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Biosynthesis of Polyhydroxybutyrate and Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Bacillus thuringiensis* R-510

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Biosynthesis of polyhydroxybutyrate and copolymer consisting of 3-hydroxybutyrate and 3-hydroxyvalerate [poly(3HB-*co*-3HV)] by *Bacillus thuringiensis* R-510 grown with glucose or with mixtures of glucose and propionate was investigated. n-Alkanoic acids other than propionate were not precursors of 3HV units. The fraction of 3HV unit in the copolymer increased from 0 to 84 mol% as the concentration of propionate in the medium increased from 0 to 0.8% (w/v). However, *B. thuringiensis* R-510 grown with only propionate (0.1%) produced poly(3HB-co-3HV) containing 42 mol% of 3HV. Polymer yield decreased as the fraction of propionate was increased but the molecular weight distribution was not affected by the composition of carbon substrate. The minimum melting temperature (around 65°C) of poly(3HB-*co*-3HV) copolymers was observed for the polymer bearing approximately 35 mol% of 3HV. Polyhydroxyalkanoates production by this organism was not dependent on nutritional limitation, but remarkably influenced by dissolved oxygen concentration in the culture medium. Low level of dissolved oxygen concentration prevented spore formation in the cells and stimulated the synthesis of polyhydroxyalkanoate. The composition of poly(3HB-*co*-3HV) produced by *B. thuringiensis* R-510 varied according to the growth time. However, there was no evidence that polymers isolated from cells were mixtures of immiscible polymers.

Key words: *Bacillus thuringiensis*, copolymer, dissolved oxygen concentartion, poly-β-hydroxyalkanoate, polyhydroxybutyrate.

Various species of bacteria produce polyesters of a wide range of different hydroxyalkanoates including poly-β-hydroxybutyrate (PHB) (1, 14). Some of these microbially-produced polyhydroxyalkanoates (PHAs), especially copolymers consisting of 3-hydroxybutyrate and 3-hydrxyvalerate [poly(3HB-*co*-3HV)], have attracted commercial interest as promising candidates for large-scale production of biodegradable and biocompatible thermoplastics (3, 6, 18, 26). The poly (3HB-*co*-3HV) has been commercialized by Zeneca BioProducts under the trade name Biopol.

PHB is synthesized by the 3-ketothiolase-mediated condensation of acetyl-CoA, followed by acetoacetyl-CoA reduction and polymerization of the resultant β-hydroxybutyryl-CoA subunits (1). However, poly-(3HB-*co*-3HV) can be produced in many bacteria only when the medium is supplemented with a secondary carbon source (cosubstrate) as the 3HV precursor (9, 13, 15). Propionate and valerate are the most frequently used cosubstrates. It is known that propionate is utilized to produce 3-ketoacyl-CoA via propionyl-CoA, which is condensed with acetyl-CoA by 3-ketothiolase (2, 22). Increased content of 3HV monomer unit in the copolymer with the increasing concentration of cosubstrate has been normally observed. However, the maximum content of 3HV in copolymer is limited (generally less than 20 mol%) by the toxic effect of cosubstrate at relatively low concentrations. For example, the growth and copolymer production in *Alcaligenes eutrophus* is inhibited by as little as 0.1% (w/v) propionic acid (6), and even *Pseudomonas cepacia*, which was known to be propionate-tolerant, cannot produce a substantial amount of polymer in the presence of 0.3% propionic acid (16). Therefore, a good control of propionate feed rate during fermentation process is essential for the copolymer production. In this respect, it is important to investigate the capability of other bacteria to synthesize poly(3HB-*co*-3HV) so as to provide a wider choice of cultures which can potentially be used in a fermentation process.

