconvoluted and integrated with a standard software package.

RESULTS AND DISCUSSION

Polyester Synthesis from Linear Primary Alcohols

The optimum concentration for cell growth was 1 %(v/v) for methanol and 0.1 %(v/v) for *n*-pentanol (7). Table 1 shows that *P. denitrificans* synthesized two homopolymers, poly(3-hydroxybutyrate)[P(3HB)] and poly(3-hydroxyvalerate)[P(3HV)], and P(3HB-co-3HV) copolyesters depending on the type of primary alcohol fed as the sole carbon source. A two-stage batch cultivation was carried out for those carbon sources from which no polyester was obtained at the given concentration in the one-stage cultivation. For some primary alcohols, even though the one-stage cultivation in the presence of nitrogen produced no polyester, nitrogen limitation in the second-stage induced the synthesis of polyesters. This means that the C/N ratio may be a critical factor in PHA

Table 1. Biosynthesis of PHAs in *P. denitrificans* using linear primary alcohols.

Carbon source	(g/l)	Cultrue time (h)	Dry cell wt. (g/l)	Polyester content (wt %)	Polyester composition[a] (mol %)		Molecular weight (M_v)[f]	Cultivation method
					3HB[b]	3HV[c]		
Methanol	7.9	48	1.48	26	100		169,000	Two-stage
Ethanol	1.9	48	0.93	4	100		nd[d]	Two-stage
Propanol	2.4	48	0.71	11	83	17	nd	One-stage
Butanol	1.6	48	2.39	22	100		88,000	Two-stage
Pentanol	0.8	48	0.84	22		100	nd	One-stage
Pentanol[e]		120	2.91	33		100	nd	Fed-batch
Heptanol	0.2	72	0.31	2		100	nd	One-stage
Nonanol	0.2		no growth					One-stage

[a]Determined by gas chromatography. [b]3HB, 3-hydroxybutyric acid. [c]3HV, 3-hydroxyvaleric acid. [d]nd, not determined. [e]0.1% (v/v) of pentanol was fed every 24 h. [f]M_v was calculated using the equation $[\eta]=kM^a$ where k is equal to 7.7×10^{-5} cm^3g^{-1} and a, 0.82 in chloroform (8).

formation.

Pentanol is a good substrate for the production of P(3HV) homopolymer in *P. denitrificans* as reported by Yamane *et al.* (17). However, since an increase in the concentration higher than 0.2 %(v/v) caused inhibition in cell growth a fed-batch technique was employed to increase the biomass and PHA content (7). After 120 h of fed-batch cultivation the biomass increased up to 3 g/l and the PHA content to 33 %(w/w) of the dry cell weight. It is also interesting to note that the growth on C_5 or C_7 odd-carbon alcohol produced P(3HV) homopolymer while only 17 mol % of 3HV was incorporated when the bacterium was grown on propanol.

Polyester Synthesis from Monocarboxylic Acids

The PHA synthesis characteristics of *P. denitrificans* from monocarboxylic acids showed carbon-number dependence similar to that observed for the primary alcohol analogues described above. Valeric acid was found to be a promising carbon source for the effective production of P(3HV) homopolymer in *P. denitrificans*. On the contrary to the case of the even-carbon acids, ethanol and butanol, interestingly, the growth on the higher even-carbon acids hexanoic or octanoic acid resulted in the production of P(3HB-co-3HV) copolymers.

P. denitrificans did not accumulate PHA in the one-stage cultivation when grown on even-carbon acid as the sole carbon, similar to that observed in alcohols. However, nitrogen limitation in the second-step cultivation induced PHA accumulation. In addition, an increase in the C/N ratio of the feed in the one-stage cultivation leaded to PHA accumulation for the most carbon sources which were utilized only for cell growth in the one-stage cultivation. As an example, when butyric acid was fed at 40 mM with the C/N ratio(mol/mol) of 5.8, 10 wt% of polyester was accumulated in cells (7). This suggests that the growth-associated synthesis of PHA could occur only when *P. denitrificans* would be fed with the carbon source having odd number of carbon except methanol. For all carbon sources with even number of carbon including methanol, nitrogen limitation was required to induce PHA synthesis in *P denitrificans*.

