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MOLECULAR WEIGHT OF POLY(3-HYDROXYBUTYRATE) DURING BIOLOGICAL POLYMERIZATION IN *ALCALIGENES EUTROPHUS*

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ABSTRACT

Poly(3-hydroxybutyrate) [P(3HB)] was produced by *Alcaligenes eutrophus* from fructose or butyric acid in nitrogen-free media at different pHs and temperatures, and the time-dependent changes in the molecular weight of P(3HB) were studied. P(3HB) polymers were accumulated within cells in the presence of a carbon source, and the rate of P(3HB) accumulation increased with temperature in the 16–35°C range but was almost independent of the pH value (5.0–8.0) of the media. The apparent activation energy for the rate of P(3HB) accumulation was 30 kJ/mol. The number-average molecular weight ($\overline{M}_n$) of P(3HB) polymers increased rapidly with time during the course of P(3HB) accumulation to reach a maximum value ($(10 \pm 2) \times 10^5$) at around 6 hours of incubation, followed by a gradual decrease in $\overline{M}_n$ with time. The polydispersities ($\overline{M}_w/\overline{M}_n$) of P(3HB) polymers increased from 1.5 to 2.0 during P(3HB) accumulation. The time-dependent changes in $\overline{M}_n$ have been accounted for in terms of the kinetic model in which a chain transfer agent is formed during the course of P(3HB) synthesis and reacts with a growing chain of P(3HB) on the active site of polymerase to regulate the length of P(3HB) chain.

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INTRODUCTION

A wide variety of bacteria synthesize an optically active polymer of (*R*)-3-hydroxybutyric acid (3HB) as an intracellular storage polymer, and P(3HB) is accumulated as granules within the cytoplasm [1–4]. *Alcaligenes eutrophus* used in this study accumulates P(3HB) in the cells in amounts up to 70% of the dry weight, when growth is limited by the depletion of an essential nutrient but the cells have an excess of carbon source [5]. The structure of native P(3HB) granules in *A. eutrophus* cells was characterized by means of ^{13}C-NMR spectroscopy [6] and x-ray diffraction [7], which demonstrated that the P(3HB) granules were completely amorphous. The P(3HB) polymer can be extracted from bacterial cells with a suitable organic solvent such as chloroform or methylene chloride. The isolated P(3HB) is a partially crystalline thermoplastic with biodegradable and biocompatible properties [3].

The pathway and regulation of P(3HB) synthesis have been studied extensively in *A. eutrophus* [8–11]. P(3HB) is synthesized from acetyl-coenzyme A (CoA) by a sequence of three enzymatic reactions. 3-Ketothiolase catalyzes the reversible condensation reaction of two acetyl-CoA molecules into acetoacetyl-CoA, the intermediate is reduced to (*R*)-3-hydroxybutyryl-CoA by NADPH-linked acetoacetyl-CoA reductase, and P(3HB) is then produced by the polymerization of (*R*)-3-hydroxybutyryl-CoA with P(3HB) polymerase (synthase). The DNA sequences of the genes encoding these enzymes have been analyzed [12–14]. Recent immunocytochemical analysis in *A. eutrophus* revealed the localization of P(3HB) polymerase at the surface of P(3HB) granules [15]. The degradation of P(3HB) is initiated by P(3HB) depolymerase to form (*R*)-3-hydroxybutyric acid [16]. In a previous paper [17] we suggested that the intracellular P(3HB) depolymerase is an *exo*-type hydrolase which hydrolyzes an ester bond of P(3HB) at the terminus of a polymer chain.

The time-dependent changes in the molecular weights of P(3HB) polymers during the course of P(3HB) synthesis have been studied in *A. eutrophus* by several groups [17–20]. The number-average molecular weights ($\overline{M}_n$) of P(3HB) polymers were found to decrease with time during the synthesis of P(3HB). However, little is known about the regulation in molecular weight of P(3HB) polymers within bacterial cells. In a previous paper [17] we studied the kinetics of synthesis and degradation of P(3HB) in *A. eutrophus* and proposed a kinetic model involving chain propagation and chain transfer reactions of P(3HB) on the active site of polymerase.

In this paper we investigate time-dependent changes in the molecular weight of P(3HB) polymers produced in *A. eutrophus* from fructose or butyric acid under various conditions and extend the kinetic model of biological polymerization.

EXPERIMENTAL SECTION

Alcaligenes eutrophus H16 (ATCC 17699) was used in this study. The cell growth and P(3HB) accumulation within cells was carried out on a two-stage batch cultivation of *A. eutrophus*. The first stage was for cell growth. *A. eutrophus* cells were grown under aerobic conditions at 30°C for 24 hours in a nutrient-rich medium

(1.0 L) containing 10 g polypetone, 10 g yeast extract, 5 g meat extract, and 5 g $(NH_4)_2SO_4$. The cells were harvested by centrifugation at 4500*g* for 10 minutes at 4°C. Under these growth conditions, P(3HB) was not accumulated in the cells. The second stage was for P(3HB) accumulation. About 4g (dry weight) of the collected cells without P(3HB) was transferred into a nitrogen-free mineral medium (1 L) containing different amounts of Na_2HPO_4 and KH_2PO_4, 0.2 g $MgSO_4$, and 1 mL microelement solution [21]. To adjust the pH value of the mineral medium, different amount of 0.5 M Na_2HPO_4 (A) and 0.5 M KH_2PO_4 (B) were mixed in the medium (1 L) as listed below. When necessary, the pH value of the medium was adjusted by the addition of 1N NaOH and H_2SO_4 solution.

	pH			
	5.0	5.8	7.0	8.0
(A) mL	159	159	39.0	7.38
(B) mL	13.8	13.8	53.6	64.2

Different amounts of fructose and butyric acid were added to the media as the sole carbon source. The cells were aerobically incubated at different temperatures from 16 to 35°C in a 2.6-L jar fermenter equipped with six conventional turbine impellers and three baffles.

