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P(3HB) Accumulation in *Alcaligenes eutrophus* H16 (ATCC 17699) under Nutrient-Rich Condition and Its Induced Production from Saccharides and Their Derivatives

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Poly(3-hydroxybutyrate)(P(3HB)) accumulation under nutrient-rich condition with various amounts of $(NH_4)_2SO_4$ was systematically investigated. The results of the electron-microscopy and the solvent extraction showed that the P(3HB) accumulation is unavoidable even under nutrient-rich condition. This indicates that in a two-step culture of *Alcaligenes eutrophus* H16, the researches should be careful in interpreting the data of polyhydroxyalkanoates(PHAs) accumulation in terms of the carbon-source fed in the second step because the two-step culture product contains the P(3HB) produced under nutrient-rich condition. The polyester production capability in a two-step batch culture of *A. eutrophus* H16(ATCC 17699) was also investigated using various saccharides and their derivatives such as glucose, fructose, gluconic acid, glucaric acid, sorbitol, lactose, galactose, and mannose. The polyesters synthesized were characterized by 500 MHz ^{1}H-NMR spectroscopy, intrinsic viscosity[η] measurement in chloroform and differential scanning calorimetry(DSC). 500 MHz ^{1}H-NMR analysis showed that all polyesters synthesized generally contained 1~2 mol% of 3HV. Another finding is that the glucose utilization can be increased by changing the autoclaving procedure of the substrate to enhance the P(3HB) production yield up to 46 wt% of P(3HB) in dry cells.

A wide variety of bacteria make and store lipophilic inclusion bodies mainly composed of poly(3-hydroxybutyrate)(P(3HB)) or other types of polyesters(polyhydroxyalkanoates; PHAs) (2, 7, 8, 17). These microbial polyesters have attracted much attention as environmentally degradable thermoplastics which can be used for a wide range of possible applications. It also has various potential medical applications such as for the uses as surgical sutures, biodegradable matrices for controlled drug release and *etc.* Many authors have been trying to find new biodegradable polyesters by changing the bacterial strain, carbon-source and culture condition (4-8, 14, 16, 18, 23).

The P(3HB) or PHAs production of *A. eutrophus* H16 has been studied extensively by Doi *et al.* using various organic carbon-sources (7, 8). A two-step culture technique was often employed in their study; the first step was to obtain the maximum amount of cells and the second step to induce the accumulation of various types of polyesters from various organic carbon sources under nitrogen limitation condition. In order to see what type of polyester derives from the carbon-source fed in the second step, first of all, the P(3HB)-free cells have to be recovered after they were grown under nutrient-rich condition.

It is well known that most bacteria accumulate P(3HB) even when they are not limited by factors such as nitrogen or phosphate, albeit at a lesser rate (2, 13, 22). Thus, the first-step culture biomass may contain some P(3HB) homopolyester before transferring to the second accumulation stage. Doi *et al.*, on the contrary, reported that in *A. eutrophus* H16, P(3HB) accumulation was not observed at the first-step with their nutrient-rich medium (9, 11, 12, 15). They assumed that most polyesters in cells may be derived from the carbon sources fed at the second-step cultivation. In this paper, we report our systematic experimental result that the P(3HB) accumulation of *A. eutrophus* H16(ATCC 17699) is unavoidable under nutrient-rich condition even in the presence of

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$(NH_4)_2SO_4$. We, also, report the result of the PHAs synthesis of *A. eutrophus* H16(ATCC 17699) from various saccharides and their derivatives such as fructose, glucose, gluconic acid, glucaric acid, sorbitol, lactose, galactose, and mannose.

