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Mass Production of Poly(3-Hydroxybutyrate) by Fed-Batch Cultures of *Ralstonia eutropha* with Nitrogen and Phosphate Limitation

RYU, HEE WOOK[1], KYUNG-SUK CHO[2], BEOM SOO KIM[3], YONG KEUN CHANG[4]*, HO NAM CHANG[4], AND HYUN JOO SHIM[5]

[1]*Department of Chemical and Environmental Engineering, Soong Sil University, Seoul 156-743, Korea*
[2]*Department of Environmental Science and Engineering, Ewha Womans University, Seoul 120-750, Korea*
[3]*Department of Chemical Engineering, Konyang University, Nonsan, Chungnam 320-711, Korea*
[4]*Department of Chemical Engineering and BioProcess Engineering Research Center, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea*
[5]*Department of Textile Engineering, Soong Sil University, Seoul 156-743, Korea*

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Abstract For mass production of poly(3-hydroxybutyrate) (PHB), high cell density cultures of *Ralstonia eutropha* were carried out in 2.5-l and 60-l fermentors by two fed-batch culture techniques of nitrogen and phosphate limitation. When the nitrogen limitation technique was employed using both an on-line glucose monitoring and control system, a high concentration level of PHB (121 g/l) was obtained in the small-scale fermentor of 2.5 l. However, the PHB concentration obtained in a large-scale fermentor of 60 l only turned out to be 60 g/l. In contrast, when another fed-batch culture technique of the phosphate-limitation employing dissolved oxygen (DO) stat glucose feeding was used, a large amount of PHB was successfully produced in both 60-l and 2.5-l fermentors. In a 2.5-l fermentor, concentrations of PHB and cells obtained in 58 h were 175 and 210 g/l, respectively, which corresponded to the PHB productivity level of 3.02 g/l/h. In a 60-l fermentor, a final cell concentration of 221 g/l and a PHB concentration of 180 g/l with PHB productivity level of 3.75 g/l/h were obtained in 48 h. PHB content and yield from glucose were 81% and 0.38 g PHB/g glucose, respectively. These data suggest that the phosphate limitation technique is more effective compared to nitrogen limitation in the mass production of PHB by *R. eutropha* of a large scale.

Key words: Poly(3-hydroxybutyrate), *Ralstonia eutropha*, fed-batch culture, phosphate limitation

Polyhydroxyalkanoates (PHAs) are biodegradable thermoplastic and biocompatible polyesters which have been drawing much attention because their physical properties are close to that of conventional plastics. Poly(3-hydroxybutyrate) (PHB) is an intracellular carbon and energy storage material accumulated by many microorganisms under unfavorable growth conditions, such as limitation of N, P, S, Mg, or O_2 [1, 8]. Numerous bacteria such as *Ralstonia eutropha* [5-7, 9, 12, 13, 21, 22], *Alcaligenes latus* [10, 33], *Azotobacter vinelandii* [23-25], *Azotobacter chroococcum* [14, 18], *Methylotrophs* [15-16, 30-32], *Pseudomonads* [26], *Rhodobacter sphaeroides* [17], and recombinant *Escherichia coli* [11, 19, 27, 34] synthesize and accumulate PHA. Depending on what type of the carbon source is used, various kinds of PHA can be produced. *R. eutropha* has been the most widely used organism for PHB production, because it accumulates a large amount of PHB (up to 80% dry cell weight) in a simple glucose-salt medium. The pathway and regulation of PHB synthesis in *R. eutropha* have been studied in full detail [27, 28].

One of the problems hindering commercialization of PHA is the high production cost compared to that of the conventional nonbiodegradable plastics. Much effort has recently been made to develop an economical process for the production of PHA [11-13, 30-32]. Improving productivity of the fermentation process and developing an efficient recovery procedure will make it economically more feasible.

Fed-batch culture has been the most popular method used to achieve a high productivity level of desired bioproduct [35]. Several strategies in which the substrate is intermittently or continuously fed into the fermentor based on monitoring of the dissolved oxygen (DO), pH, or a carbon source concentration as a feedback parameter have been developed. DO-stat and pH-stat are simple, but these two methods cannot effectively maintain the substrate concentration at a desired level because of their nature being an indirect indication of the substrate concentration.