Each 20 mL of culture was collected periodically during the incubation. The supernatant was removed by centrifugation at 4500*g* for 10 minutes at 4°C, and the collected cells were washed with distilled water and lyophilized. P(3HB) was extracted from the lyophilized cells into chloroform by stirring for 48 hours at room temperature, and the cell material was removed by filtration. P(3HB) samples were purified by reprecipitation with hexane and dried in vacuo.

The concentrations of fructose in nitrogen-free media were determined at 25°C by the UV-method with a biochemical analysis kit (Boehringer Mannheim GMBH Biochemica) on a Hitachi U-2000 spectrophotometer. The concentration of butyric acid was determined by gas chromatography analysis as described in a previous paper [21] and by dissolved organic carbon (DOC) analysis using a Shimadzu TOC-5000 analyzer.

To determine the P(3HB) content in bacterial cells, 8–10 mg lyophilized cells was reacted with a solution containing 2 mL chloroform, 1.7 mL methanol, and 0.3 mL sulfuric acid for 4 hours at 100°C. After the reaction, 1 mL distilled water was added and the tube was shaken for 1 minute. After phase separation the organic phase was removed and used for GC analysis. The resulting methyl esters in the organic phase were measured with *n*-octanoic acid methyl ester as a standard by gas chromatography using a Shimadzu GC-14A with a Neutra Bond-1 column and a flame ionization detector.

Molecular weight data of P(3HB) samples were obtained at 40°C by gel permeation chromatography (GPC) using a Shimadzu LC-9A system equipped with Shodex 80M and K-802 columns and RID-6A refractive index detector. Chloroform was used as the eluent at a flow rate of 0.8 mL/min, and a sample concentration of 1 mg/mL was used. The number-average and weight-average molecular weights

($\overline{M}_n$ and $\overline{M}_w$) were calculated by a Shimadzu Chromatopac C-R4A with a GPC program. A molecular weight calibration curve of P(3HB) was obtained on the basis of the universal calibration method [22] with polystylene standards of low polydispersities.

RESULTS

The effect of the initial concentration (5–20 g/L) of the carbon source (fructose or butyric acid) on the rate of P(3HB) accumulation by *A. eutrophus* was reported in a previous paper [17]. The rates of fructose consumption and of P(3HB) accumulation were independent of the concentration of fructose. In contrast, a high concentration (20 g/L) of butyric acid caused a decrease in the rates of butyrate consumption and of P(3HB) accumulation. In this study the effects of pH and temperature on the rate of P(3HB) accumulation were studied in nitrogen-free media of *A. eutrophus*. A two-stage batch fermentation was used for the kinetic analysis of P(3HB) accumulation in *A. eutrophus* cells. In the second stage, no cell growth took place in nitrogen-free media, and P(3HB) polymers accumulated within cells.

P(3HB) Production from Fructose

Figure 1 shows the time courses of P(3HB) accumulation and degradation in *A. eutrophus* cells in the nitrogen-free media containing 20 g/L fructose as the sole carbon source at 30°C. The pH values of the media remained constant at 5.0, 5.8, 7.0, and 8.0, respectively, during the incubation. The concentration of fructose in the media decreased linearly with time and reached zero at around 30 hours of incubation (Fig. 1C). In contrast, the amounts of P(3HB) in cells increased proportionally to time while fructose was present in the medium (Fig. 1B). These results suggest that the concentration of monomer ((*R*)-3-hydroxybutyryl-CoA) in cells remains constant during the synthesis of P(3HB). After the carbon source was exhausted, P(3HB) gradually degraded with time, indicating that P(3HB) was utilized for energy generation under conditions of carbon starvation.

Figure 2 (A and B) shows the time-dependent changes in the number-average molecular weight ($\overline{M}_n$) and polydispersities ($\overline{M}_w/\overline{M}_n$) of P(3HB) produced from fructose at different pH values. Figure 2(C) shows the time-dependent changes in the number of P(3HB) polymer chains ([*N*]) during incubation. The number of P(3HB) polymer chains at time *t* ($[N]_t$) can be calculated by

$$[N]_t = Y_t/\overline{M}_{n,t} \tag{1}$$

where Y_t and $\overline{M}_{n,t}$ are the yield and number-average molecular weight of P(3HB) at time *t*, respectively. The $\overline{M}_n$ value of P(3HB) increased rapidly with time and reached a maximum value (800,000–1,100,000) at around 6 hours of incubation, followed by a gradual decrease in $\overline{M}_n$ during the course of P(3HB) accumulation (Fig. 2A). The polydispersities ($\overline{M}_w/\overline{M}_n$) of P(3HB) decreased from 2.2 ± 0.3 to 1.6 ± 0.2 during the initial stage of incubation for 4 hours, followed by a gradual increase to 2.0 ± 0.3 with time (Fig. 2B). On the other hand, the [*N*] value increased with time during the course of P(3HB) accumulation (Fig. 2C).