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. Gluconic and glucaric acids were purchased from Sigma Chemical Co. and used as received. Glucose, fructose, sorbitol, lactose, mannose, $(NH_4)_2SO_4$, 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 $(NH_4)_2SO_4$ 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 polyesters 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 KH_2PO_4, 7.3 g of $Na_2HPO_4 \cdot 12H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $Fe(NH_4)_2$-citrate or $FeCl_3 \cdot 12H_2O$, 0.5 g of $NaHCO_3$, 0.2 g of $CaCl_2 \cdot 2H_2O$ 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 $ZnSO_4 \cdot 7H_2O$, 1 g of $MnCl_2 \cdot 4H_2O$, 0.3 g of H_3BO_3, 0.8 g of $CoSO_4 \cdot 7H_2O$, 0.04 g of $CuCl_2 \cdot 2H_2O$, 0.02 g of $NiCl_2 \cdot 6H_2O$ and 0.03 g of $NaMoO_4 \cdot 2H_2O$ 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 polyester 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.

Electron Microscopy

A 10 ml of culture broth was centrifuged and washed with distilled water. The washed cells were fixed doubly with 2.5% glutaraldehyde and 1% osmium tetraoxide (OsO_4) (19) and the fixed cells were washed with a phosphate buffer (pH 7.3). The cells were dehydrated in a graded series of ethanol and acetone, and embedded in a epon resin (19). Ultrathin sectioning was performed with a LKB-Ultratome with a diamond knife. Sections were 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.

Polyesters Extraction and Purification

Dried cells were weighed and transferred onto an extraction thimble filter (28×100 mm, Advantec; Toyo Roshi 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.

Determination of Remaining Glucose and Biomass Increase with Time

To measure the change in biomass during cell cultivation, 10 ml of culture broth was centrifuged at 6,000 rpm for 10 mins, suspended in 10 ml of distilled water and recentrifuged. The pellet was then transferred onto a preweighed aluminum dish and dried to a constant mass under vacuum at 100°C. For measuring the remaining glucose in a culture solution, 3,5-dinitrosalicylic acid (DNS) method was used (21).

Determination of P(3HB) Accumulation with Time

About 10 mg of dried cells was sampled from the culture under consideration every 4 hrs and transferred onto a test tube with teflon-lined screw cap(Pyrex). A 5 ml of chloroform was added to this test tube to extract P(3HB) from the dried cells. It was placed in a temperature-controlled water bath(EYELA; SB-9 bath) at 50°C for 12 hrs and cooled to room temperature. 50~100 μl aliquots of the solvent extract were transferred onto

another test tube and evaporated to dryness at 60°C. Then, 5 m*l* of concentrated sulfuric acid was added to the test tube containing the dried P(3HB), and the solution was heated in a boiling-water bath for 10 mins. Finally, the solution was cooled to room temperature and shaken thoroughly. The absorbance of the solution was measured at 235 nm (Abs_{235}) with concentrated sulfuric acid as a blank.

Five standards containing 20, 40, 60, 80 and 100 μg of P(3HB), respectively were prepared in the same manner as those in the above sample solution. The obtained standard curve shows a linear relation between the absorbance and the P(3HB) weight within the range of 0~80 μg of P(3HB). Compared to the absorbance on the standard curve, the amount of P(3HB) in the cells was determined.

Nuclear Magnetic Resonance(NMR) Spectroscopy

The ^{1}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}H-NMR spectra were recorded at 25°C using $CDCl_3$ solutions of the polyester(5 mg/m*l*) 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(Tm) was measured by using a DuPont 9900 differential scanning calorimeter (DSC V2.2 A) 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 the relation between molecular weight and intrinsic viscosity. The intrinsic viscosities of various PHB 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 (20):

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

k; 7.7×10^{-5} ($cm^3 g^{-1}$) (in chloroform)
a; 0.82

RESULTS AND DISCUSSION

Polyesters Accumulation under Nutrient-Rich Condition

Table 1. Effect of ammonium sulfate on P(3HB) accumulation of *A. eutrophus* H16 under nutrient-rich condition[a]

batch No.	amount of ammonium sulfate (g/*l*)	culture time (hr)	wt. of dry cells(g/*l*)	wt% of PHB in dry cells	average wt% of PHB for 22hrs culture
1	0	18	2.4	15.0	
1	0	22	3.0	16.4	
2	0	22	3.8	17.0	16.7
3	2.5	22	2.9	12.2	12.2
4	5	18	3.0	5.0	
4	5	22	3.2	9.2	
5	5	22	3.3	9.0	
6	5	22	3.4	7.0	
7	5	22	3.4	8.7	
8	5	22	3.3	8.4	8.5
9	7.5	22	3.3	8.0	8.0
10	10	22	3.0	1.7	
11	10	22	3.0	5.0	
12	10	22	2.8	3.2	
13	10	22	2.9	2.8	
14	10	22	3.2	4.3	3.4
15	12.5[b]	64	3.0	5.9	5.9
16[c]	5	22	3.1	16.8	
17[c]	5	22	3.8	20.0	18.4

