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Production of Poly-(β-Hydroxybutyric-Co-β-Hydroxyvaleric) Acids

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***Alcaligenes latus*, *Alcaligenes eutrophus*, *Bacillus cereus*, *Pseudomonas pseudoflava*, *Pseudomonas cepacia*, and *Micrococcus halodenitrificans* were found to accumulate poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acid [P(HB-co-HV)] copolymer when supplied with glucose (or sucrose in the case of *A. latus*) and propionic acid under nitrogen-limited conditions. A fed-batch culture of *A. eutrophus* produced 24 g of poly-β-hydroxybutyric acid (PHB) liter^{-1} under ammonium limitation conditions. When the glucose feed was replaced with glucose and propionic acid during the polymer accumulation phase, 17 g of P(HB-co-HV) liter^{-1} was produced. The P(HB-co-HV) contained 5.0 mol% β-hydroxyvaleric acid (HV). Varying the carbon-to-nitrogen ratio at a dilution rate of 0.15 h^{-1} in a chemostat culture of *A. eutrophus* resulted in a maximum value of 33% (wt/wt) PHB in the biomass. In comparison, *A. latus* accumulated about 40% (wt/wt) PHB in chemostat culture under nitrogen-limited conditions at the same dilution rate. When propionic acid was added to the first stage of a two-stage chemostat, *A. latus* produced 43% (wt/wt) P(HB-co-HV) containing 18.5 mol% HV. In the second stage, the P(HB-co-HV) increased to 58% (wt/wt) with an HV content of 11 mol% without further addition of carbon substrate. The HV composition in P(HB-co-HV) was controlled by regulating the concentration of propionic acid in the feed. Poly-β-hydroxyalkanoates containing a higher percentage of HV were produced when pentanoic acid replaced propionic acid.**

Poly-β-hydroxyalkanoates (PHAs) are of commercial value as thermoplastics (10, 11). Certain PHA copolymers have more generally useful thermomechanical properties than do poly-β-hydroxybutyric acid (PHB) homopolymers (2, 12). At present, the PHA copolymer of greatest industrial interest is poly-(β-hydroxybutyric-co-β-hydroxyvaleric) acid [P(HB-co-HV)]. The major costs of PHA production are the substrate (5) and the separation process.

Because of its high yield and rapid production rate, *Alcaligenes eutrophus* has been the species of choice for P(HB-co-HV) production (P. A. Holmes, S. H. Collins, and W. F. Wright, European patent 69,497, April 1987). However, this culture uses only a limited variety of sugars as its carbon source, and although mutants of *A. eutrophus* grow on both glucose and fructose, they cannot hydrolyze sucrose (8). The optimal growth temperature of *A. eutrophus* is only 30°C. Therefore, low-temperature cooling water or a fermentor with very good heat exchange capacity would be required to cool the high-density, rapidly growing cultures that would be used industrially. In addition, it has been reported that strains of *A. eutrophus* are inhibited by as little as 0.1% (wt/wt) propionic acid (5), which must be added to the fermentation medium to allow the production of P(HB-co-HV). Until recently, little had been done to examine the ability of other microorganisms to produce P(HB-co-HV) (9). Thus, it is important to investigate the capability of other bacteria to produce P(HB-co-HV) so as to provide a wider choice of cultures which can potentially be used in a production process.

PHB can be produced by heterotrophic microorganisms in quantities up to only about 10 g liter^{-1} by using simple batch fermentation techniques. The maximum quantity obtainable is limited by the yield of biomass from substrate and by the maximum initial concentration of carbon source that can be used without adversely affecting growth. Propionic or pentanoic acids are required as substrates in the production of P(HB-co-HV). Since these organic acids are toxic at relatively low concentrations (7, 13), simple batch fermentations cannot produce significant quantities of P(HB-co-HV). Fed-batch culture has been reported to produce over 25 g of PHB liter^{-1} from glucose, but few details of the feeding regimen were given (1). Fed-batch culture of the methylotroph *Protomonas extorquens* has been studied extensively and was reported to produce up to 149 g of PHB liter^{-1} (16–19). Theoretically, high concentrations of PHA could be produced in continuous-cultivation systems. Such systems would be especially desirable commercially because they are potentially more productive than batch systems. Unfortunately, there is a lack of published information relating either to the fed-batch production of P(HB-co-HV) or to the continuous production of any PHA. This paper reports on a variety of techniques for the production of P(HB-co-HV) copolymers by heterotrophic bacteria. These production methods can potentially be used in the laboratory or as a basis for the development of commercial processes. In addition, several species of bacteria known to accumulate PHB were tested for their abilities to produce P(HB-co-HV).