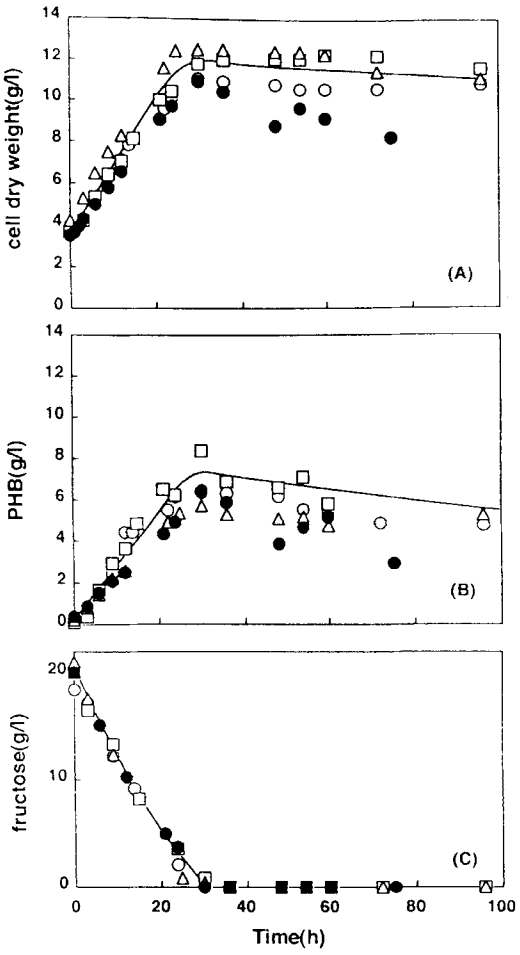

FIG. 1. Time courses of P(3HB) accumulation and degradation during the batch incubation of *A. eutrophus* in nitrogen-free media (1 L) containing fructose (20 g/L) as a sole carbon source at 30°C. The pH values of media remained constant at (○) 5.0, (□) 5.8, (△) 7.0, and (●) 8.0.

Table 1 lists the rate of P(3HB) accumulation (R_p), the rate of P(3HB) degradation (R_d), and the rate of increase in the number of P(3HB) polymer chains (R_N) at different pH values of the nitrogen-free media (Runs 1, 2, 3, and 4) as determined from Figs. 1(B) and 2(C). The rate of P(3HB) accumulation was almost independent of the pH value of the medium, but the rate of increase in the number of P(3HB) polymer chains decreased slightly with an increase in pH value.

Figure 3 shows the time courses of P(3HB) accumulation and degradation in *A. eutrophus* cells in nitrogen-free media containing 20 g/L fructose at pH 7.0. The temperatures of the media were kept constant at 16, 23, 27, 30, and 35°C, respectively, during the incubation. The rates of P(3HB) accumulation and fructose consumption increased with temperature. The maximum weights of P(3HB) accumulated in cells are listed in Table 1. The maximum weights (7.0 ± 1.3 g/L) of

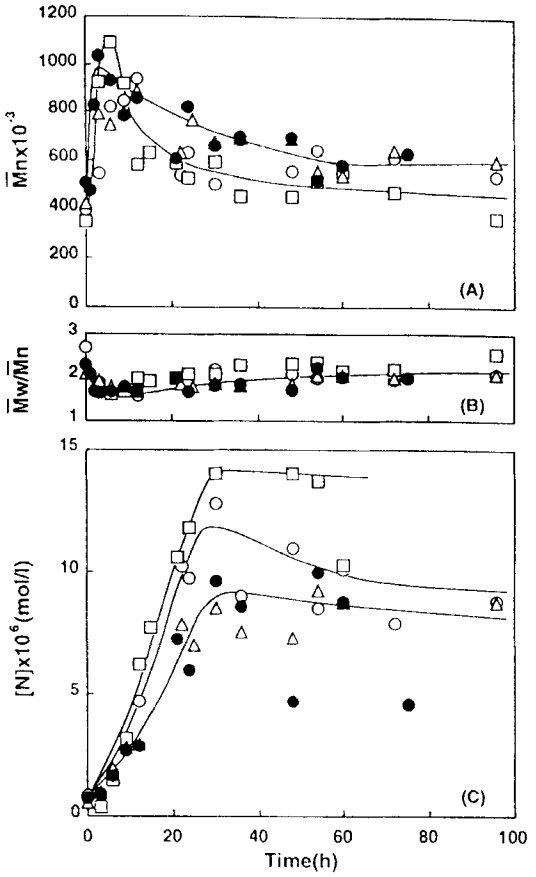

FIG. 2. Time-dependent changes in the number-average molecular weights ($\overline{M}_n$) (A), polydispersities ($\overline{M}_w/\overline{M}_n$) (B), and the chain number ([N]) (C) of P(3HB) polymers produced from fructose in nitrogen-free media (1 L) at pH values of (○) 5.0, (□) 5.8, (△) 7.0, and (●) 8.0.