[a] Culture medium contained 10 g of yeast extract, 15 g of nutrient broth and the corresponding amount of ammonium sulfate perliter, [b] No cell growth occurred in 24 hrs, [c] Culture medium contained 10 g of yeast extract, 10 g of polypeptone, 5 g of beef extract perliter (the medium composition proposed by Doi *et al.* to harvest polyester-free cells).

In the two-step cultivation for polyester synthesis, it is necessary to harvest healthy and polyester-free cells to investigate what type of polyester derives from the carbon-source fed in the second induction stage. As shown in Table 1 and Fig. 1, when the cells were cultivated in the nutrient-rich medium containing additional $(NH_4)_2SO_4$(see the footnote of Table 1 for the composition), increasing ammonium sulfate content decreased the P(3HB) production yield while the dried total biomass weights remained relatively constant. However, when high $(NH_4)_2SO_4$ concentration(10 g/L) was used, a lot of lysed cells were seen in the second-step medium. Furthermore, our ATCC(17699) strain produced 18 wt% of P(3HB) when we used the culture medium which had the same composition as in Doi *et al*'s experiment (Table 1). Therefore, 5 g of $(NH_4)_2SO_4$ was added to the first step exponential culture medium of 1 liter to minimize the P(3HB) production in a nutrient-rich medium. The cells harvested under this condition contained 8.5 wt% of P(3HB) in dry cells (Table 1). Fig. 2(a) shows the electron micrographs of P(3HB) granules in *A. eutrophus* H16 which was cultivated in a nutrient-rich medium for 22 hrs. A stronger evidence of the P(3HB) production at the early stage of growth in a nutrient-rich cultivation is the appearance of P(3HB) granules in the cell division phase (Fig. 2(b)). These results clearly demonstrate that in *A. eutrophus* H16 the P(3HB) accumulates during cell growth even under well balanced nutrient-rich condition, and can be stimulated under unbalanced conditions such as a high C:N ratio and a nitrogen deficiency (2, 17).

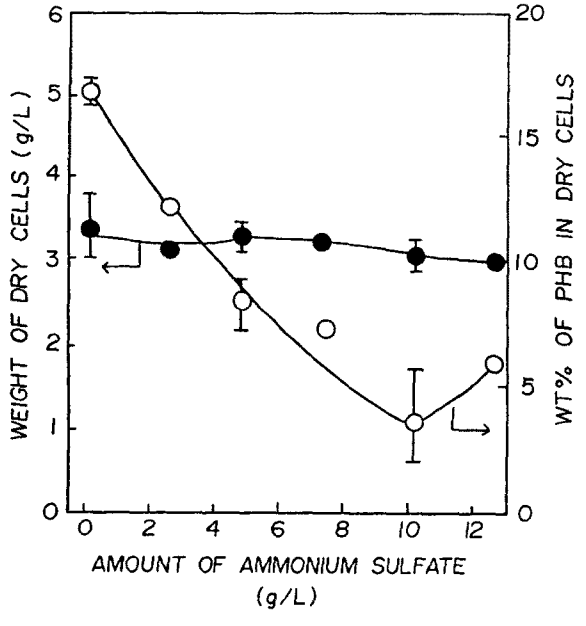

Fig. 1. Effect of ammonium sulfate on production of total biomass (●) and P(3HB) (○) by *A. eutrophus* H16 under nutrient-rich condition.

