Synthesis and Characterization of Polyesters Produced by *Paracoccus* sp. 12-A from Formic Acid

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Introduction

Poly(3-hydroxybutyrate), P(3HB), is known to be an intracellular energy and carbon storage material of bacteria,[1,2] and it is also known as a biodegradable thermoplastic. In addition to one-carbon compounds (methanol[3,4] and carbon dioxide[5]), fatty acids,[4,6] sugars,[4,6] corn syrup,[7] and molasses[7] are used as carbon sources or feedstock for the production of P(3HB). *Alcaligenes eutrophus* produces P(3HB) from hydrogen and carbon dioxide, and the content of P(3HB) reaches around 80% of the dry-cell weight (DCW) when the bacterium is incubated under nitrogen-deficient conditions after it has grown to the stationary growth phase.[5] On the other hand, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), P(3HB-*co*-3HV), a family of polyesters, offers a range of thermoplastics varying in toughness and flexibility.[8] Although *A. eutrophus*, *Paracoccus denitrificans*,[9] *Rhodospirillum rubrum*,[4] and *Methylobacterium extorquens*[4,9] synthesize P(3HB-*co*-3HV) from carbon dioxide or methanol of one-carbon compounds, there has been no report about microbial synthesis of the polyester from formate.

Paracoccus sp. 12-A can assimilate formate as the sole carbon source. This strain is not a methanol-assimilating bacterium and grows poorly with methane.[10] Recently, we found that *Paracoccus* sp. 12-A accumulated P(3HB-*co*-3HV) when the cells grew on formate, and the ratio of 3-hydroxyvalerate (3HV) in P(3HB-*co*-3HV) remarkably increased when formate-grown cells of this strain were incubated with 2-hydroxyoctanoic acid (2HO) under nitrogen-deficient conditions.

Results and Discussion

Production of Polyester by Growing Cells. In electron micrographs of thin sections, polyester electron-opaque granules were contained, in various sizes, in the cells cultured in a formate-containing medium (F medium)[10] for 73 h. As shown in Figure 1, the polyester production was found to be associated with cell growth, and its yield leveled out (0.25 g/L culture broth which corresponded to 24% of DCW) at the stationary growth phase (dry cell yield: 1.03 g/L). Polyester contents, however, varied with batches, and the highest level that occurred in this study was about 60% of the DCW (0.21 g/0.35 g of dried cells) in a 1 L fermentor. The melting points of the polyester (POL-1) obtained from the cells cultured for 73 h and the standard P(3HB) were 168 and 180 °C, respectively. In P(3HB-*co*-3HV), the melting point of the polyester decreases with increasing 3HV content.[8] Therefore, this result suggests that POL-1 contains fatty acids other than 3HB. The molecular weight of POL-1 was determined to be 6.4×10^5 from HPLC analyses. In GC analysis of the fatty acid methyl esters obtained from methanolysate of POL-1, two peaks, peak 1 (R_t: 5.7 min) and peak 2 (R_t: 7.0 min), were detected. In GC−MS spectra of the methyl esters obtained, the characteristic peaks (*m*/*z* 103 [$C_4H_7O_3^+$], *m*/*z* 74 [$C_3H_6O_2^+$], *m*/*z* 71 [$C_3H_3O_2^+$], *m*/*z* 43 [C_2H_3O]) for the 3-hydroxy functional group were identified. Since the respective [M − 1]s of peak 1 and peak 2 were found at *m*/*z* 117 and *m*/*z* 131, their respective molecular weights must be 118 and 132. These R_t's and fragmentation patterns coincided with those of the standard compounds of 3HB and 3HV, respectively.[11] POL-1 gave the ^{1}H-NMR spectrum [δ (ppm): 0.89 (CH_3−, t), 1.27 (CH_3−, d), 1.62 (CH_2−, m), 2.53 (CH_2−, m), and 5.24 (CH−, m)] and ^{13}C-NMR spectrum [δ (ppm): 9.4 (C5), 19.9 (C9), 27.0 (C4), 38.9 (C2), 40.9 (C7), 67.7 (C8), 72.0 (C3), 169.2 (C1, C6)] shown in Figures 2 and 3. These chemical shifts and spin−spin couplings nearly coincided with those of the standard compound of P(3HB-*co*-3HV) containing 30 mol % 3HV, P(3HB-*co*-30 mol % 3HV).[12,13] Therefore, POL-1 was found to contain two fatty acid components, 3HB and 3HV, in the ratio 95:5 (mol %); *i.e.*, POL-1 was identified to be P(3HB-5 mol % 3HV). The mole fractions of the two monomeric units were determined from the intensive ratio of the triplet CH_3− proton resonance 5 at 0.89 ppm to the doublet CH_3− proton resonance 9 at 1.27 ppm. This is the first report showing the microbial formation of copolymer P(3HB-*co*-3HV) from formate. In the culture of *Paracoccus* sp. 12-A with formate as the sole carbon source, P(3HB-*co*-3HV) copolymer was accumulated during cell growth without intentional nitrogen-source limitation or air-supply restriction.