C/N Ratio Effect on the 3HV Homopolymer Preparation

Yamane *et al.* recently reported the production of P(3HV) from *n*-pentanol by *P. denitrificans* using a fed-batch culture technique (17). PHA was synthesized in the growth phase. In the early growth phase having enough nitrogen, 3HB/3HV copolymer was produced. The 3HB content was decreased with cultivation time and then, the P(3HV) homopolymer was obtained around 20 h of cultivation. But, further cultivation after nitrogen depletion incorporated 3HB monomer units again. Thus, the P(3HV) production is a function of the cultivation time. This may indicate that the P(3HV) production depends on the C/N ratio of the culture medium. Therefore, we investigated C/N ratio dependence on the P(3HV) synthesis in the batch cultivation of *P. denitrificans* using valeric acid. The concentration of valeric acid was fixed at 92 mM and the concentrations of ammonium sulfate were 3.8, 7.6, 9.1 and 15.1 mM whose C/N molar ratio corresponding to 24.2, 12.1, 10.1 and 6.1, respectively (Fig. 1). The highest dry biomass was obtained at the C/N molar ratio of 6.1 and the highest PHA content at the C/N ratio 9.1. Futher increases in C/N molar ratio to 24.1 and 12.1 induced the incorporation of 10 mol% of 3HB unit into the polymer. The optimum C/N ratio for the 3HV homopolymer production was found to be 10 when the strain was grown on valeric acid for 96 h.

Thus, the C/N ratio plays a critical role on the formation of 3HV homopolymer. In order to investigate the effect in detail the time courses for cell growth, ammonia consumption, PHA production and monomer composition were followed (Fig. 2). The dry biomass reached to 4 g/l around 90 h of cultivation. The polyester content within cells reached a maximum at the

Table 2. Biosynthesis of PHAs in *P. denitrificans* using various monocarboxylic acids.

Carbon source (g/l)		Culture time (h)	Dry cell wt. (g/l)	Polyester content (wt %)	Polyester composition[a] (mol %)		Molecular weight (M_v)[f]	Cultivation method
					3HB[b]	3HV[c]		
Acetic acid	2.4	48(48)[e]	0.98	67	100		64,000	Two-stage
Propionic acid	4.0	48(48)	2.18	16	82	18	nd[d]	Two-stage
Butyric acid	3.8	48(48)	4.26	38	100		34,000	Two-stage
Valeric acid	9.4	96	6.38	29		100	nd	One-stage
Hexanoic acid	2.8	48(48)	1.18	61	90	10	nd	Two-stage
Heptanoic acid	1.4	72	0.84	9		100	nd	One-stage
Octanoic acid	0.8	48(48)	0.54	49	92	8	nd	Two-stage
Nonanoic acid	0.2	72	0.28	3		100	nd	One-stage
Decanoic acid	0.4	120	0.32	0				One-stage

[a]Determined by gas chromatography. [b]3HB, 3-hydroxybutyric acid. [c]3HV, 3-hydroxyvaleric acid. [d]nd, not determined. [e]The cultivation time in the first-step culture. [f] M_v was calculated using the equation $[\eta]=kM^a$ where k is equal to 7.7×10^{-5} cm^3g^{-1} and a, 0.82 in chloroform (8).