P(3HB) in *A. eutrophus* cells were not influences by pH and the temperature of the media. Thus, the yields of P(3HB) from fructose were 35 ± 6 wt%, independent of incubation conditions.

Figure 4 (A and B) shows the time-dependent changes in the values of $\overline{M}_n$ and $\overline{M}_w/\overline{M}_n$ of P(3HB) produced from fructose at different temperatures. The $\overline{M}_n$ values increased rapidly with time to reach a maximum value (900,000–1,400,000) at around 6 hours of incubation, followed by a gradual decrease in $\overline{M}_n$ with time. The values of $\overline{M}_w/\overline{M}_n$ decreased with time to reach a minimum value (1.5 ± 0.2) at around 6 hours of incubation, followed by a gradual increase to 1.9 ± 0.2 with time. Figure 4(C) shows the time-dependent change in the number of P(3HB) polymer chains ([N]) during incubation at different temperatures.

Table 1 gives the rates of R_p, R_d, and R_N at different temperatures, determined from Figs. 3(B) and 4(C). The temperature dependences of R_p and R_N are plotted in Fig. 5. The apparent activation energies of the rate of P(3HB) accumulation (R_p)

TABLE 1. Maximum Weight of P(3HB), Content of P(3HB) in Dry Cell, Rate (R_p) of P(3HB) Accumulation, Rate (R_d) of P(3HB) Degradation, and Rate (R_N) of Increase in the Number of P(3HB) Polymer Chains under Different Incubation Conditions in Nitrogen-Free Media Containing Fructose or Butyric Acid as the Sole Carbon Source for *A. eutrophus*

Run	Concentration of substrate, (g/L)	pH	Temperature, °C	Maximum weight, g/L	Content, wt%	$R_p \times 10^3$, mol/h·L	$R_d \times 10^4$, mol/h·L	$R_N \times 10^7$, mol/h·L
	Fructose							
1	20	5.0	30	6.4	58	2.6 ± 0.3	3.3 ± 0.4	4.3 ± 0.3
2	20	5.8	30	8.3	73	3.3 ± 0.5	7.1 ± 0.8	5.5 ± 1.0
3	20	7.0	30	5.7	46	2.3 ± 0.5	3.2 ± 0.2	3.2 ± 0.3
4	20	8.0	30	6.3	58	2.3 ± 0.3	7.7 ± 0.6	3.0 ± 0.4
5	20	7.0	16	7.3	72	1.5 ± 0.3	n.d.	2.1 ± 0.5
6	20	7.0	23	6.2	52	1.7 ± 0.3	1.6 ± 0.2	2.0 ± 0.1
7	20	7.0	27	7.4	62	2.8 ± 0.5	4.4 ± 1.0	2.9 ± 0.2
8	20	7.0	35	7.2	60	3.2 ± 0.5	5.7 ± 0.2	3.0 ± 0.8
	Butyric acid							
9	10	7.0	20	3.5	42	0.82 ± 0.2	3.7 ± 0.5	2.1 ± 0.2
10	10	7.0	25	4.2	50	2.7 ± 0.4	6.9 ± 0.2	4.1 ± 0.5
11	10	7.0	30	4.3	55	3.2 ± 0.5	3.2 ± 0.6	4.5 ± 1.0
12	10	7.0	35	4.2	47	4.0 ± 0.2	4.9 ± 0.5	6.0 ± 1.5

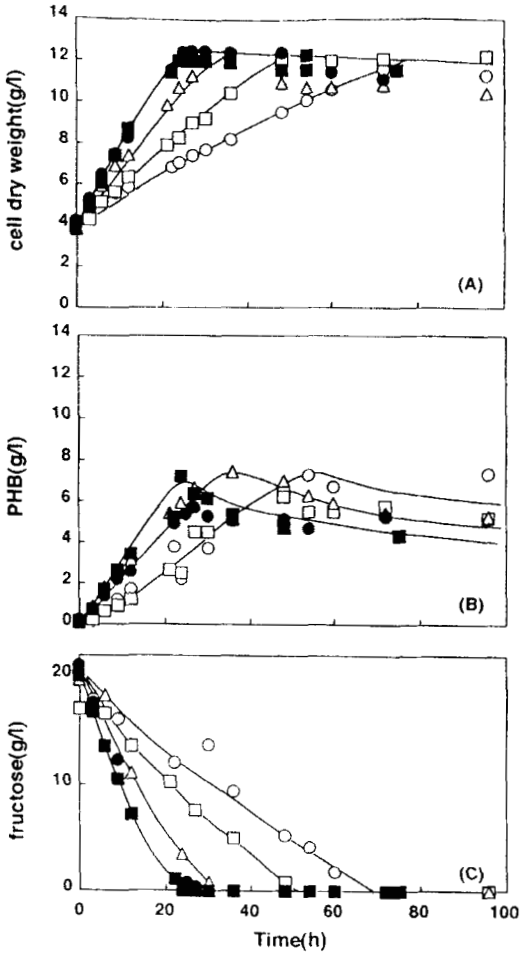

FIG. 3. Time courses of P(3HB) accumulation and degradation during the batch incubation of *A. eutrophus* in nitrogen-free media containing fructose (20 g/L) as a sole carbon source at pH 7.0. The temperatures of media remained constant at (○) 16, (□) 23, (△) 27, (●) 30, and (■) 35°C.

and of the increase in the number of P(3HB) polymer chains (R_N) were determined as 30 and 20 kJ/mol, respectively.