*Corresponding author
Phone: 82-42-869-3927, 8812; Fax: 82-42-869-8800;
E-mail: ychang@sorak.kaist.ac.kr

On-line methods which can directly measure and control the substrate concentration in the culture broth at a desired level seem to be more desirable. Several fed-batch culture methods using an on-line glucose monitoring system were used for the PHA production [12, 13, 30-32]. Previously, a report was made in regards to the fed-batch culture of *R. eutropha* with an on-line glucose concentration monitoring and control system which gave a high productivity level of 2.42 g/l/h in a 2.5-l fermentor under nitrogen limitation [12]. Using the same cultivation strategy, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] could be efficiently produced from glucose and propionic acid [13]. Although various methods for an efficient production of PHA have been developed in academia, most of them have not been evaluated in a large-scale fermentor.

In this study, a nitrogen limitation was applied to fed-batch cultures of *R. eutropha* in a large-scale fermentor of 60 l. PHB production was also studied by applying phosphate limitation in fermentors of 2.5 l and 60 l, and the results were compared with those by nitrogen limitation.

MATERIALS AND METHODS

Organism and Media

The strain used in this study was identified as *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) NCIMB 11599. The medium composition for seed cultures and the initial medium compositions for fed-batch cultures are shown in Table 1. The trace element solution contained (per liter of 5 N HCl solution) 10 g $FeSO_4 \cdot 7H_2O$, 2 g $CaCl_2 \cdot 2H_2O$, 2.25 g $ZnSO_4 \cdot 7H_2O$, 0.5 g $MnSO_4 \cdot 4\text{-}5H_2O$, 1.0 g $CuSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}$, and 0.2 g $Na_2B_4O_7 \cdot 7H_2O$.

Fed-Batch Cultures

The seed cultures were prepared in 250-ml flasks on a reciprocal shaker and in a 5-l fermentor (Korea Fermentor Co., Ltd, Korea) with a 2.25-l working volume at 34°C for 24-30 h, and then used to inoculate the fermentors. The initial volumes for the 2.5-l and 60-l fermentors were 0.8 and 18 l, respectively. Temperature and pH were controlled at 34°C and 6.8, respectively. Dissolved oxygen (DO) concentration was measured with a dissolved oxygen concentration meter (DKK Corporation, Japan) and was maintained over 10% of air saturation by manipulating both the agitation speed and aeration rate. Pure oxygen was used when required.

Fed-batch cultures were performed under nitrogen limitation or phosphate limitation. The nitrogen limitation fed-batch cultures with an on-line glucose control were carried out in a 60-l fermentor. A phosphate-rich salt medium was used as the initial medium [12]. Culture broth was continuously taken out and recycled by using a peristaltic pump at a speed of ca. 100 ml/min through a homemade cross-flow filtration unit with a ceramic tubular membrane (pore size 0.4 mm, 6 mm×80 mm, Ashai Kasei Co., Japan). This was done to analyze filtrate for glucose. Glucose concentration in the culture broth was monitored every 10 min by using automatic injection of a fixed volume of the filtrate to a glucose analyzer (Model 2700, Yellow Springs Instruments, U.S.A.). A concentrated glucose solution (700 g/l) was fed to control the glucose concentration of the culture broth at a desired value using a peristaltic pump (Cole-Parmer Instrument Co., U.S.A.) that was interfaced to a personal computer. The pH was controlled with 2 N HCl and 28% NH_4OH solution. The latter was replaced by a 10 N NaOH/KOH (1:1) solution throughout the nitrogen limitation period. The fed-batch cultures with phosphate limitation were carried out in 2.5-l and 60-l fermentors. The phosphate-free salt medium (Table 1) was used as the initial medium with an addition of desired amounts of phosphate. The pH was controlled with 2 N HCl and 28% NH_4OH throughout the entire fermentation process. The feeding of glucose was done by employing a DO-stat method. When glucose became exhausted and the DO level suddenly increased, then a concentrated glucose solution (800 g/l) was fed into the fermentor. An appropriate amount of the feed solution, which was equivalent to 15-g glucose/l in the culture broth, was added each time.