Electron Microscopy for Polyester Granules in Cells

Fig. 3a and 3b show the electron micrographs of P(3HB) granules in *A. eutrophus* H16 which was grown on crotonic acid and gluconic acid, respectively, under P(3HB) production condition. The size of individual granules in the cells was estimated to be in the range of 300 to 500 nm in diameter and they appeared to occupy more than 80% of the cell volume. They are generally spherical in shape. The 6 hrs of extraction was

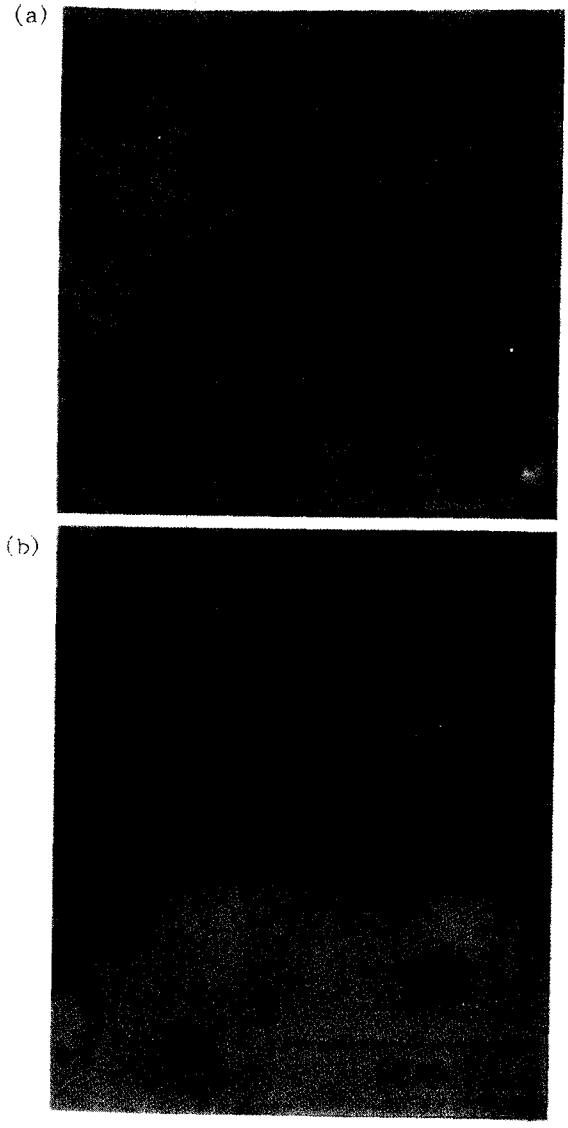

Fig. 2. Electron micrographs of P(3HB) granules in *A. eutrophus* H16; (a) cultured in a nutrient-rich medium; (b) during cell division.