We have previously reported the isolation of a PHA-producing strain, *Bacillus thuringiensis* R-510, that is resistant to relatively high concentration of propionate (11). This organism accumulates poly-(3HB-*co*-3HV) bearing 3HV monomer units in high-

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er amounts than other organisms when grown with mixtures of glucose and propionate. Even though copolymers with a high 3HV content are not necessarily more useful individually, alteration of 3HV content in poly(3HB-*co*-3HV) is desirable from an industrial viewpoint because it may offer the opportunity of producing different thermoplastics with various degrees of flexibility and toughness. In this study, we describe the growth behavior and kinetics of poly(3HB-*co*-3HV) production in *B. thuringiensis* R-510 grown with mixtures of glucose and propionate. The effect of culture conditions on the PHA yield and the compositions of copolymers are also described.

Materials and Methods

Microorganism and culture media

The microorganism, *B. thuringiensis* R-510, was isolated from a soil sample (11). Stock cultures were maintained at 4°C by periodical transfer onto solid medium (yeast extract 1%, polypeptone 1%, beef extract 0.5%, NaCl 0.5%, and agar 2%). A liter of basal medium contained 30 g of glucose, 4 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 1 g of K_2HPO_4, 1.7 g of Na_2HPO_4, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, and 2 ml of trace-element solution. Each liter of trace-element solution contained 0.2 g of $ZnSO_4$ $7H_2O$, 0.6 g of H_3BO_3, 0.06 g of $MnCl_2 \cdot 4H_2O$, 0.4 g of $CoCl_2 \cdot 6H_2O$, 0.02 g of $CuSO_4 \cdot 4H_2O$, and 0.06 g of $NaMoO_4 \cdot 2H_2O$. Culture media containing propionate in various concentrations were prepared and pH adjusted to 6.0.

Shake flask experiments

The organism was grown aerobically in 250 ml Erlenmeyer flasks containing 50 ml of basal medium. The medium was inoculated with a 1% (v/v) inoculum of an overnight culture in the same medium with 1.5% glucose. Once the flasks were inoculated, they were incubated at 33°C with agitation at 180 rpm for 30 h on a rotary shaker. The cells were then harvested by centrifugation and their PHA content was determined as described below. All experiments were repeated at least three times.

Fermentator experiments

All batch fermentations were carried out in a 5 L jar fermentor (Korea Fermentor Co., Korea) with a working volume of 3 L. The culture media used were the same as described above. The medium was inoculated with a 5%(v/v) inoculum and the cells were cultivated for 27 h. Temperature and pH were automatically controlled at optimal values, 33°C and 6.0, respectively. The air flow rate was 1.0 vvm and unless otherwise stated, agitation speed was set at 350 rpm.

Preparation of poly(3HB-*co*-3HV)

Cells containing the copolymer were harvested by centrifugation (7,500 rpm for 10 min), and the pellet was washed twice with deionized water. The resulting concentrated cells were lyophilized and extraction with boiling chloroform was performed overnight using a Soxhlet extractor. The copolymer solution in chloroform was added dropwise to diethyl ether. The resulting precipitate was redissolved in chloroform; and the precipitation was carried out two more times. The final precipitate was filltered through Watman filter paper and dried at atmosphere.

Analytical methods

Cell growth was monitored photometrically by measuring optical density of the culture at 660 nm. Dry cell weight (DCW) was measured by drying the harvested cells to constant weight at 105°C. Concentrations of glucose and ammonium ions remaining in the broth were determined using a Yellow Springs Instrument (YSI) biochemistry analyzer and an ion meter (Cole Parmer, USA) in coupling with ammonia electrode, respectively. PHA content and its composition were determined by gas chromatography (Hewlett Packard 5890, USA) using PHA standards containing known proportions of 3HB and 3HV monomers after methanolysis of freeze-dried cells for 140 min at 100°C to yield the methyl esters of the constituent 3-hydroxyalkanoic acids (5). Benzoic acid was used as an internal standard. The total amount of PHA was quantified by summing the amounts of 3HB and 3HV units detected.