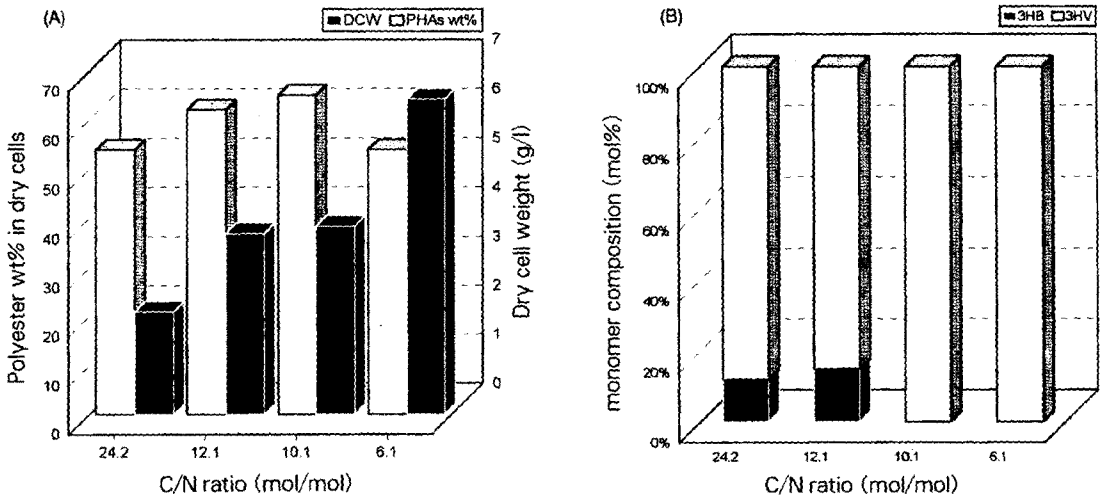

Fig. 1. Effect of C/N ratio on (A) cell growth and polyester content in cells and (B) monomer composition of the polyesters.
P. denitrificans was cultivated on valeric acid (10 ml/l) for 96 h.

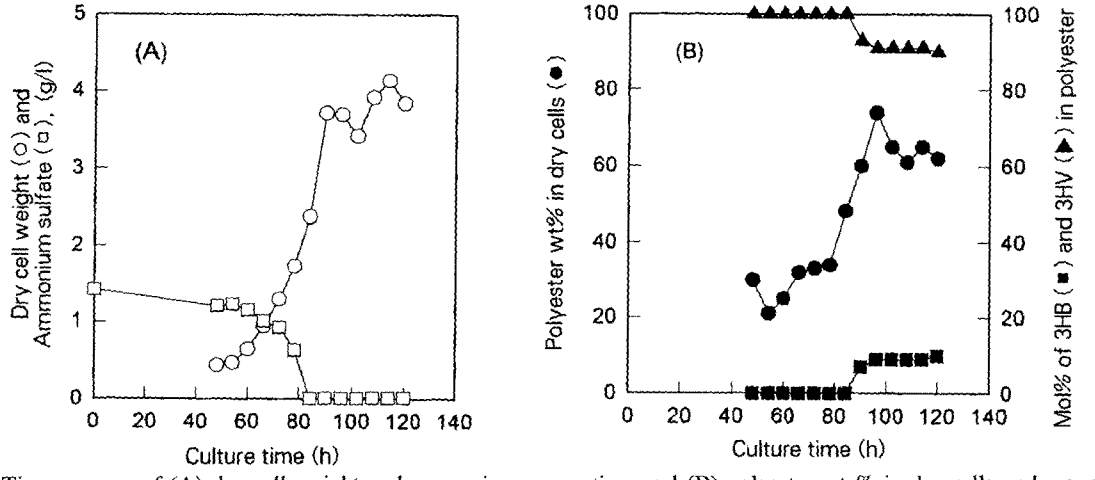

Fig. 2. Time courses of (A) dry cell weight and ammonia consumption, and (B) polyester wt % in dry cells and monomer composition in polyester.
P. denitrificans was cultivated with 10 ml/l of valeric acid and 1.5 g/l of ammonium sulfate.

time when the nitrogen was depleted. This implies that PHA synthesis is closely associated with cell growth. During the growth phase in which nitrogen is available for cell growth, only 3HV-units are synthesized while 3HB unit starts to be polymerized upon depletion of nitrogen. The nitrogen deficiency opens the pathway of D(-)-3-hydroxybutyryl-CoA synthesis from two acetyl-CoA's, but the pathway to TCA cycle is blocked. However, *C. violaceum*, which is also known to synthesize P(3HV) homopolyester from valeric acid, synthesizes P(3HV) under nitrogen starvation condition (15).