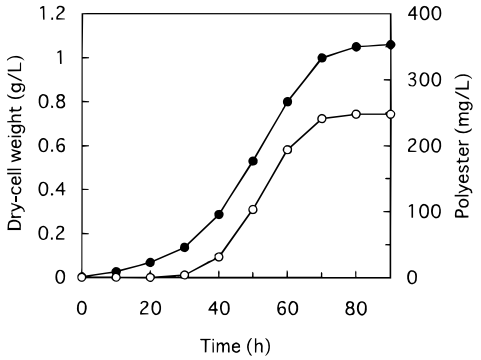

Figure 1. Growth of *Paracoccus* sp. 12-A and polyester production by it in the presence of formate. The growth was evaluated by measuring the dry-cell weight (DCW), and the polyester production was determined by the method of Braunegg *et al.*[16] Key: (●) DCW; (○) polyester production.

Production of Polyesters by Cells Grown on Formate. *Alcaligenes* sp. AK 201 produces P(3HB-*co*-47 mol % 3HV) copolymers from hydroxylated fatty acids, such as 2HO and 12-hydroxystearate, as the sole carbon source.[14] Since *Paracoccus* sp. 12-A could not assimilate 2HO, however, formate-grown cells were incubated with 2HO to produce the copolymer having a high content of 3HV units. As a result, 80.2 mg (18.4% of 437 mg of DCW) of polyester (POL-2) and 88.6 mg (21.3% of 416 mg of dry-cell weight) of polyester (POL-3) were extracted after incubation for 24 and 48 h, respectively. From the control cells, which were incubated under the same conditions without 2HO for 24

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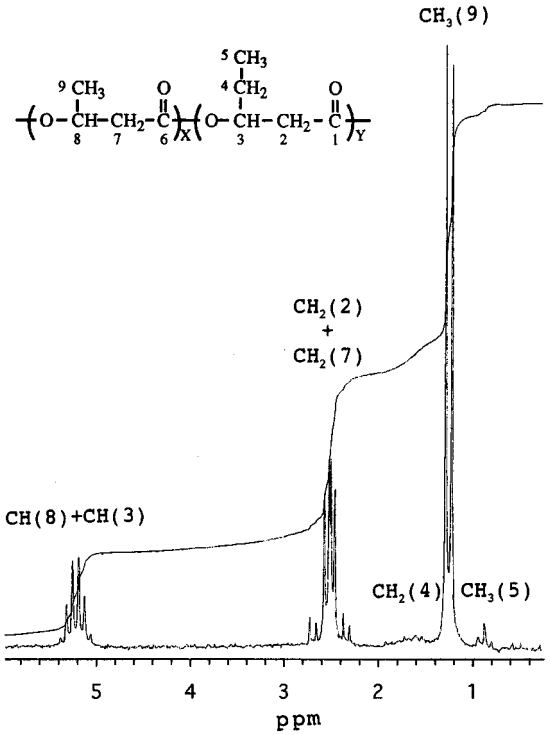

Figure 2. 100 MHz ^{1}H-NMR spectrum of polyester (POL-1) extracted from formate-grown cells of *Paracoccus* sp. 12-A.