The concentration of propionate remaining in the culture supernatant fluid was determined as follows. Two volumes of 2% aqueous sulfuric acid solution was mixed with one volume of culture supernatant in screw-cap tubes and agitated vigorously. One volume of each of distilled water and dichloromethane were added. Then the contents of the tubes were mixed vigorously. The organic phase was separated and analyzed by gas chromatography. Butyric acid was used as the internal standard. A capillary column (HP-1, 25 m length by 0.2 mm inside diameter) was used for the analysis of propionic acid. The initial oven temperature was 25°C and was raised to 180°C at a ramp of 10°C/min. Initial time was 3 min and the final time was 5 min. The injector temperature was 190°C and the flame ionization detector temperature was 250°C.

Molecular weights of PHAs were determined by a gel permeation chromatography (GPC) system as described previously (27). Molecular weights of standard polystyrenes were 2.75×10^6, 2.0×10^5, 3.5×10^4, and 3.6×10^3. The eluent was chloroform; the concentration of the sample was approximately 1 wt%, and 100 µl was injected. Samples for GPC analysis were prepared by dissolving crude extracts from dry cells. Chromatograms were recorded and molecular weights were calculated using a PC compatible equipped with Chromate Data Analysis Kit (Interface Co. Ltd., Korea).

Differential scanning calorimeter (DSC) measurement was carried out using a Thermal Analysis Model 2510. The temperature range for the DSC measurement was -100 to 210°C. Polymer samples were heated at a rate of 10°C/min. DSC samples were taken from polymers annealed at room temperature for several days. The weights of polymer used for DSC measurement was 7 to 10 mg.

Results and Discussion

Biosynthesis of copolymer from alkanoic acids

Many bacterial strains have been reported to synthesize copolymers containing 3HB and other hydroxy acid monomer units when short-chain fatty acids are added to a medium as precursors of monomers other than 3HB. To elucidate the ability of *B. thuringiensis* R-510 to produce polymers bearing repeating units other than 3HB, several n-alkanoic acids were added singlly to the basal medium at a concentration of 1.0 g/L. The DCW yield, PHA content and monomeric constituents of the polymers produced were determined after 30 h of incubation (Table 1). *B. thuringiensis* R-510 accumulated a PHB homopolymer within the cells when grown with only basal medium. Only the mixture containing propionate supported the production of polymers bearing 3HV units. Valerate is known to be one of the most effective precursors for the production of 3HV in many bacterial strains (7, 9, 13). However, *B. thuringiensis* R-510 did not utilize valerate as a precursor for 3HV units. Relatively short alkanoic acids ranging from butyrate (C4) to heptanoate (C7) had inhibitory effects on cell growth and polymer accumulation. The monomeric constituent other than 3HB was not detected from the PHAs synthesized in the presence of these alkanoic acids. C4 to C7 alkanoic acids used as sole carbon sources at concentrations lower than 0.4 % supported slight cell growth and accumulation of PHB homopolymer (data not shown). Recently, production of medium-chain-length PHAs by *B. thuringiensis* grown with nonanoic acid was reported (19). However, cell growth of *B. thuringiensis* R-510 was completely inhibited by octanoate and nonanoate; these acids did not support polymer production. It is known that β-oxidation is involved in the metabolism of uneven-chain-length alkanoic acids to produce acetyl-CoA and propionyl-CoA (22). In this study, the production of PHB homopolymer by *B. thuringiensis* R-510 from valerate and heptanoate suggests that propionyl-CoA formed after β-oxidation of these alkanoic acids may be directed into general metabolic pathways rather than into those producing intermediates for 3HV monomer unit. Similarly, intracellular PHA synthetic system with very low substrate specificities towards these acids was reported in a mutant of *Sphaerotilus natans* (24).