P(3HB) Production from Butyric Acid

In this experiment, butyric acid was used as the sole carbon source for *A. eutrophus* in nitrogen-free media. Figure 6 shows the time courses of P(3HB) accumulation and degradation in *A. eutrophus* cells in the nitrogen-free media containing 10 g/L butyric acid at pH 7.0. The temperature of the medium remained constant at 20, 25, 30, and 35°C, respectively, during the incubation. The rates of P(3HB) accumulation and butyrate consumption increased with temperature. The maximum weights of P(3HB) accumulated in cells are given in Table 1. The values

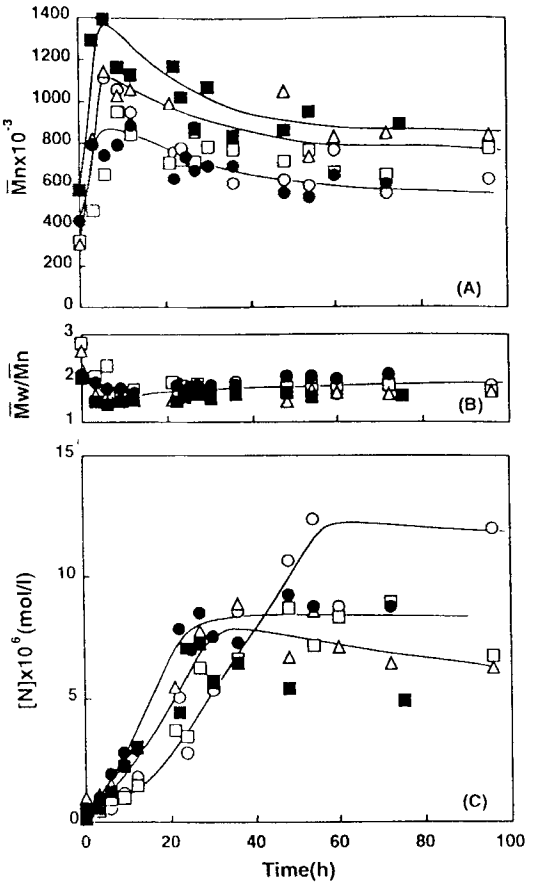

FIG. 4. Time-dependent changes in the number-average molecular weights ($\overline{M}_n$) (A), polydispersities ($\overline{M}_w/\overline{M}_n$) (B), and the chain number ([N]) (C) of P(3HB) polymers produced from fructose in nitrogen-free media (1 L) at temperatures of (○) 16, (□) 23, (△) 27, (●) 30, and (■) 35°C.

(4.2 ± 0.1 g/L) were independent of temperature in the 25–35°C range. The yields of P(3HB) from butyric acid were 42 wt% at temperatures above 25°C, while the yield at 20°C was 35 wt%.

Figure 7 shows the time-dependent changes in $\overline{M}_n$ (A), $\overline{M}_w/\overline{M}_n$ (B), and the number of polymer chains [N] (C) of P(3HB) produced from butyric acid at different temperatures. The $\overline{M}_n$ values increased with time to reach a maximum value at 5 hours and then decreased to a constant value with time. It is important to note that the $\overline{M}_n$ values of P(3HB) remain almost constant during the degradation of P(3HB) in the absence of butyric acid. This result suggests that a gradual decrease in $\overline{M}_n$ during the course of P(3HB) accumulation is not related to the degradation of P(3HB) by the intracellular depolymerase. The $\overline{M}_w/\overline{M}_n$ values increased to a constant value (2.0 ± 0.3) with time. The [N] values increased with time during the course of P(3HB) accumulation in the presence of butyric acid. The rates of increase in the [N] value in the initial stage for 5 hours were slower than the rates

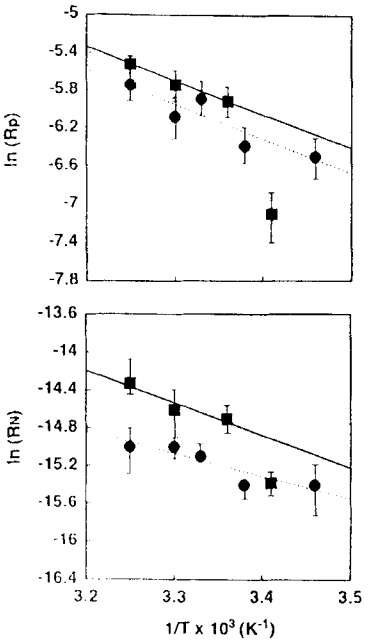

FIG. 5. Arrhenius plots of ln R_p (A) and ln R_N (B) against $1/T$. The filled squares (■) and circles (●) are the data obtained from butyric acid and fructose, respectively.

after 5 hours. After the carbon source was exhausted, the $[N]$ values decreased with time. The rates of decrease in $[N]$ values in Fig. 7(C) are higher than the rates in Fig. 4(C).

Table 1 lists the rate of P(3HB) accumulation (R_p), the rate of P(3HB) degradation (R_d), and the rate of increase in the $[N]$ value (R_N) at different temperatures, determined from Figs. 6(B) and 7(C). The temperature dependence of R_p and R_N are shown in Fig. 5. The apparent activation energies of R_p and R_N were determined as 30 and 28 kJ/mol, respectively. Thus, the activation energy of P(3HB) accumulation by *A. eutrophus* from butyric acid was consistent with the value (30 kJ/mol) from fructose.