Microstructure of P(3HB-co-3HV) Synthesized from *n*-Propanol

It is usually required to feed bacteria two or more precursor carbons to synthesize bacterial copolyesters. However, in this method microstructurally heterogeneous copolyesters are generally synthesized because of their different assimilation rates (3, 9). The product is mostly a mixture of homopolymers and their comonomer-rich copolyesters. It was expected that any resulting copolyester could be a random copolyester if prepared from the cultivation using a single carbon source.

P. denitrificans synthesized P(3HB-co-3HV) copolyester from n-propanol as the single carbon source. In order to find any relation between microstructural heterogeneity of the synthesized copolyester and growth characteristics we investigated the biomass increase, PHA wt%, and

monomer composition with cultivation time (Fig. 3A and B). The biomass increased exponentially for the initial 30 h when the strain was grown on 10 ml/l of *n*-propanol and 3 g/l of ammonium sulfate. The PHA content increased up to 12 wt% of dry cell weight during the exponential growth phase. Until the initial 18 h, only 3HB-unit was detected in cells, and thereafter, 3HV-unit started to be incorporated, eventually reaching up to 20 mol%. The time course data for the change of monomer composition suggested the microstructural heterogeneity of the copolyester which was confirmed by ^{13}C-NMR dyad sequence analysis (5) described below. The D value of the copolyester, obtained by analyzing the splitted ^{13}C-NMR chemical shifts, was calculated to be 2.5 (7), where D is defined as $[F_{VV} \cdot F_{BB}]/[F_{VB} \cdot F_{BV}]$. Here, F_{VV} is defined as the fraction of -V-V- dyad sequence, and the other F's are defined similarly. Each fraction for the corresponding dyad sequence was determined from the integrated area ratios (Fig. 4). When D equals to 1 the copolymer is considered to be a random copolyester. Thus, the copolyester prepared from *n*-propanol is not a random copolyester, but probably a mixture of 3HB homopolymer, 3HV homopolymer, 3HB-rich copolymer and 3HV-rich copolymer.

Such structural heterogeneity of the copolyester from *n*-propanol was also confirmed by differential scanning calorimetry (DSC) analysis. The DSC thermogram for the sample exhibited two endothermic peaks at 117 and 150°C, associated with the melting of 3HV-rich and 3HB-rich chains, respectively. P(3HB) homopolymer from methanol melted at 175°C with the enthalpy of 84.9 J/g. And P(3HV) homopolymer from valeric acid melted at 112°C with the enthalpy of 58.2 J/g (7). The copolyester had a glass transition temperature at 0°C. The structural heterogeneity of the copolymer sample could be more clearly seen through fractionation followed by DSC and