Table 1. Synthesis of PHA by *B. thuringiensis* R-510 in glucose medium containing n-alkanoic acids

n-Alkanoate	DCW (g/L)	PHA (%DCW)	PHA composition	
			3HB	3HV
None	3.0	18.5	100	0
Propionate	2.9	21.1	59	41
Butyrate	1.7	15.3	100	0
Valerate	1.6	8.5	100	0
Hexanoate	0.5	10.1	100	0
Heptanoate	0.2	ND[a]	ND	ND
Octanoate	NG[b]	NG	–	–
Nonanoate	NG	NG	–	–

Sodium salts of alkanoic acids were added to basal medium at a concentration of 1.0 g/L and cultivations were done in flasks for 30 h

[a] Not determined, [b] No growth

Effect of propionate concentrations

Table 2 shows the relationship between the concentration of propionate, cell growth, and copolymer composition. The addition of increased propionate concentrations in glucose medium resulted in an increase of molar fraction of 3HV in the copolymer. The 3HV content in the copolymer was as high as 84 mol% when the concentration of pro-

Table 2. Synthesis of poly(3HB-*co*-3HV) in response to increased propionate concentrations

Propionate (%)	DCW (g/L)	PHA (%DCW)	PHA composition (mol%)	
			3HB	3HV
0	2.8	38.8	100	0
0.1	2.7	38.7	66	34
0.2	2.6	30.0	40	60
0.3	2.1	20.4	25	75
0.5	1.9	17.4	19	81
0.8	1.5	5.3	16	84

Table 3. Molecular weights of poly(3HB-*co*-3HV) in response to 3HV mol%

Mol% of 3HV	$\overline{M}_n \times 10^3$	PDI
0	269	4.0
5	244	3.7
34	329	3.0
42	280	2.7
50	298	2.7
60	300	2.4
75	257	3.0
84	286	2.9

pionate was 8 g/L. However, it was interesting that the fraction of 3HV in the copolymer isolated from the cells grown with a medium containing 0.1% of propionate as a sole carbon source was as low as 42 mol%. This result suggests that *B. thuringiensis* R-510 is able to convert propionate to acetyl-CoA via propionyl-CoA, methylmalonyl-CoA, and succinyl-CoA (8).

When the concentration of propionate was higher than 0.3%, cell growth and PHA production were inhibited severly. Compared with other well known PHA-producing microorganisms, *B. thuringiensis* R-510 was highly resistant to the toxic effects of propionate. The extreme tolerance to propionate allows easier control of the fermentation processes (15). Moreover, this organism produced poly(3HB-*co*-3HV) containing much higher molar fraction of 3HV monomer than other bacterial strains when grown with the same carbon sources (4, 6, 13, 15, 21). The production of poly(3HB-*co*-3HV) with high molar fraction of 3HV at lower concentrations of propionate is a useful advantage in industrial viewpoint since propionate is a relatively expensive substrate (12, 17, 18, 20).

Properties of poly(3HB-*co*-3HV)

The molecular weights of the poly(3HB-*co*-3HV) with a wide range of compositions (from 0 to 84 mol% 3HV) were measured by gel permeation chromatography. As shown in Table 3, the number average molecular weights (M_n) of polymers did not vary significantly and stayed in the range of 2.7×10^5 to 3.3×10^5. The polydispersity indices were between 2 and 4. The molecular weight distribution of the copolymers obtained in this study was quite different from those of poly(3HB-*co*-3HV) isolated from the cells of *A. eutrophus* (7) and *Alcaligenes* sp. SH-69 (27). The M_n of copolymers produced by these microorganisms decreased gradually as the 3HV fraction increased to 50 mol%.

The melting temperatures (Tm) of poly(3HB-*co*-3HV) copolymers obtained in this study are shown in Fig. 1. The Tm of PHB homopolymer was 175°C;