MATERIALS AND METHODS

Microorganisms. *Alcaligenes latus* ATCC 29714, *A. eutrophus* DSM 545, *Bacillus cereus* NRC 9008, *Pseudomonas pseudoflava* ATCC 33668, *Pseudomonas cepacia* ATCC 17759, and *Micrococcus halodenitrificans* NRC 14024 were the microorganisms used in this study.

Shake flask experiments. Each liter of mineral salts medium contained 6.7 g of $Na_2HPO_4 \cdot 7H_2O$, 1.5 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 60 mg of ferrous ammonium citrate, 10 mg of $CaCl_2 \cdot 2H_2O$, and 1 ml of trace-element solution. Each liter of trace-element solution contained 0.3 g of H_3BO_3, 0.2 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $ZnSO_4 \cdot 7H_2O$, 30 mg of $MnCl_2 \cdot 4H_2O$, 30 mg of

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$NaMoO_4 \cdot 2H_2O$, 20 mg of $NiCl_2 \cdot 6H_2O$, and 10 mg of $CuSO_4 \cdot 5H_2O$. After different concentrations of propionic acid were added to flasks containing 90 ml of mineral salts medium, the pH was adjusted to 7.0 and the flasks were autoclaved for 20 min at 121°C. Once the medium was cooled, 10 ml of glucose (or sucrose for *A. latus*) solution was added to give a final concentration of 10 g liter^{-1}. Portions (10 ml each) of exponential-phase cells grown in nutrient broth (Lab-Lemco CM15; Oxoid Canada, Inc., Nepean, Ontario, Canada) containing 1 g of glucose (or sucrose) and 1 g of yeast extract liter^{-1} were used as inocula. Once the flasks were inoculated, they were incubated at 30°C and 160 rpm for a period of 48 to 72 h.

Fermentation conditions. To produce PHB, fed-batch fermentations of *A. eutrophus* were conducted in a 20-liter Bioengineering reactor at 30°C and pH 7.0. The agitation and aeration were manually controlled to keep the dissolved-oxygen concentration above 40% air saturation. The mineral salts medium was the same as that described above, except that there were 2.5 g of $Na_2HPO_4 \cdot 7H_2O$ and 0.83 g of KH_2PO_4 liter^{-1}. Initially, there were 30 g of glucose and 9 g of $(NH_4)_2SO_4$ liter^{-1} in the reactor. Once the glucose and ammonium neared exhaustion, more was added to bring the concentrations to 30 g of glucose and 3 g of ammonium liter^{-1}. When the glucose and ammonium neared exhaustion a second time, a concentrated glucose solution (500 g liter^{-1}) was added continuously to keep the concentration in the reactor between 5 and 16 g liter^{-1}.

The fed-batch fermentation to produce P(HB-co-HV) with *A. eutrophus* was performed in a 3.5-liter Chemap reactor. The growth medium, growth conditions, and feeding regimen were the same as for PHB production, except that after the second exhaustion of glucose and ammonium, 132 g of glucose and 18 g of propionic acid liter^{-1} were added at a constant rate of 35 ml h^{-1}.

Chemostat culture of *A. latus* was done in a 3.5-liter Chemap fermentor at 33°C and pH 7.0. The airflow rate was set at 1.25 liter min^{-1}, and the agitation was between 450 and 600 rpm. The mineral salts medium was the same as that used in the shake flask experiments, except that it contained 3.7 g of $Na_2HPO_4 \cdot 7H_2O$, 0.83 g of KH_2PO_4, and 2.0 g of $(NH_4)_2SO_4$. The medium also contained 20 g of sucrose liter^{-1}. The concentration of propionic acid was varied at a dilution rate of 0.15 h^{-1}. Samples were taken after at least 6 fermentor volumes of medium containing a specific amount of propionic acid has been added to the fermentor. The fermentor volume was maintained at 2.1 liters by a weight control device.