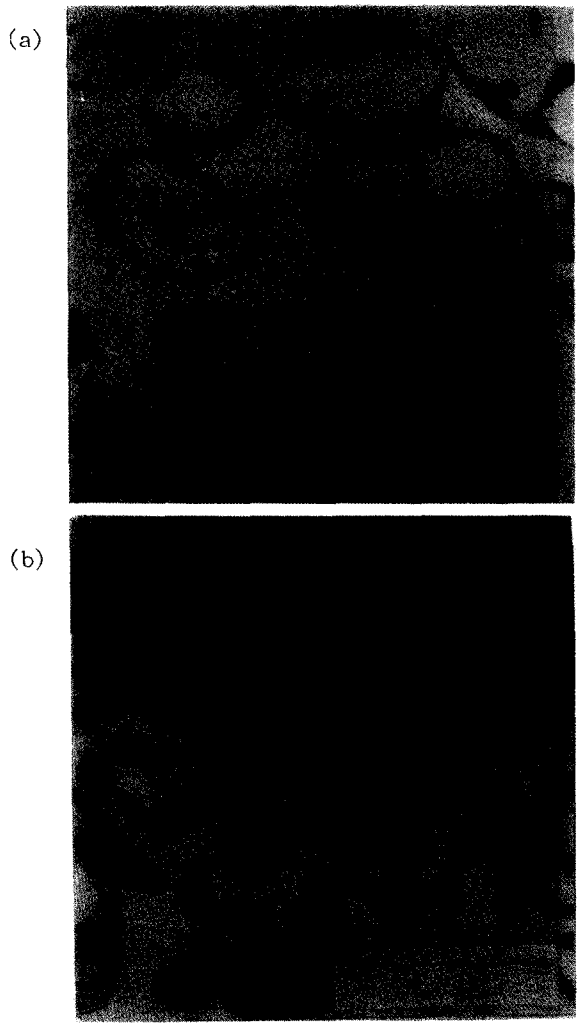

Fig. 3. Electron micrographs of P(3HB) granules in *A. eutrophus* H16 cultured on (a)crotonate and (b)gluconate under P(3HB) accumulation condition.

long enough to extract 98% or more of PHAs from the cells. The solvent extraction of the cells in Fig. 3a revealed the existence of 61 wt% of P(3HB) in them, rather lower than expected from the electron microscopic data. Recent NMR (1, 3) and X-ray diffraction (1) studies showed that the P(3HB) granules in vivo are in a mobile state and amorphous in which water acts as a plasticizer for the polymer.

NMR Spectroscopy for Polyesters Produced under Nutrient-Rich Condition

The 500 MHz ^{1}H-NMR spectrum of P(3HB) synthesized in a nutrient-rich medium is shown in Fig. 4. The doublet resonance at 1.28 ppm is assignable to methyl protons[HB(4)], and the methine proton[HB(3)] attached to the asymmetric carbon is a sextet centered at 5.26 ppm. The methylene [HB(2)] multiplet centered

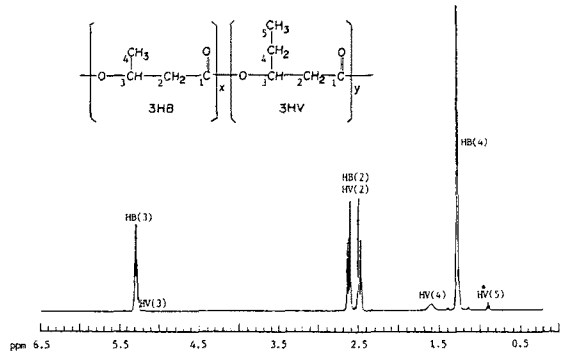

Fig. 4. 500 MHz ^{1}H-NMR spectrum of polyester produced under nutrient-rich condition by *A. eutrophus* H16.

at 2.55 ppm consists of eight peaks due to the ABX type coupling of two magetically inequivalent hydrogens (10). The ratio of integrated areas was in good agreement with the number of each type of proton in 3HB within ± 1% deviation. It was 3.0 (CH_3) : 2.0 (CH_2) : 1.0 (CH). As shown in Fig. 4, beside the three resonances from P(3HB), two minor additional resonances can be seen. These are the resonances of methylene protons [HV(4)] and methyl protons [HV(5)] in HV unit and each chemical shift value of these protons are 1.63 and 0.90 ppm, respectively (9). The resonance of methylene protons [HV(2)] overlaps the methylene resonance in 3HB unit. Thus, the monomer composition of copolyesters can be determined by comparing the areas of the peaks representing each constituent monomer. The methylene proton resonance [HV(4)] peak in the HV unit was not used for the quantitation of monomer composition since it overlapped the proton resonance (~1.6 ppm) of water which probably existed in deutrated solvent as impurity (Fig. 4). The spectrum shows that the polyester accumulated under nutrient-rich condition also contains a minor amount of 3HV unit (less than 1 mol%).

Polyesters Accumulation on Saccharides and Their Derivatives

Table 2 shows the polyesters synthesis data for the carbon sources of several saccharides and glucose derivatives such as sorbitol, gluconic acid, and glucaric acid. All polyester products from unrelated carbon sources contained 2 mol% or less of 3HV monomer unit. The polyesters accumulation time was allowed for 48 hrs long enough to reach the steady state of accumulation, which is suitable to common natural substrates. The steady-state accumulation time will be discussed in the next paragraph. It was reported that most saccharides can not be utilized by *A. eutrophus* H16 (7). Fructose is the only utilizable sugar (7), which is confirmed by the polyester production yield data in Table 2. As shown