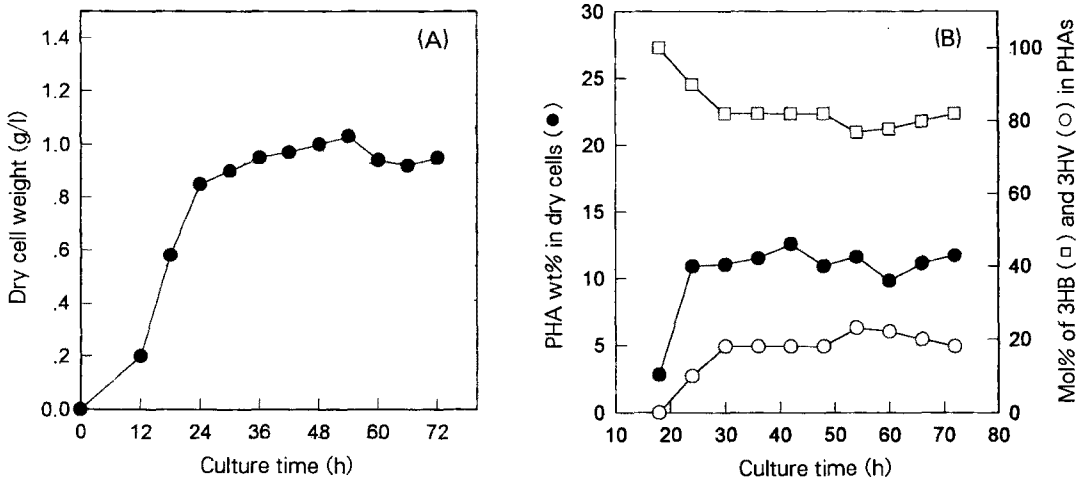

Fig. 3. Time courses of (A) dry cell weight and (B) polyester wt % in dry cells and monomer composition in polyester when *P. denitrificans* was cultivated with 10 ml/l of *n*-propanol and 3.0 g/l of ammonium sulfate.

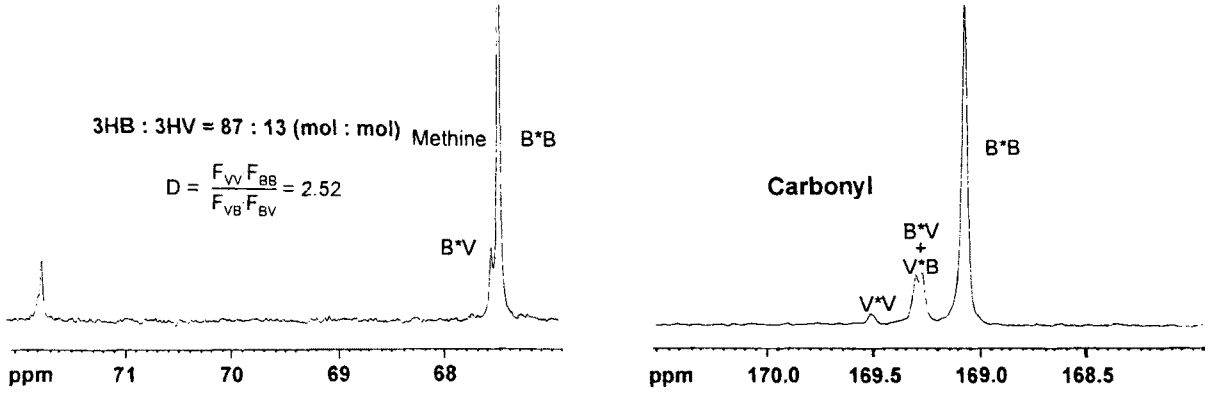

Fig. 4. Determination of the NMR parameter D from the analysis of splittings in the ^{13}C-NMR spectrum of P(3HB-co-3HV) copolymer synthesized by *P. denitrificans* from *n*-propanol.

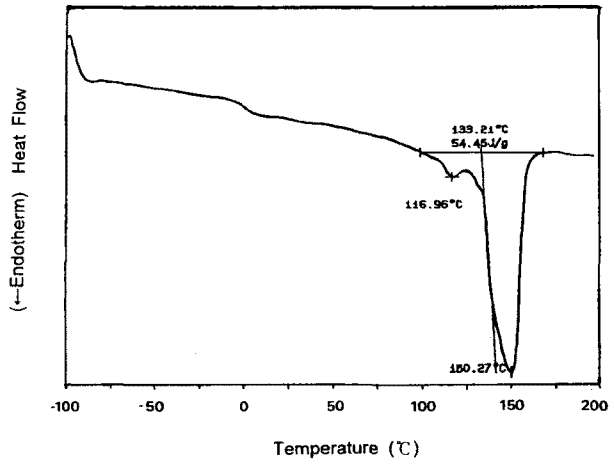

Fig. 5. Differential scanning calorimetric thermogram of P(3HB-co-3HV) copolymer synthesized by *P. denitrificans* from *n*-propanol.