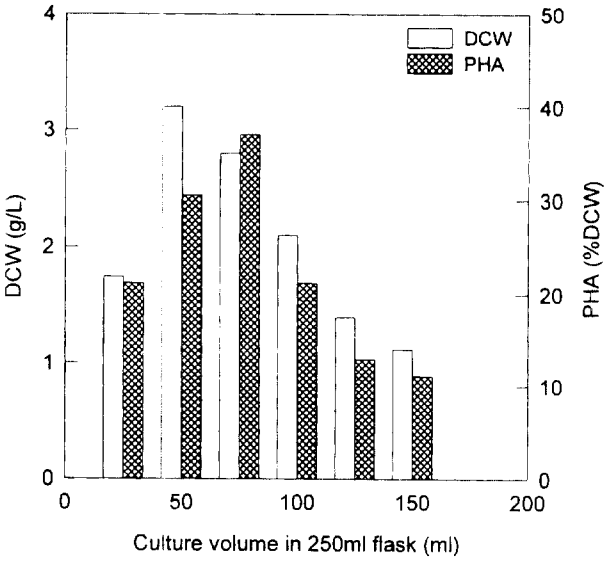

Fig. 1. Effect of culture volumes on cell growth and PHA accumulation. *B. thuringiensis* R-510 was grown in 250 ml flasks containing different volumes of medium for 24 h.

the Tm of copolymer with 84 mol% 3HV was approximately 100°C. The minimum Tm (around 65°C) was observed for the polymer bearing approximately 35 mol% of 3HV. X-ray diffraction study showed that the copolymers bearing 3HV units of 35 mol% and less than that had crystal lattice of PHB; and the copolymers bearing 3HV in higher fractions had crystal lattice of polyhydroxtvalerate. These results correspond to those reported elsewhere (3, 7).

Effect of aeration conditions on copolymer production

Under the circumstances that terminal electron acceptor is not sufficiently supplied, the synthesis of PHA provides a sink for reducing equivalents for aerobic bacteria, and the accumulation of PHA is promoted if the cells are cultivated under conditions of limited aeration (18, 25). To clarify the effect of aeration conditions on the cell growth and PHA accumulation, cultivations were done using flasks of the same size supplemented with varied volumes of basal medium containing 20 g/L glucose. The results after 24 h incubation are depicted in Fig. 2. Both DCW and PHA yield reached the maximum of 3.2 g/L and 1.0 g/L, respectively, when the organism was grown in 50 ml medium. However, the highest accumulation of PHA (37% DCW) was obtained from the cells grown in 75 ml medium, suggesting that optimum aeration conditions for cell growth and PHA accumulation are different.

Similar experiments were extended to batch cultures in a jar fermentor using combined carbon

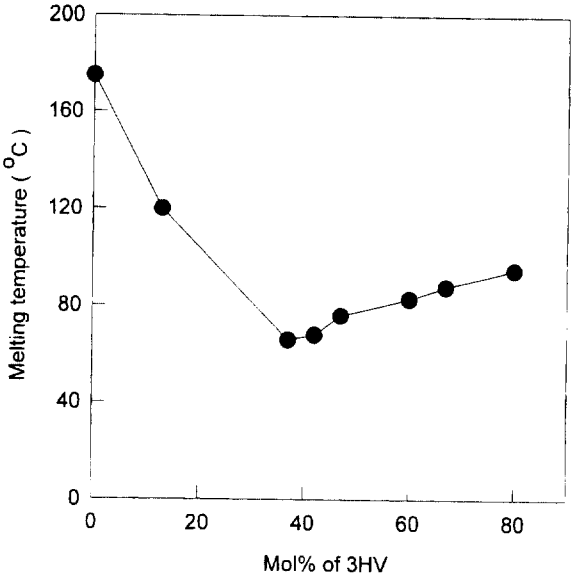

Fig. 2. Melting temperature (Tm) versus composition curves for poly(3HB-*co*-3HV) produced by *B. thuringiensis* R-510.

sources-glucose (30 g/L) and propionate (1 g/L). The rate of aeration was controlled by varing the agitation speed during the fermentation. When the agitation speed was set at 350 rpm throughout the experiment (Experiment 1), high amount of DCW exceeding 3.3 g/L was achieved within 14 h (Fig. 3A). However, the weight fraction of PHA in the cells was less than 18%. This low fraction of PHA in the cells was presumably caused by the increased rate of $NAD(P)H_2$ oxidation (1) and/or by the sporulation of the cells. In this case, glucose in the medium was consumed very slowly at a rate of 0.35 g/L/h. When the agitation speed was maintained at 200 rpm throughout the experiment (data not shown), the weight fraction of PHA in the cells increased to 25%, but cell yield was very poor (less than 2.0 g/L). On the other hand, when the agitation speed was changed from 350 rpm to 100 rpm after 6 h of growth (Experiment 2), the accumulation of PHA was stimulated immediately and the weight fraction of PHA in the cells reached a maximum of 38% after 27 h with continued increase of biomass (Fig. 3B). In this case, the spore formation in the cells did not take place throughout the fermentation, and the consumption rates of glucose and propionate were 0.60 g/L/h and 0.31 mM/L/h, respectively.