Table 1. Medium compositions for seed and fed-batch cultures.

Components	Seed culture	Fed-Batch culture[a]	
		Phosphate-rich	Phosphate-free
Glucose (g/l)	10	20	20
$(NH_4)_2SO_4$ (g/l)	1	4	4
$MgSO_4 \cdot 7H_2O$ (g/l)	0.2	1.2	1.2
KH_2PO_4 (g/l)	1.5	13.3	-
$K_2HPO_4 \cdot 12H_2O$ (g/l)	9	-	-
Citric acid (g/l)	-	1.7	1.7
Trace element soln (ml/l)	1	10	10

[a]Initial composition.

Analytical Procedures

Cell growth was monitored by measuring the optical density at 600 nm with a spectrophotometer (Beckman, U.S.A.). Cell concentration was determined by measuring the dry cell weight (DCW) of a 5-ml culture broth. The broth sample was centrifuged, washed with distilled water, and dried under a vacuum at 60°C until no further decrease in weight was shown. The phosphate concentration was measured by ion chromatography with a conductivity detector (Waters 431, U.S.A.) using KH_2PO_4 as a standard. PHB content was determined by using a gas chromatograph (Varian 3300, U.S.A.) with benzoic

acid as the internal standard [3]. Measurement of glucose concentration in the medium was carried out with a glucose analyzer (Model 2700, Yellow Springs Instruments, U.S.A.).

RESULTS

PHB Production with Nitrogen Limitation

Figure 1 shows time courses of the cell, PHB, and residual biomass (total cell mass minus PHB) concentrations when nitrogen limitation was applied at a relatively low cell concentration of 25 g/l. After a short period, ammonium in the culture broth became depleted and, when this occurred, PHB started to accumulate significantly. The final cell concentration, PHB concentration, and PHB content at 55 h were 85 g/l, 60 g/l, and 70% of the dry cell weight, respectively. The PHB productivity was 1.1 g/l·h. To obtain a higher concentration of PHB, nitrogen limitation was applied at a cell concentration of 50 g/l at 30 h (Fig. 2). After the nitrogen feeding stopped, the cell concentration increased up to 83 g/l at 39 h and thereafter decreased. A significant cell lysis was observed. The final PHB content was only 58% with a PHB productivity of 1.2 g/l/h. Similar results in other runs were obtained, but production of the appreciable amount of PHB was not possible (data not shown).

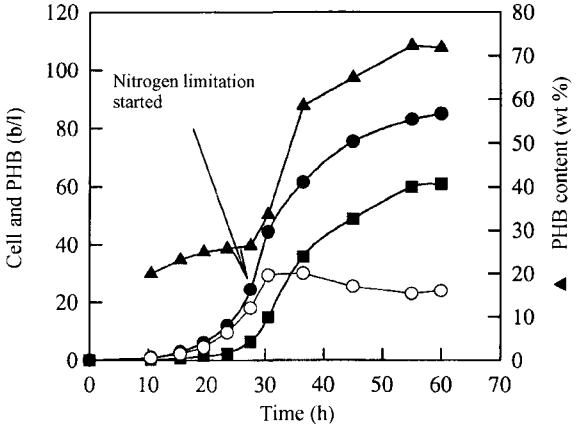

Fig. 1. Fed-batch culture of *R. eutropha* with nitrogen limitation and on-line glucose control in a 60-l fermentor.
Ammonia feeding was stopped when the cell concentration reached 25 g/l at 28 h. Cell concentration, ● ; PHB concentration, ■ ; residual biomass concentration, ○ ; PHB content, ▲ .