Table 2. Biosynthesis of PHAs by *A. eutrophus* H16 from saccharides and their derivatives at 30°C.

carbon source (g/l culture medium)		wt. of dry cells (g/l)	wt. of PHAs (g/l)	wt% of PHAs in dry cells	PHAs composition mol%[a]		physical properties of PHAs		
					3HB	3HV	Tm (°C)[b]	[η][c]	Mv×10^{-5}[d]
fructose	20	9.5	5.3	56.0	99	1			
glucose	20	5.7	0.44	7.7	99	1	171	5.04	7.4
gluconic acid	10	6.2	2.84	45.8	98	2	172	2.27	2.8
sorbitol	10	3.1	0.08	2.6	99	1	167	2.16	2.7
glucaric acid	10	4.7	1.30	27.9	99	1	167	2.16	2.7
L-arabinose	10	2.7	0.20	6.1	99	1			
lactose	10	2.6		<1.0					
galactose	10	2.8		<1.0					
mannose	10	2.3	0.023	1.0					

[a] Calculated from NMR data, [b] Peak temperature from DSC data, [c] Intrinsic viscosity, [d] Viscosity molecular weight.

in Table 2, the polyester production yields from glucose and sorbitol were less than 10wt% [(wt. of polyester/wt. of dry cells)×100] whereas those from gluconic acid and glucaric acid were about 46 and 28 wt%, respectively. This indicates that the incorporation of carboxyl group into the glucose molecule may generally enhance the accumulation of polyesters. Compared to the average 8.5 wt% of residual P(3HB) accumulated under nutrient-rich condition, the bacterium accumulated a negligible amount of P(3HB) from glucose during the second induction period. This is reasonable because of the incapability (7) of glucose utilization for growth by *A. eutrophus* H16. This was also substantiated in the time course study for the glucose consumption, O.D., biomass increases, aand P(3HB) production yield (Fig. 5). Only 1g of glucose was consumed during the second step cultivation. However, Doi *et al.* (8) reported 21 wt% accumulation on glucose.

In addition, when 6 g of glucose and 4 g of gluconate in 500 m*l* were used as cosubstrates, the amount of glucose consumption(~1 g) was comparable to the case in which glucose was an only carbon source. The increasing rates of the biomass, the O.D. value and the P(3HB) production yield were very similar for initial 28 hrs. Finally they all reached the stationary phase (Fig. 6). This indicates that the P(3HB) production yield data obtained after 48 hrs of cultivation are steady-state results. This also shows that the biomass increase from 1.7 to 3.8 g/500 m*l* with time is almost totally due to the P(3HB) accumulation(net P(3HB) weight increase of ~1.2 g). It can be seen from Fig. 6 that the P(3HB) accumulation also affects the O.D. (at 660 nm) value.

For other carbon sources of sorbitol, arabinose, lactose, galactose, and mannose, the polyester contents in cells were even lower than the control value of 8.5 wt% obtained under nutrient-rich condition. For the carbon sources showing such a lower or similar level of polyester content

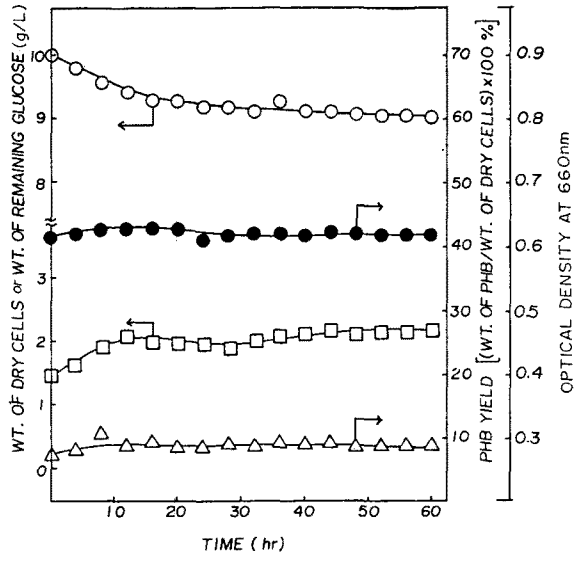

Fig. 5. Time courses of biomass (□), remaining glucose (○), O.D. at 660 nm (●) and P(3HB) production yield (△) during the second step culture of *A. eutrophus* H16 in the nitrogen-free medium containing 20 g of glucose per liter.

to the control value during the accumulation period, it can be surmised that when using these substrates, a concomitant synthesis and degradation of polyesters in cells would occur (8).