For two-stage chemostat operation, the second stage was fed with the effluent from the first stage. Operating conditions and reactor type were the same as in the first stage. No additional medium was added to the second stage.

Biomass and cellular protein measurement. To measure biomass, a volume of culture broth between 2 and 20 ml was centrifuged, suspended in 10 ml of 0.9% (wt/vol) saline solution, recentrifuged, suspended in 10 ml of distilled water, and recentrifuged. The pellet was then transferred to a preweighed aluminum dish and dried to a constant mass at 105°C. For protein measurements, 2 to 10 ml of culture broth was centrifuged. The pellet was washed with distilled water, recentrifuged, and analyzed for protein content by the biuret reaction (15) with bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) as the standard.

PHA analysis. PHA samples were prepared by the method of Braunegg et al. (4). The resulting methyl esters of the PHA monomers were quantified by gas chromatography. The helium flow rate through the 25-m-long HP5 capillary column (Hewlett-Packard Co., Palo Alto, Calif.) was 0.9 ml min^{-1}. The flame ionization detector was supplied with 500 ml of air, 30 ml of hydrogen, and 30 ml of auxillary helium min^{-1}. The injection split ratio was 120:1, and 1-μl infections were made. The injection port temperature was 180°C, and the detector temperature was 200°C. The initial oven temperature was 90°C (maintained for 1 min), with increases of 8°C min^{-1} to a final temperature of 150°C (maintained for 5 min). The internal standard was benzoic acid, and the external standards were β-hydroxybutyric acid (Sigma) and a P(HB-co-HV) copolymer (Biopol; Imperial Chemical Industries). The retention times of the methyl esters of β-hydroxybutyric (HB), β-hydroxyvaleric (HV), and benzoic acids were 3.7, 4.9, and 7.2 min, respectively.

Propionic acid analysis. Two volumes of methanol acidified with 2% (vol/vol) concentrated H_2SO_4 were mixed with one volume of supernatant (obtained from the biomass analysis) in screw-cap tubes. Once securely closed, these tubes were placed in an oven at 60°C for 1 h. After cooling, 1 volume each of distilled water and dichloromethane was vigorously mixed with the contents of the tubes. The organic phase was separated and analyzed by gas chromatography. The internal standard was butyric acid, and the external standard was propionic acid. The capillary column and conditions were the same as those used for PHA analysis, except that the injection temperature was 170°C and the initial oven temperature was 40°C (maintained for 1 min), with a rate of increase of 2.5°C min^{-1} to a final temperature of 60°C.

Carbohydrate analysis. Sucrose, glucose, and fructose concentrations were determined by high-pressure liquid chromatography with an Aminex 87P column [Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada] at 80°C and a Waters R-401 refractive-index dectector, with water as the mobile phase.

Other analyses. Ammonium ion concentration in the culture broth was quantified by using a modified Berthelot reaction (14).

RESULTS

PHA production by diverse microorganisms. Table 1 shows the ability of six bacterial strains which accumulate PHB to also accumulate P(HB-co-HV) in shake flasks with glucose (or sucrose for *A. latus*) and propionic acid as carbon substrates. The effect of propionic acid on growth and accumulation of P(HB-co-HV) varied with each microorganism. The P(HB-co-HV) produced by *A. latus* had 3.4 mol% HV monomers, while that of *B. cereus* contained up to 50 mol% HV monomers, although relatively little polymer was produced. While the minimum concentration of propionic acid which reduced P(HB-co-HV) production by *A. latus* was only 0.01% (wt/vol), PHA production was completely inhibited above 0.05% (wt/vol) propionic acid. On the other hand, with *B. cereus*, inhibition of PHA production began at 0.1% (wt/vol) propionic acid and was totally stopped above a concentration of 3% (wt/vol). The ability of *B. cereus* to produce P(HB-co-HV) in the presence of up to 3% (wt/vol) propionic acid may account for the high amounts of HV in its polymer compared with that of *A. latus*. *A. eutrophus* and *P. pseudoflava* accumulated similar amounts of P(HB-co-HV) (43% [wt/wt]) with the same ratio of HV monomers (45 mol%). However, for *A. eutrophus*, growth and production were inhibited by 0.3% (wt/vol) propionic acid, while for *P. pseudoflava*, they were inhibited by 0.1% (wt/vol) propionic acid.