DISCUSSION

In a previous paper [17] we proposed the reaction scheme of initiation, chain propagation, and chain transfer in the enzymatic polymerization of (*R*)-3-hydroxybutyryl-CoA with P(3HB) polymerase as follows.

Initiation:

$$\text{E-SH} + (R)\text{-CH}_3\text{CH(OH)CH}_2\text{CO-SCoA} \xrightarrow{k_i}$$
$$(R)\text{-CH}_3\text{CO(OH)CH}_2\text{CO-S-E} + \text{CoA-SH} \qquad (2)$$

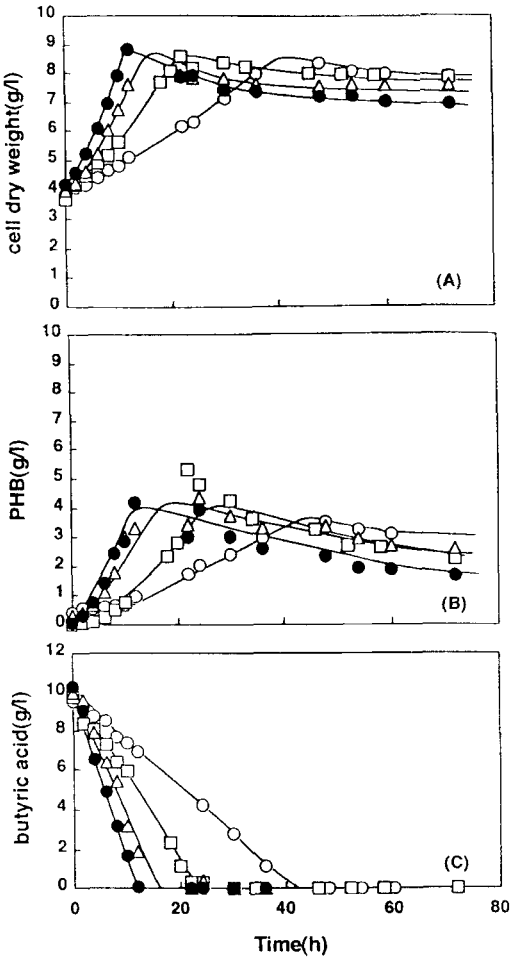

FIG. 6. Time courses of P(3HB) accumulation and degradation during the batch incubation of *A. eutrophus* in nitrogen-free media (1 L) containing butyric acid (10 g/L) as a carbon source at pH 7.0. The temperatures of media remained constant at (○) 20, (□) 25, (△) 30, and (●) 35°C.

Chain propagation:

$$P(3HB)_{n-1}OCH(CH_3)CH_2CO\text{-}S\text{-}E + (R)\text{-}CH_3CH(OH)CH_2CO\text{-}SCoA \xrightarrow{k_p} P(3HB)_nOCH(CH_3)CH_2CO\text{-}S\text{-}E + CoA\text{-}SH \quad (3)$$

Chain transfer:

$$P(3HB)_{n-1}OCH(CH_3)CH_2CO\text{-}S\text{-}E + X(H_2O) \xrightarrow{k_t} P(3HB)_{n-1}OCH(CH_3)CH_2COOH + E\text{-}SH + X \quad (4)$$

where E-SH, CoA-SH, (R)-$CH_3CH(OH)CH_2CO$-SCoA, $P(3HB)_n$, and X denote P(3HB) polymerase with thiol groups as the active site, coenzyme A, (R)-3-

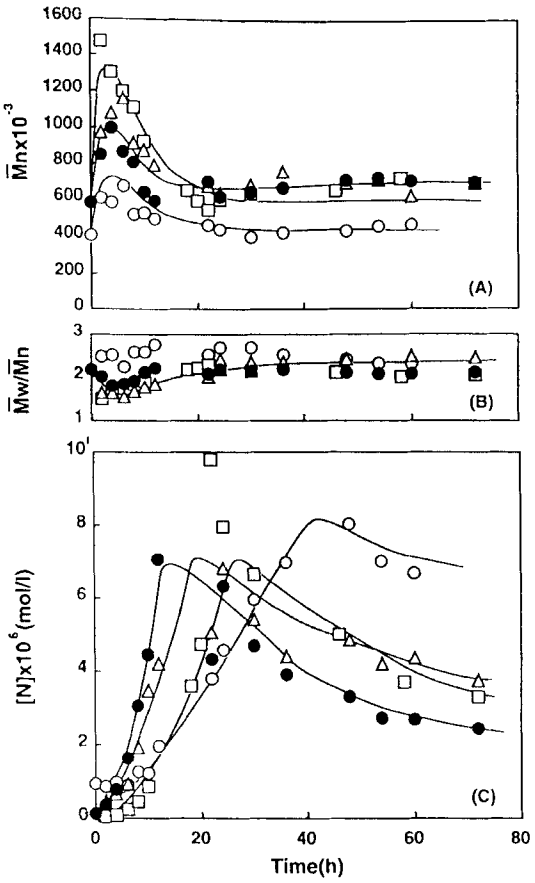

FIG. 7. Time-dependent changes in the number-average molecular weights ($\bar{M}_n$) (A), polydispersities ($\bar{M}_w/\bar{M}_n$) (B), and the chain number ([N]) (C) of P(3HB) polymers produced from butyric acid in nitrogen-free media (1 L) at temperatures of (○) 20, (□) 25, (△) 30, and (●) 35°C.

hydroxybutyryl-CoA as monomer, a polymer chain with an n polymerization degree of 3HB units, and a chain-transfer agent, respectively, and k_i, k_p, and k_t are the pseudo-first-order rate constants for initiation, propagation, and chain-transfer reactions. The chain transfer agent X may bind a water molecule as an active form.