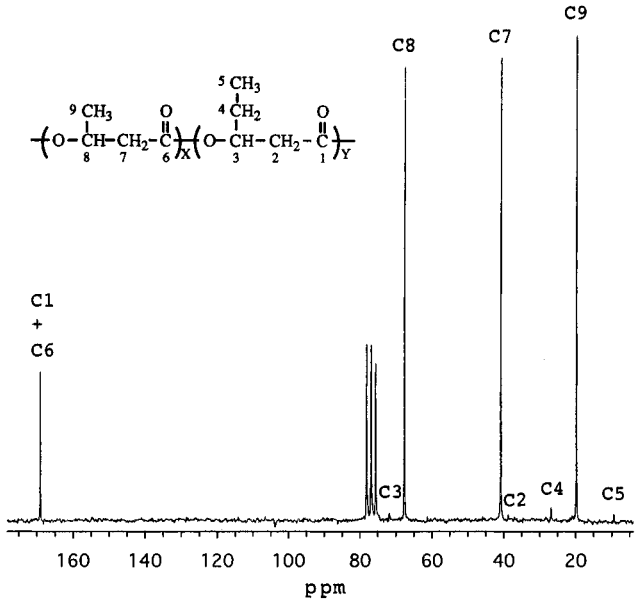

Figure 3. 100 MHz ^{13}C-NMR spectrum of polyester (POL-1) extracted from formate-grown cells of *Paracoccus* sp. 12-A.

and 48 h, 60.4 mg (14.8%) of polyester (POL-2C) and 36 mg (8.8%) of polyester (POL-3C) were obtained, respectively. The melting points of POL-2 and POL-3 were 146 and 76 °C, respectively. The molecular weights of POL-2 and POL-3 were determined to be 3.7 $\times 10^5$ and 7.4 $\times 10^5$, from the results of HPLC analyses. In the GC, GC−MS, ^{1}H-NMR, and ^{13}C-NMR analyses, the signals for POL-2 and POL-3 occurred at the same positions as those for POL-1. As a result, POL-2 and POL-3 were found to contain two fatty acid components, 3HB and 3HV, in the ratios of 80:20 and 59:41 (mol %, by ^{1}H-NMR analyses), respectively. Therefore, POL-2 and POL-3 were determined to be P(3HB-*co*-20 mol % 3HV) and P(3HB-*co*-41 mol % 3HV). On the other hand, the melting point of both POL-2C and POL-3C was 168 °C and they were identified as P(3HB-*co*-5 mol % 3HV) by the above analyses.

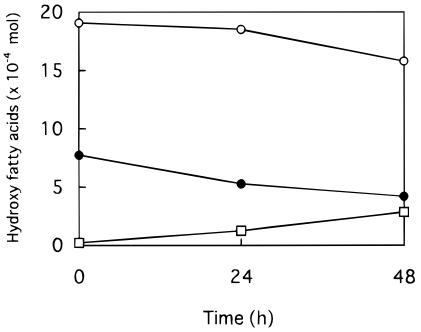

Figure 4. Effect of incubation with 2-hydroxyoctanoic acid (2HO) on 3-hydroxylated fatt acids composition of P(3HB-*co*-3HV) copolymer in *Paracoccus* sp. 12-A. After methanolysis of the copolymer, each 3-hydroxylated fatty acid methyl ester was analyzed by GC. Key: (○) 2HO; (●) 3HB; (□) 3HV.