Enhancement of Glucose Utilization for P(3HB) Production

It is well known that *A. eutrophus* H16 does not grow on glucose (7). However, it is interesting to note a significant difference of P(3HB) production yield between two autoclaving procedures; the separate autoclaving of glucose and buffer medium and the at once-autoclaving of glucose-in-buffer (Table 3). The simultaneous autoclaving of glucose-in-buffer increased the P(3HB) produc-

Table 3. Enhancement of P(3HB) production owing to changing autoclaving procedure[a].

autoclaving procedure	amount of glucose (g/l)	wt%. of dry cells (g/l)	wt. of P(3HB) (g/l)	wt% of P(3HB) in dry cells
separate	20	5.7	0.44	7.7
autoclaving	20	4.3	0.44	10.1
of glucose	20	4.8	0.44	9.3
and buffer	20	3.9	0.26	6.4
	20	5.4	0.47	8.9
			average	(8.5)
at once-	20	6.5	3.0	46.3
autoclaving	20	7.6	3.2	42.5
of glucose-in-buffer	20	7.4	2.6	34.8
			average	(41.2)

[a] No additional $(NH_4)_2SO_4$ was added in the first step culture under nutrient-rich condition.

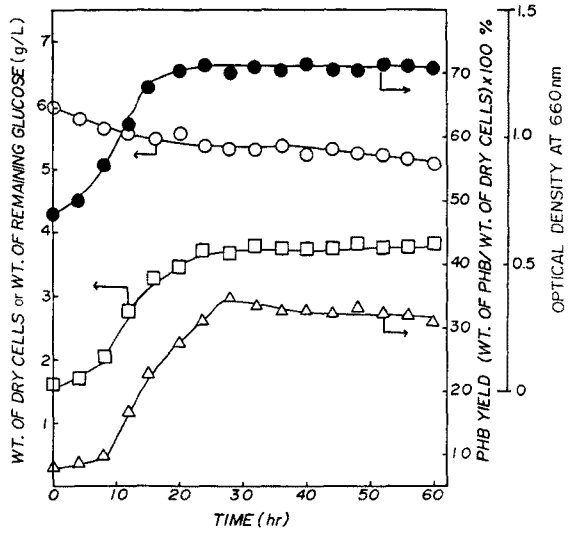

Fig. 6. Time courses of biomass (□), remaining glucose (○), O.D. at 660 nm (●) and P(3HB) production yield (△) during the second step culture of *A. eutrophus* H16 in the nitrogen-free medium containing 12 g of glucose and 8 g of gluconate per liter.

tion yield up to 46 wt%, very high in comparison with the 8 wt% obtained when glucose and buffer were autoclaved separately as usual. It is uncertain what happened to cause such a significant increase in P(3HB) production. Further study on this accidental finding is presently under way.