In this paper we extend our kinetic model proposed in a previous paper [17]. A key enzyme of P(3HB) synthesis is P(3HB) polymerase with thiol groups as the active site [11, 13, 23]. The polymerase active of *A. eutrophus* was found to remain almost constant during P(3HB) accumulation [11]. This result suggests that the number of polymerase molecules remains constant during P(3HB) accumulation. As shown in Figs. 2, 4, and 7, the number of P(3HB) polymer chains [N] increased with time during the accumulation of P(3HB) with an induction period of several hours, and the polydispersities ($\bar{M}_w/\bar{M}_n$) of molecular weight distribution of P(3HB) polymers increased to 2.0 with time. These results indicate that a chain-transfer reaction of the propagating chain takes place on the P(3HB) polymerase.

As a result of the chain-transfer reaction, the length of the growing P(3HB) chain is limited, and the $[N]$ value increases with time.

In this study an induction period with an increase in the number of P(3HB) polymer chains $[N]$ was observed for several hours in the initial stage of P(3HB) accumulation (see Figs. 2, 4, and 7), which suggests that the rate of chain-transfer reaction increases with time in the initial stage of biological polymerization. Here we propose a reaction scheme in which the concentration of a chain-transfer agent (X) increases with time to reach a constant value, resulting in an increase in the rate of chain transfer. A chain-transfer agent (X) may be formed from an inactive compound A after the synthesis of P(3HB) is initiated within cells as,

$$\mathrm{A} \xrightarrow{a} \mathrm{X} \qquad (d[\mathrm{X}]/dt = a[\mathrm{A}]) \tag{5}$$

where a is the rate constant of the formation of chain-transfer agent X. The concentration of chain-transfer agent X at time t is represented by

$$[\mathrm{X}] = [\mathrm{X}]_0(1 - e^{-at}) \tag{6}$$

with $[\mathrm{X}]_0 = [\mathrm{A}] + [\mathrm{X}]$. Then the rate of chain-transfer reaction (R_t) at time t is given by

$$R_t = k_t[\mathrm{E}][\mathrm{X}] = k_t[\mathrm{E}][\mathrm{X}]_0(1 - e^{-at}) \tag{7}$$

where [E] is the concentration of P(3HB) polymerase molecules and k_t is the rate constant of the chain-transfer reaction. The number of P(3HB) polymer chains $[N]_t$ at time t is given by Eq. (8) from Eq. (7):

$$[N]_t = [E] + \int_0^t R_t dt = [E] + k_t[E][X]_0\{t + (e^{-at} - 1)/a\} \tag{8}$$

TABLE 2. Rate Constants of Chain Propagation (k_p), Chain Transfer Reaction ($k_t[\mathrm{X}]_0$), and Formation of a Chain Transfer Agent (a)

Run	k_p, h^{-1}	$k_t[\mathrm{X}]_0$, h^{-1}	a, h^{-1}
1	5200	0.86	0.20
2	6600	1.1	0.20
3	4600	0.64	0.20
4	4600	0.60	0.23
5	3000	0.40	0.11
6	3400	0.40	0.20
7	5600	0.58	0.25
8	6400	0.60	0.21
9	1600	0.42	0.09
10	5400	0.82	0.13
11	6400	0.90	0.19
12	8000	1.2	0.30