Figure 4 shows the relationship between incubation time and each level of 2HO, 3HB, and 3HV. The decrease rate of 2HO during the incubation was related to the increase rate of 3HV, as shown in Figure 4. DCW hardly changed during the incubation with or without 2HO. Although the polyester contents of cells in the control decreased remarkably during the incubation (0 h, 22.9% of DCW; 24 h, 14.8%; 48 h, 8.7%), the contents of polyester in the presence of 2HO scarcely changed throughout the incubation. 3HB may be consumed gradually and replaced by 3HV, which seems to be formed from 2HO by α- and β-oxidation during the incubation. We have already reported that POL-3 was degraded 94% by the PHB depolymerases from *Agrobacterium* sp. K-03 at 30 °C for 72 h.[15]

In conclusion, *Paracoccus* sp. 12-A accumulated P(3HB-*co*-5 mol % 3HV) copolymer (the polyester content maximum was 60% of DCW) in the presence of formate, as the sole carbon source, during cell growth without intentional limitation of any nutrient or air-supply restriction. By incubation of the formate-grown cells with 2HO for 48 h, the 3HV content in P(3HB-*co*-3HV) copolymer increased to 41 mol %.

Experimental Section

Materials. The P(3HB) (M_w = 640k) and P(3HB-*co*-30 mol % 3HV) (M_w = 800k) used as the standard polyesters were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Formic acid (GR) purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) was used without further purification.

Polyester Production. *Paracoccus* sp. 12-A was isolated from a sewage sample as a facultative formate-utilizing bacterium. Strain 12-A was cultivated in the presence of formate as the sole source of carbon in F medium, as stated previously.[10] The F medium was composed of 0.7% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.004% $FeSO_4 \cdot 7H_2O$, 0.1% HCOONa, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.01% NaCl, and 0.1% trace element solution, at pH 7.4. The trace element solution was composed of 0.03% H_3BO_4, 0.02% $MnCl_2 \cdot 4H_2O$, 0.075% $ZnCl_2$, 0.02% $CuSO_4 \cdot 5H_2O$, 0.25% $FeCl_3 \cdot 6H_2O$, 0.01% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 0.015% $CoSO_4 \cdot 7H_2O$. Cultivation in a 7 L fermentor (Takasugi Co. Ltd., Tokyo; stirring by a four fans disk turbine, 200 rpm; 3 baffles) containing 3 L of F medium was carried out at 30 °C with feeding of formic acid to maintain the pH of the culture at around 7.2. The air flow rate was maintained at 3.0 L/min (1.0 L/L of medium/min). Another cultivation in a 1 L fermentor (Tokyo Rikakikai Co. Ltd., Tokyo; stirring only by aeration; 3 baffles) containing 600 mL of F medium was carried out under the same condition as that in the 7 L fementor except for the aeration rate (2.4 L/min, *i.e.*, 4.0 L/L of medium/min). The cells were harvested in the

stationary growth phase (3 days) by centrifugation (7000*g*) at 4 °C. After washing with sterilized distilled water, the cells were lyophilized to yield the dry cells. The polymer was extracted from the lyophilized cells in a Soxhlet extractor with chloroform, filtered through a cotton plug, and precipitated from chloroform by addition of 10 volumes of *n*-hexane. The polymer obtained was redissolved in chloroform, filtered, reprecipitated by addition of *n*-hexane, washed with acetone, and dried *in vacuo* for 20 h.

Incubation of Formate-Grown Cells with 2HO. The cells harvested in the stationary growth phase were washed with sterilized distilled water and resuspended (corresponding to 400 mg of dry cells) in 100 mL of modified F medium. The modified F medium, in which $(NH_4)_2SO_4$ as a nitrogen source was omitted and the concentration of KH_2PO_4 was changed to 0.27%, newly contained 0.43% K_2HPO_4 and 320.4 mg of 2HO, instead of formate, as the sole carbon source. The suspension was incubated on a rotary shaker (160 rpm) at 30 °C for 24 or 48 h. After the incubation, cells were harvested, washed with distilled water, and lyophilized. The polymer was extracted with chloroform in the same manner as mentioned above. The remaining 2HO in the incubation mixture was extracted with diethyl ether, esterified by diazomethane, dissolved with chloroform, and quantified by GC.