TABLE 1. Accumulation of P(HB-co-HV) by diverse bacterial species

Species	Maximum PHA concn (% [wt/wt] of biomass [dry wt])	Maximum HV content (mol% of total PHA)	Minimum propionic acid concn (% [wt/vol]) inhibiting:		Concns tested (% [wt/vol])	Incubation time (h)
			PHA accumulation[a]	Growth[b]		
B. cereus	13	50	0.1	>0.5	0–1	72
M. halodenitrificans	21	9	0.3	>0.5	0–1	48
P. cepacia	64	30	0.03	0.5	0–0.7	48
P. pseudoflava	43	45	0.1	0.1	0–2	48
A. latus	53	3	0.01	0.01	0–1	48
A. eutrophus	43	45	0.3	0.3	0–1	48

[a] Lowest concentration at which the amount of PHA accumulated was less than that of the control, to which no propionic acid was added.
[b] Lowest concentration at which the biomass was less than that of the control.

Fed-batch production of PHA. *A. eutrophus* was found to produce up to 24 g of PHB liter^{-1} in fed-batch culture (Fig. 1). During the accumulation phase, the glucose concentration in the reactor was kept between 5 and 16 g liter^{-1} by the constant addition of a 50% (wt/vol) glucose solution. The maximum rate of PHB production was about 2 g liter^{-1} h^{-1}, and the maximum specific rate of PHB production was 0.14 g liter^{-1} h^{-1} g of cellular protein^{-1}.

In a similar fermentation (Fig. 2), a concentrated mixture of 1.8% (wt/vol) propionic acid and 13.2% (wt/vol) glucose was added to a fermentor containing high-protein biomass. This time, the additional carbon source was added at a lower rate to keep the propionic acid from reaching an inhibitory concentration. This resulted in the production of 17 g of PHA liter^{-1} containing 5.0 mol% HV monomer. The maximum rate of PHA accumulation was 0.74 g liter^{-1} h^{-1}, or about 0.05 g h^{-1} g of cellular protein^{-1}. The rate of accumulation was limited by the rate of substrate addition until about the 50-h mark in the fermentation, when the glucose concentration in the fermentor began to rise. At that time, the rate of P(HB-co-HV) accumulation began to decrease progressively. This change in the rate of accumulation was also observed with PHB production (Fig. 1). No measurable quantity of propionic acid was ever accumulated in the production of P(HB-co-HV). About 0.1 g of HV monomer was produced for each gram of propionic acid metabolized. This corresponds to a molar yield of 0.074 mol mol of propionic acid^{-1}.

One-stage chemostat production of PHA. *A. eutrophus* was grown in a chemostat at a dilution rate of 0.15 h^{-1}, well below the critical dilution rate (μ_{max}) of 0.35 h^{-1}. No measurable PHB was accumulated under carbon-limited conditions (Fig. 3). Ammonium became limiting at a glucose concentration of just over 8 g liter^{-1}. Above this concentration, the production of cellular protein remained constant and the rate of PHB accumulation increased as the glucose concentration in the feed was increased. Above a glucose feed concentration of 18 g liter^{-1}, no further increase in PHB