X-ray diffraction (9).

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REFERENCES

1. Anderson, A. J. and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54**: 450-472.
2. Choi, M. H. and S. C. Yoon. 1994. Polyester biosynthesis characteristics of *Pseudomonas citronellolis* grown on various carbon sources, including 3-methyl-branched substrates. *Appl. Environ. Microbiol.* **60**: 3245-3254.
3. Choi, M. H., J. J. Song, and S. C. Yoon. 1995. Biosynthesis of copolyesters by *Hydrogenophaga pseudoflava* from various lactones. *Can. J. Microbiol.* **41**(Suppl. 1): 60-67.
4. Dawes, E. A. 1990. *Novel Biodegradable Microbial Polymers*, Kluwer Academic.
5. Doi, Y. 1990. *Microbial polyesters.* VCH Publisher, Inc. U.S.A.
6. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. An unusual bacterial polyester with a phenyl pendent group. *Makromol. Chem.* **191**: 1957-1965.
7. Kim, B. K. 1997. MS thesis, Gyeongsang National University, Chinju, Korea.
8. Marchessault, R. H., K. Okamura, and C. J. Su. 1970. Physical properties of poly(β-hydroxybutyrate). II. Conformational aspects in solution. *Macromolecules* **3**: 735-740.
9. Mitomo, H., N. Morishita, and Y. Doi. 1993. Composition range of crystal phase transition of isodimorphism in poly (3-hydroxybutyrate-co-3-hydroxyvalerate). *Macromolecules* **26**: 5809-5811.
10. Pearce, R. P. and R. H. Marchessault. 1994. Melting and crystallization in bacterial poly(β-hydroxyvalerate), PHV, and blends with poly(β-hydroxybutyrate-co-β-hydroxyvalerate). *Macromolecules* **27**: 3869-3874.
11. Saito, Y. and Y. Doi. 1994. Microbial synthesis and properties of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in *Comamonas acidovorans. Int. J. Biol. Macromol.* **16**: 99-104.
12. Song, J. J. and S. C. Yoon. 1994. Isolation of *Pseudomonas putida* BM01 accumulating high amount of PHA_{MCL}. *J. Microbiol. Biotechnol.* **4**: 126.
13. Song, J. J., Y. C. Shin, and S. C. Yoon. 1993. P(3HB) accumulation in *Alcaligenes eutrophus* H16(ATCC 17699) under nutrient-rich condition and its induced production from saccharides and their derivatives. *J. Microbiol. Biotechnol.* **3**: 115-122.
14. Song, J. J. and S. C. Yoon. 1996. Biosynthesis of novel aromatic copolyesters from insoluble 11-phenoxyundecanoic acid by *Pseudomonas putida* BM01. *Appl. Environ. Microbiol.* **62**: 536-544.
15. Steinbüchel, A., E.-M. Debzi, R. H. Marchessault, and A. Timm. 1993. Synthesis and production of poly(3-hydroxyvaleric acid) homopolymer by *Chromobacterium violaceum. Appl. Microbiol. Biotechnol.* **39**: 443-449.
16. Ueda, S., S. Matsumoto, A. Takagi, and T. Yamane. 1992. Synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from methanol and *n*-amyl alcohol by the methylotrophic bacteria *Paracoccus denitrificans* and *Methylobacterium extorquens. Appl. Environ. Microbiol.* **58**: 3574-3579.
17. Yamane, T., X.-F. Chen, and S. Ueda. 1996. Growth-associated production of poly(3-hydroxyvalerate) from n-pentanol by a methylotrophic bacterium, *Paracoccus denitrificans. Appl. Environ. Microbiol.* **62**: 380-384.

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