PHAs are usually biosynthesized if carbon sources are provided to the cells in excess and one nutrient essential for growth, such as nitrogen, phosphorus, or sulfur, is depleted (1, 14, 23). When the pre-cultured *B. thuringiensis* R-510 cells were transferred to media in which each of NH_4^+, PO_4^{3-}, SO_4^{2-}, K^+, Mg^{2+}, and Fe^{2+} was eliminated, the highest PHA production was obtained from the control culture in which all nutrients are supplemented (data not shown). This result reveals that PHA production by this organism is not so dependent upon nutritional limitations. From the present results it is apparent that low level of dissolved oxygen concentration (DOC) is more effective for PHA formation while high DOC level is more favorable for the cell growth in this organism. Moreover, these results suggest that low level of DOC triggers the synthesis of PHA by *B. thuringiensis* R-510.

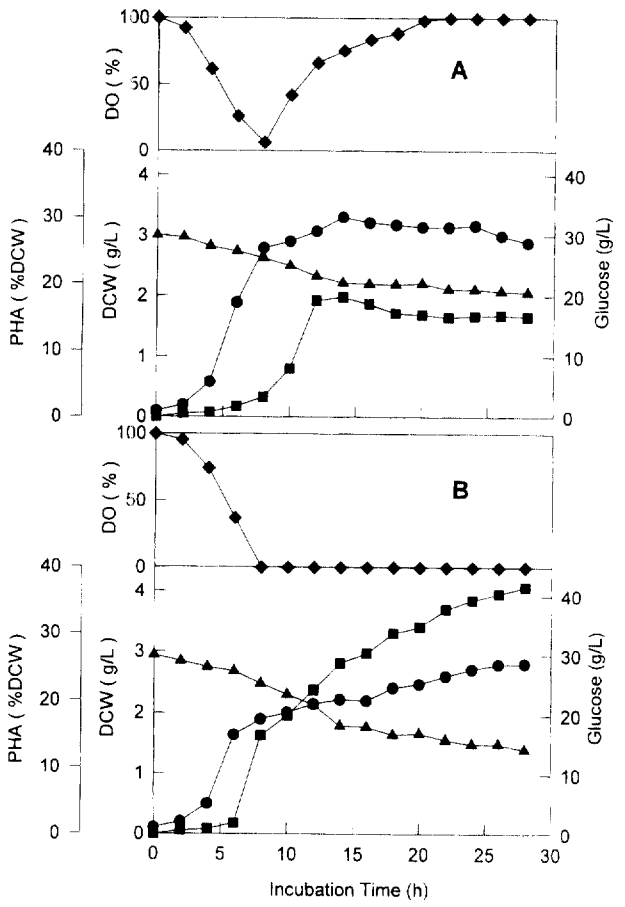

Fig. 3. Time courses of cell growth and poly(3HB-*co*-3HV) accumulation in fermentor cultures with different agitation speeds. In Experiment 1 (A), the agitation speed was set at 350 rpm throughout the fermentation. In Experiment 2 (B), the agitation speed was changed from 350 rpm to 100 rpm after 6 h of incubation to maintain DOC level in culture broth below 5% of air saturation. Symbols : ◆, DO (%); ▲, glucose; ●, DCW; ■, PHA (%DCW).