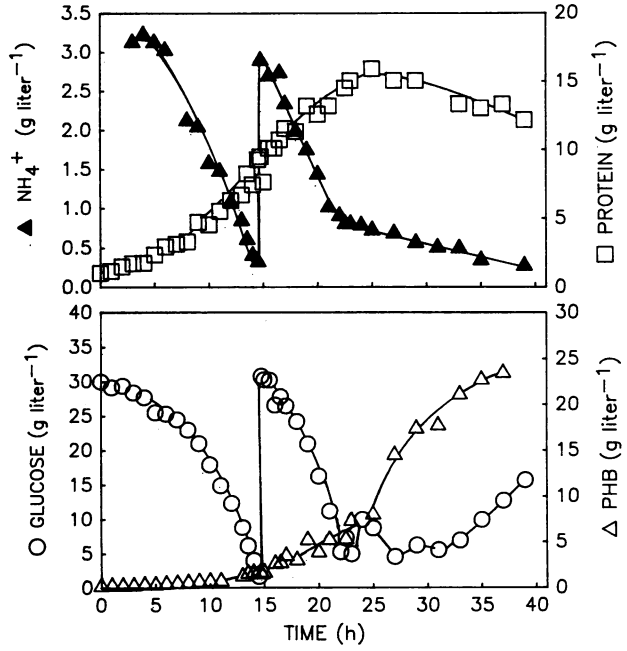

FIG. 1. Production of PHB by *A. eutrophus* by using a fed-batch mode of fermentation. Once the initial 30 g of glucose and 9 g of $(NH_4)_2SO_4$ liter^{-1} neared exhaustion at 15 h, their concentrations were increased to original levels. When the glucose and ammonium neared exhaustion a second time at 23 h, glucose was added continuously to keep the concentration in the reactor between 5 and 16 g liter^{-1}.

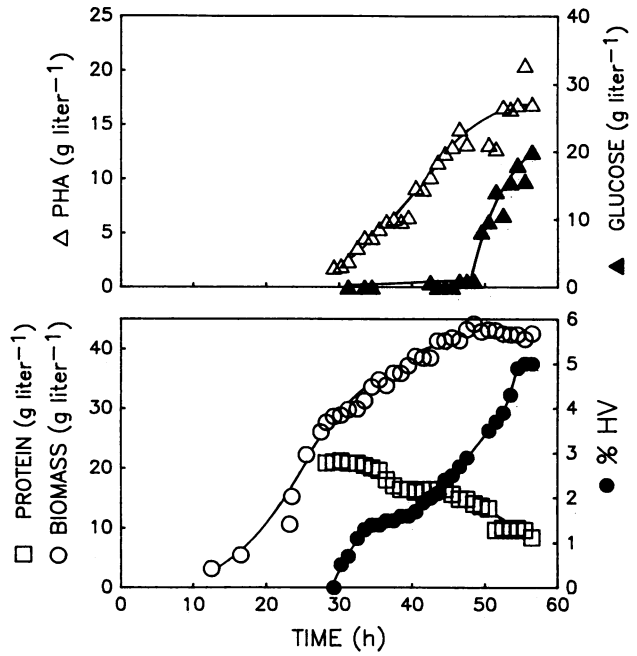

FIG. 2. Production of P(HB-co-HV) by *A. eutrophus* in a fed-batch fermentation. The feed regimen was the same as in PHB production, except that after the second exhaustion at 30 h, 132 g of glucose and 18 g of propionic acid liter^{-1} were added at a constant rate of 35 ml h^{-1}.

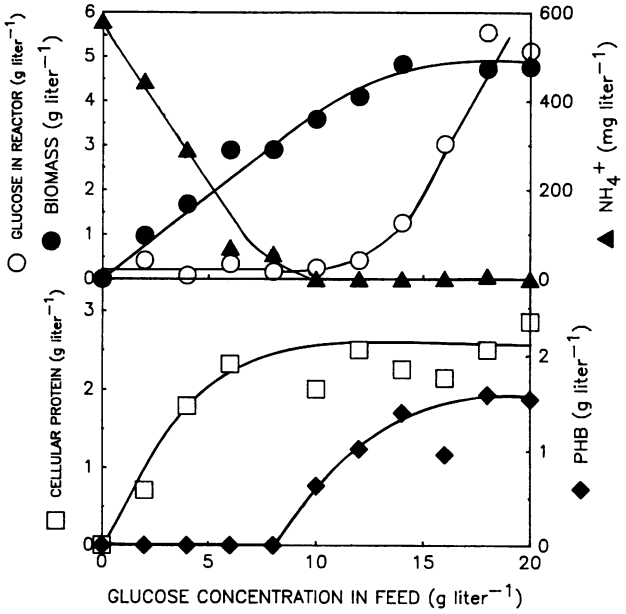

FIG. 3. Effect of glucose feed concentration on chemostat production of PHB by *A. eutrophus* (dilution rate, 0.15 h^{-1}).

accumulation occurred. At that point, 0.6 g of PHB g of cellular protein^{-1} (equivalent to 33% [wt/wt] of the total biomass) was produced.