Partial Methanolysis. After 6.0 mg of each sample was dissolved in 2.0 mL of chloroform, 2.0 mL of 3% sulfuric acid in methanol was added. The mixture was heated at 100 °C for 3.5 h in capped tubes.[16] After cooling to room temperature, 1.0 mL of distilled water was added to the mixture, followed by vigorous shaking. The lower layer was dried over anhydrous sodium sulfate and used as a sample for GC and GC−MS analyses.

Measurements. The melting points of the polyester samples were measured on a Shimadzu MM-2 micro melting point determination apparatus at a heating rate of 2 °C/min. The molecular weights were determined by gel permeation chromatography (GPC) with a Tosoh Model HLC-8020 high-speed GPC apparatus, a Model SC-8020 super system controller, and a Model RI-8012 refractive index detector with a TSKgel GMH_{HR}-H column. Chloroform was used as the eluent at 40 °C at a flow rate of 1.0 mL/min. A sample concentration of 0.5 mg/mL and an injection volume of 200 μL were used. A calibration curve was generated with 13 polystyrene standards of low dispersity (Tosoh, Tokyo). GC was carried out with a Hitachi 163 gas chromatograph with a column packed with 15% DEGS (3 mm × 1.5 m) at 130−150 °C (2 °C/min). N_2 was used as a carrier gas (40 mL/min). The GC−MS spectra were measured on a Hitachi M-80B double-focus mass spectrometer (EI; ion source temperature, 180 °C; interface temperature, 220 °C) with a Hewlett Packard 5792A gas chromatograph (column, DB-WAX 0.25 mm × 60 m, 100−240 °C, 2 °C/min; carrier gas, He, 1.0 mL/min). The 100 MHz ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 25 °C in $CDCl_3$ on a JEOL JNM FX 100 instrument.

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References and Notes

(1) Anderson, A. J.; Dawes, E. A. *Microbiol. Rev.* **1990**, *54*, 450.
(2) Doi, Y. *Microbial polyesters*; VCH: New York, 1990.
(3) Yamane, T. *FEMS Microbiol. Rev.* **1992**, *103*, 257.
(4) Yamane, T. *Biotechnol. Bioeng.* **1993**, *41*, 165.
(5) Heinzle, E.; Lafferty, R. M. *Eur. J. Appl. Microbiol. Biotechnol.* **1980**, *11*, 8.
(6) Koizumi, F.; Abe, H.; Doi, Y. *J. Macromol. Sci. Pure Appl. Chem.* **1995**, *A32*, 759.
(7) Page, W. J. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 329.
(8) Marchessault, R. H.; Bluhm, T. L.; Deslandes, Y.; Hamer, G. K.; Orts, W. J.; Sundararajan, P. R.; Taylor, M. G.; Bloembergen, S.; Holden, D. A. *Makromol. Chem., Macromol. Symp.* **1988**, *19*, 235.
(9) Ueda, S.; Matsumoto, S.; Takagi, A.; Yamane, T. *Appl. Environ. Microbiol.* **1992**, *58*, 3574.
(10) Iida, M.; Kitamura-Kimura, K.; Maeda, H.; Mineki, S. *Biosci. Biotechnol. Biochem.* **1992**, *56*, 1966.
(11) Slater, S.; Gallaher, T.; Dennis, D. *Appl. Environ. Microbiol.* **1992**, *58*, 1089.
(12) Doi, Y.; Kunioka, M.; Nakamura, Y.; Soga, K. *Macromolecules* **1986**, *19*, 2860.
(13) Bloembergen, S.; Holden, D. A.; Hamer, G. K.; Bluhm, T. L.; Marchessault, R. H. *Macromolecules* **1986**, *19*, 2865.
(14) Akiyama, M.; Doi, Y. *Biotechnol. Lett.* **1993**, *15*, 163.
(15) Nojima, S.; Mineki, S.; Iida, M. *J. Ferment. Bioeng.* **1996**, *81*, 72.
(16) Braunegg, G.; Sonnleitner, B.; Lafferty, R. M. *Eur. J. Appl. Microbiol. Biotechnol.* **1978**, *6*, 29.

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