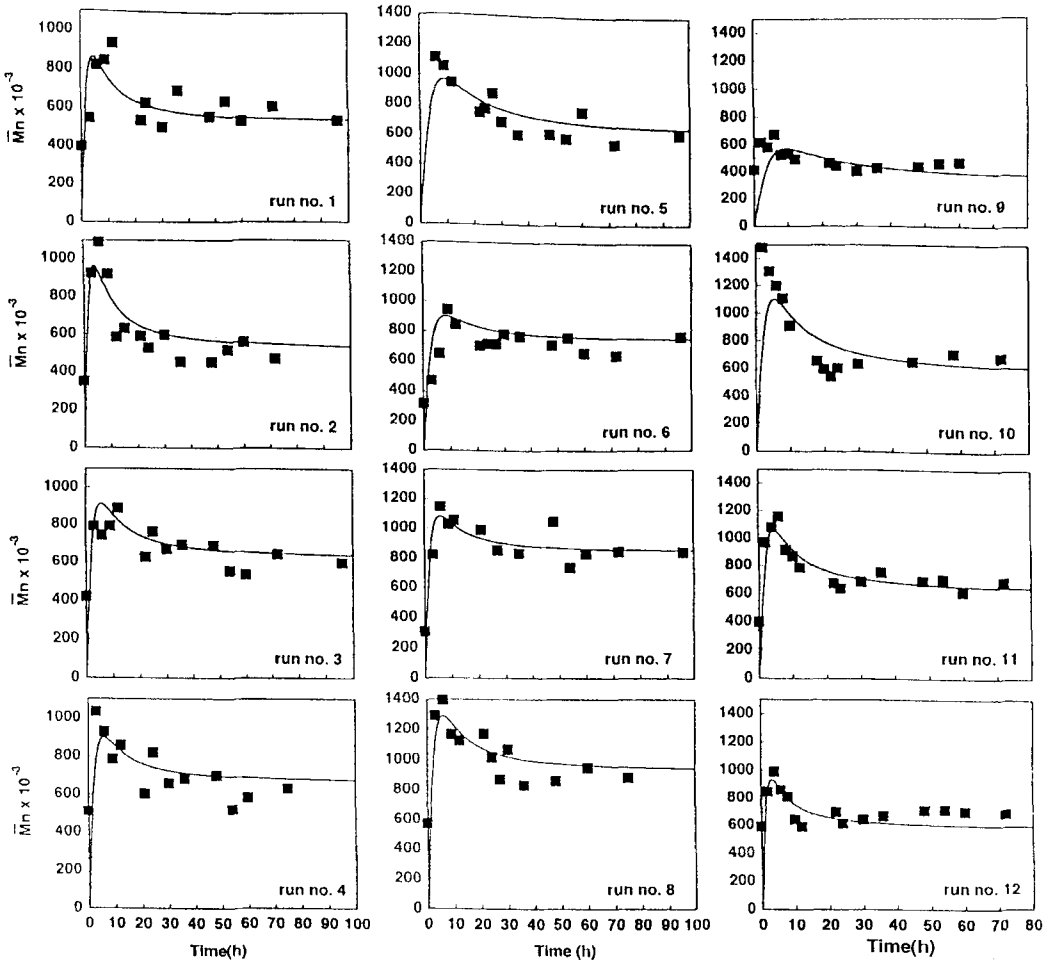

FIG. 8. Time courses of the number-average molecular weights ($\bar{M}_n$) of P(3HB) polymers obtained in Runs 1 to 12. The experimental data are shown by filled squares, and the values calculated by Eq. (11) with the rate constants in Table 2 are represented by solid lines.

Here we make the assumptions that 1) there is a rapid initiation reaction and 2) the number of polymerase molecules [E] remains constant during P(3HB) synthesis. From Eq. (8), we have $[N] = [E]$ at $t = 0$ and $[N] = k_t[E][X]_0 t$ at $t = \infty$. The value [E] may be determined by extrapolation of $[N]_t$ values to time zero, using the plots of $[N]_t$ vs t in Figs. 2, 4, and 7. It was estimated that the number of polymerase molecules was $(5 \pm 2) \times 10^{-7}$ mol/L. The value of [E] is the same value as reported in a previous paper [17]. The values of $k_t[X]_0$ and of the rate constant a for the formation of chain-transfer agent X were determined from the values of R_N (Table 1) and the relations of $[N]_t$ vs t plots in Figs. 2, 4, and 7 by using Eq. (8). The values of $k_t[X]_0$ and a are given in Table 2.

The yield Y_t of P(3HB) polymers produced for time t is given by

$$Y_t = \int_0^t R_p dt = k_p[E]t \tag{9}$$

where R_p and k_p denote the rate of chain propagation and the pseudo-first-order rate constant for propagation, respectively. The values of k_p were determined from the values of R_p (Table 1) and the [E] value (5×10^{-7} mol/L), and they are listed in Table 2.

The number-average polymerization degree ($\overline{P}_{n,t}$) of P(3HB) polymers produced for time t is given by Eq. (10):

$$\overline{P}_{n,t} = \frac{Y}{[N]_t} = \frac{\int_0^t R_p dt}{[\mathrm{E}] + \int_0^t R_t dt} = \frac{k_p[E]t}{[E] + k_t[E][X]_0\{t + (e^{-at} - 1)/a\}} \tag{10}$$

The rate constants k_p and k_t are assumed to be constant during polymerization. Then, the number-average molecular weight ($\overline{M}_{n,t}$) of P(3HB) polymers produced in time t is given by

$$\overline{M}_{n,t} = \frac{86k_p[E]t}{[\mathrm{E}] + k_t[\mathrm{E}][\mathrm{X}]_0\{t + (e^{-at} - 1)/a\}} \tag{11}$$

where 86 is the molecular weight of a 3HB unit.

The $\overline{M}_{n,t}$ values at time t were calculated from Eq. (11) by using the values of k_p, $k_t[\mathrm{X}]_0$, and a in Table 2. The results are shown in Fig. 8. The experimental data of $\overline{M}_n$ for Runs 1 to 12 are given by closed squares, and the calculated values of $\overline{M}_n$ are represented by solid lines. The calculated values of $\overline{M}_n$ are in good agreement with the experimental data for all runs. Thus, an unusual time-dependent change in $\overline{M}_n$ during the synthesis of P(3HB) may be interpreted in terms of the model that a chain-transfer agent X is formed from an inactive compound A during the synthesis of P(3HB). The chain-transfer agent X may be a coenzyme. More research is needed to identify the chain-transfer agent in this biological polymerization.

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