A. latus was grown in a chemostat at the same dilution rate of 0.15 h^{-1} with sucrose and various amounts of propionic acid. The sucrose concentration in the reactor was about 5 g liter^{-1}, with no detectable NH_4^+ or propionic acid. When no propionic acid was added, the dry biomass contained 40% (wt/wt) PHB, and approximately 0.8 g of PHB g of cellular protein^{-1} was produced (Fig. 4). The amount of HV monomer present in the PHA increased from 0 to 20 mol% as the propionic acid feed concentration increased from 0 to 5 g liter^{-1}.

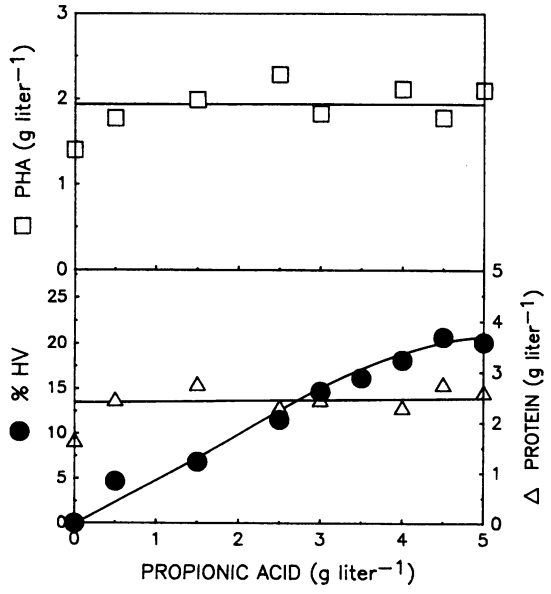

FIG. 4. Effect of propionic acid concentration in the sucrose feed on the composition and production of P(HB-co-HV) in an ammonium-limited chemostat culture of *A. latus* (dilution rate, 0.15 h^{-1}). No residual propionic acid was present in the reactor.

Two-stage chemostat production of PHA. At a feed concentration of 8.5 g of propionic acid liter^{-1}, all the acid was metabolized but there was 5.8 g of unused sucrose liter^{-1} in the first reactor. Only 0.6 g of PHA g of cellular protein^{-1} was produced. The PHA contained 18.5 mol% HV and was 43% (wt/wt) of the total biomass.

The contents of the reactor were continuously fed into a second chemostat in which the sucrose was totally consumed to increase the total production to 1.2 g of PHA g of cellular protein^{-1}, or 58% (wt/wt) of the total biomass. Since no propionic acid was fed into the second stage, there was no further production of HV monomer, resulting in a PHA containing 11 mol% HV.

P(HB-co-HV) production by using pentanoic acid. In a single-stage chemostat with sucrose and 3 g of propionic acid liter^{-1} in the feed, *A. latus* produced a PHA with 15 mol% HV. When propionic acid was replaced with 3 g of pentanoic acid liter^{-1}, the same amount of PHA was produced but the HV content had increased to 38 mol%.

DISCUSSION

All six PHB-accumulating bacterial strains tested showed the ability to synthesize P(HB-co-HV) copolymers when propionic acid was supplied in the medium. This indicates that the capacity to produce P(HB-co-HV) is a characteristic of most PHB-accumulating microorganisms. This hypothesis is supported in a recent publication by Haywood et al. (9) which reported that although two *Bacillus* spp. and a *Methylobacterium* sp. accumulated PHB but not P(HB-co-HV), 13 other bacterial strains (representing six genera) capable of producing PHB were able to produce P(HB-co-HV). The reporting of the six cultures in this paper adds four microorganisms to the list begun by Haywood et al. (9).

Although no kinetic study was done, the shake flask experiments indicated that significant differences exist between different species in their propensities for HV synthesis or incorporation into the polymer chain or both. For example, at lower concentrations of propionic acid, *B. cereus* had a much higher percentage of HV monomer in its PHA than did the other bacterial strains. The affinity constants of one or more key enzymes involved in copolymer synthesis may be substantially lower for this *B. cereus* strain than for the other microorganisms tested. A high yield of HV monomer from a given amount of propionic acid is a distinct advantage in an industrial production process. Since propionic acid is a relatively expensive substrate, its use for other purposes such as energy production or formation of acetyl-coenzyme A leading to HB monomer synthesis increases production costs.