Acknowledgments

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REFERENCES

1. **Amor, S.R., T. Rayment and J.K.M. Sanders.** 1991. Poly (hydroxybutyrate) *in vivo*: NMR and X-ray characterization of the elastomeric state. *Macromolecules.* **24**: 4583-4588.
2. **Anderson, A.J. and E.A. Dawes.** 1990. Occurrence, metabolism, metabolic role and industrial uses of bacterial polyhydroxyalkanoates. *Microbiol. Rev.* **54**: 450-472.
3. **Barnard, G.N. and J.K.M. Sanders.** 1989. The poly-β-hydroxybutyrate granule *in vivo*. *J. Biol. Chem.* **264**: 3286-3291.
4. **Brandl, H., R.A. Gross, R.W. Lenz and R.C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Appl. Environ. Microbiol.* **54**: 1977-1982.
5. **Choi, M.H., J.J. Song and S.C. Yoon.** "in press". Biodegradable polyesters biosynthesis by *Pseudomonas citronellolis* (ATCC 13674) using various carbon sources. *Int. J. Biol. Macromol.*
6. **Choi, M.H. and S.C. Yoon.** "in press". A novel copolyester with alkenyl and methyl-branched R-groups from *Pseudomonas citronellolis* (ATCC 13674). *Int. J. Biol. Macromol.*
7. **Dawes, E.A.** 1990. *Novel Biodegradable Microbial Polymers*, Kluwer Academic.
8. **Doi, Y.** 1990. *Microbial polyesters.* VCH Publisher, Inc. U.S.A.
9. **Doi, Y., M. Kunioka, Y. Nakamura and K. Soga.** 1986. Nuclear magnetic resonance studies on poly(β-hydroxybutyrate) and a copolyester of β-hydroxyvalerate isolated from *Alcaligenes eutrophus* H16. *Macromolecules,* **19**: 2860-2864.
10. **Doi, Y., M. Kunioka, Y. Nakamura and K. Soga.** 1986. ^{1}H and ^{13}C NMR analysis of poly(β-hydroxybutyrate) isolated from *Bacillus megaterium*. *Macromolecules.* **19**: 1274-1276.
11. **Doi, Y., A. Tamaki, M. Kunioka and K. Soga.** 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hyd-

roxyvalerate, and 5-hydroxyvalerate in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. *Makromol. Chem., Rapid Commun.* **8**: 631-635.

12. **Doi, Y., A. Tamaki, M. Kunioka and K. Soga.** 1988. Production of copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate by *Alcaligenes eutrophus* from butyric and pentanoic acids. *Appl. Microbiol. Biotechnol.* **28**: 330-334.
13. **Hughes, L.Y. and K.R. Richardson.** 1984. Fermentation process for the production of poly(β-hydroxybutyric acid). US Patent No. 4,433,053.
14. **Huisman, G.W., O. Leeuw, G. Eggink and B. Witholt.** 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent Pseudomonads. *Appl. Environ. Microbiol.* **55**: 1949-1954.
15. **Kawaguchi, Y. and Y. Doi.** 1992. Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. *Macromolecules.* **25**: 2324-2329.
16. **Kim, Y.B., R. Lenz and R.C. Fuller.** 1991. Preparation and characterization of poly(β-hydroxyalkanoates) obtained from *Pseudomonas oleovorans* grown with mixtures of 5-phenylvaleric acid and n-alkanoic acids. *Macromolecules,* **24**: 5256-5260.
17. **Lafferty, R.M., B. Korsatko and W. Korsatko.** 1988. Microbial production of poly-β-hydroxybutyric acid. in Biotechnology, A comprehensive treatise, ed. by H.J. Rehm and G. Reed, Vol. **66**: 137-166.
18. **Lageveen, R.G., G.W. Huisman, H. Preusting, P. Ketelaar, G. Eggink and B. Witholt.** 1988. Formation of polyesters by *Pseudomonas oleovorans*: Effect of substrates on formation and composition of poly-(R)-3-hydroxyalkanoates and poly-(R)-3-hydroxyalkenoates. *Appl. Environ. Microbiol.* **54**: 2924-2932.
19. **Mauruschat, A.W., M. Aragno, F. Mayer and H.G. Schlegel.** 1977. Micromorphology of Gram-negative hydrogen bacteria. II. Cell envelope, membranes, and cytoplasmic inclusions. *Arch. Microbiol.* **114**: 101-110.
20. **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.
21. **Miller, G.L.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chem.* **31**: 426-428.
22. **Ramsay, B.A., K. Lomaliza, C. Chavarie, B. Dub , P. Bataille and J.A. Ramsay.** 1990. Production of poly-(β-hydroxybutyric-co-β- hydroxyvaleric) acids. *Appl. Environ. Microbiol.* **56**: 2093-2098.
23. **Steinb chel, A. and S. Wiese.** 1992. A Pseudomonas strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. *Appl. Microbiol. Biotechnol.* **37**: 691-697.

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