To evaluate the effect of DOC level on the composition of PHA, specific yields of 3HB per residual biomass ($Y_{3HB/RB}$) and 3HV per residual biomass ($Y_{3HV/RB}$) were determined with respect to culture time (Fig. 4). Residual biomass (RB) was obtained by substracting PHA weight from the DCW. The fractions of 3HV monomer in the copolymer

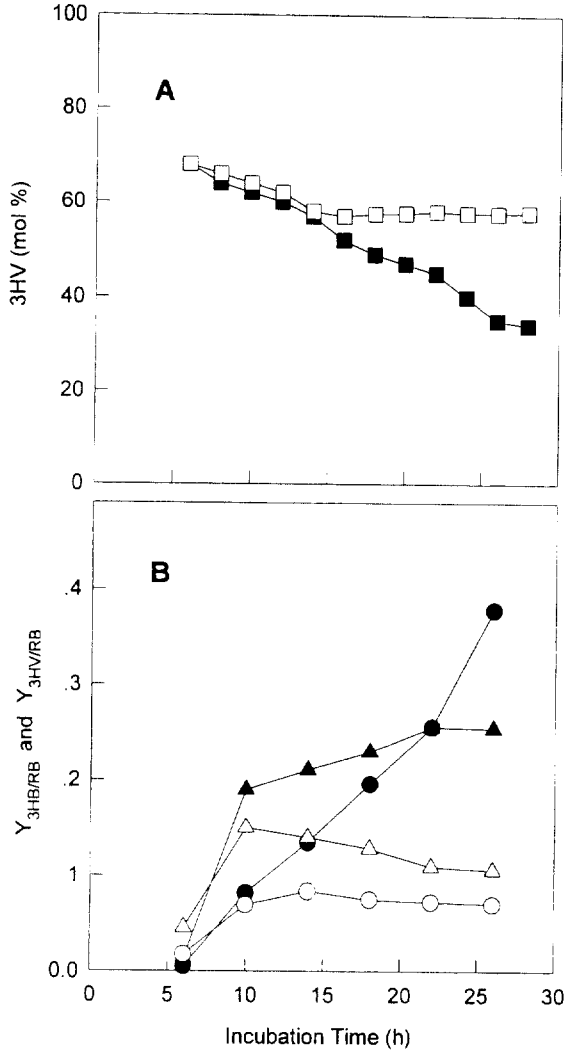

Fig. 4. (A) The molar fraction of 3HV in copolymers produced in Experiment 1 (□) and Experiment 2 (■) (see Fig. 3). (B) Profiles of 3HB and 3HV incorporation into copolymer during the fermentation processes of Experiment 1 (open symbols) and Experiment 2 (closed symbols). Symbols: ○ and ●, $Y_{3HB/RB}$; △ and ▲, $Y_{3HV/RB}$.

produced during Experiment 1 did not vary significantly and were maintained between 58 and 68 mol%. In contrast, the fractions of 3HV in Experiment 2 declined as synthesis of PHA continued, and the final 3HV content was 34 mol% after 27 h of incubation. During active PHA synthesis in Experiment 2 (after 10 h of incubation), significant change in RB did not occur, but a rapid increase of $Y_{3HB/RB}$ with slight increase of $Y_{3HV/RB}$ was observed, which were quite different from those in Experiment 1, in which $Y_{3HB/RB}$ and $Y_{3HV/RB}$ were either unchanged or decreased slightly after 10 h of incubation (Fig. 4B). From these results the decrease of 3HV content during PHA synthesis in Experiment 2 is attributable to more active incorporation of 3HB into the polymer than that of 3HV. Recently, a *Pseudomonas* sp. strain has been reported to possess two types of PHA synthase with different substrate specificities (10). This organism produces a blend of PHB homopolymer and copolymers of short- or medium-chain-length 3-hydroxyalkanoate units under certain culture conditions. In this study, it is apparent that compositions of copolymers produced by *B. thuringiensis* R-510 change according to incubation time. However, DSC thermograms of polymers isolated from fully grown cells showed one glass transition and one melting temperature (data not shown), which suggest that these polymers are random copolymers of 3HB and 3HV.

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