Ability to retain viability in the presence of high concentrations of propionic acid is another desirable characteristic of microorganisms to be used in industrial P(HB-co-HV) production. Again, *B. cereus* performed well, growing in the presence of at least 5 g of propionic acid liter^{-1}, although PHA synthesis was inhibited at lower concentrations.

A large percentage of the cost of P(HB-co-HV) production is due to the separation process. For this reason and to reduce substrate costs, it is imperative that the production strain be able to accumulate at least 60% (wt/wt) of its dry-weight biomass as P(HB-co-HV). No gram-positive bacterium has been shown to accomplish this. Although *B. cereus* has several desirable qualities, it has not been shown

to accumulate large amounts of PHA and hence would not be considered for an industrial P(HB-co-HV) production process. The reasons that, under optimal conditions, some bacterial species accumulate as much as 90% (wt/wt) (6) of their dry-weight biomass as PHB while others cease accumulation at 10% (wt/wt) or less (9) have never been elucidated.

Either fed-batch or continuous systems could be used for commercial PHB production. Simple batch systems produce low biomass concentrations, since there is a limit to the initial substrate concentration that can be used. In the fed-batch approach, the fermentor can be initially charged with all the medium components necessary for growth and production except the carbon and nitrogen sources, whose levels become critically low after a certain time. For example, during PHB production (Fig. 1), the carbon and nitrogen substrates approached growth-limiting levels after 15 h of fermentation. At this point, additional carbon and nitrogen sources were quickly added to allow the culture to produce more high-protein biomass. In order to minimize PHA accumulation during the growth phase, the concentration of ammonium in the fermentor should not be allowed to fall below 200 mg liter^{-1}. Otherwise, the final yield is diminished. During the accumulation phase, concentrated glucose is continually added to produce PHB. A mixture of glucose and propionic acid (or pentanoic acid) may be added for P(HB-co-HV) production.

Most bacteria accumulate PHB even when they are not limited by such factors as nitrogen or phosphate, albeit at a lesser rate. Thus, the bacterial biomass contained some PHB homopolymer before any propionic acid was added (Fig. 2). This explains why the amount of HV monomer increased from 0 to 5.0 mol% during the P(HB-co-HV) accumulation phase of *A. eutrophus*. Such production would result in a mixture of PHA polymers with various HV contents. To achieve a more homogeneous population, the culture should be starved of carbon before the onset of the PHA accumulation phase. The endogenous PHB reserves would then be reduced to an insignificant concentration. As shown by the chemostat study, the HB/HV monomer ratio depends on the sugar source/propionic acid ratio that is fed into the fermentor. The rate of feeding in fed-batch culture should be less than the maximum possible rate of substrate utilization to avoid a buildup of toxic substrate (in this case, propionic acid) and to ensure a constant HB/HV production ratio.

The commercial production of PHA copolymers by a continuous process is attractive, since it eliminates the downtime which occurs between batch and fed-batch fermentations and since productivity is high for much longer periods. In the chemostat experiments previously described, copolymer composition was controlled by the concentration and type of organic acid in the medium feed. *A. eutrophus* accumulated 33% (wt/wt) of its biomass as PHB, while *A. latus* produced about 20% (wt/wt) more PHA in a single-stage chemostat than did *A. eutrophus*. Most bacteria, such as *A. eutrophus*, do not accumulate high concentrations of PHA in a single-stage chemostat, because high growth rate and maximum PHA accumulation cannot occur simultaneously. Some bacteria, such as *A. latus*, which constitutively amass substantial quantities of PHA during their growth phases when not under nutrient limitation (3), are better suited for single-stage-chemostat PHA production. However, even with *A. latus*, the addition of a second stage to the chemostat resulted in an increase in the PHA concentration in the cells.

In terms of overall performance, the chemostat and fed-batch modes of production make similar amounts of PHA gram of substrate consumed^{-1}. However, the chemostat is a much better system for studying the factors which control growth and PHA production, because the physiological conditions in the reactor are kept constant and hence it is possible to vary one parameter at a time. The chemostat is also a relatively simple method of producing substantial quantities of different PHA copolymers for research purposes.

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