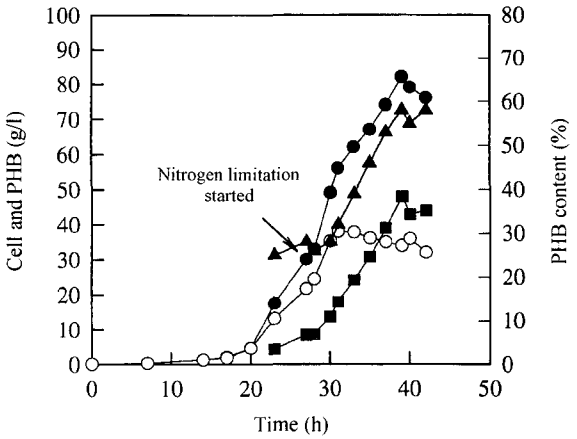

Fig. 2. Fed-batch culture of *R. eutropha* with nitrogen limitation and on-line glucose control in a 60 l fermentor.
Ammonia feeding was stopped when cell concentration reached 50 g/l at 30 h. Cell concentration, ● ; PHB concentration, ■ ; residual biomass concentration, ○ ; PHB content, ▲ .

PHB Production with Phosphate Limitation

PHB production under the phosphate limitation was performed in both 2.5-l and 60-l fermentors. When an initial phosphate concentration was 4.3 g/l, the different time courses of concentrations of the cell, PHB, residual biomass, and minerals (phosphate, sulfate, and nitrogen) in the 2.5-l fermentor were observed (Fig. 3). The phosphate concentration in the culture broth decreased exponentially as the residual cell mass increased and reached a low level (0.2-0.4 g/l) in 32 h. No significant decrease in phosphate concentration was observed during PHB accumulation. The sulfate and nitrogen concentrations in the culture broth were maintained at sufficient levels for cell growth during

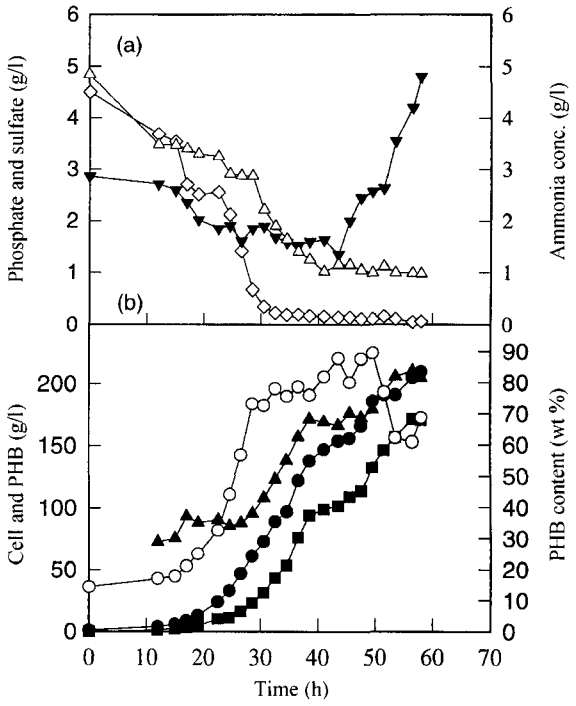

Fig. 3. DO-stat fed-batch culture of *R. eutropha* with phosphate limitation in 2.5-l fermentor.
The initial phosphate concentration was 4.3 g/l. (a) Ammonium concentration, ▼ ; sulfate concentration, △ ; phosphate concentration, ◇ . (b) PHB content, ▲ ; cell concentration, ● ; PHB concentration, ■ ; residual biomass concentration, ○ .

the whole fermentation period. At 32 h, cell growth became negligible and the PHB content started to increase rapidly. The residual biomass concentration reached 43 g/l and then decreased slightly during the PHB accumulation. The final cell and PHB concentrations obtained were 210 and 175 g/l (PHB content of 83%), respectively. The PHB productivity was 3.02 g/l/h. When the same initial phosphate concentration was used for the 60-l fermentor (Fig. 4), the final cell concentration, PHB concentration, and PHB content were 221 g/l, 180 g/l, and 81%, respectively. The PHB productivity was 3.75 g/l/h. The final culture volume was 43.5 l. The glucose concentration in the fermentation broth fluctuated from 0 to 20 g/l. No considerable cell lysis was observed even at very high cell concentration levels of over 200 g/l.

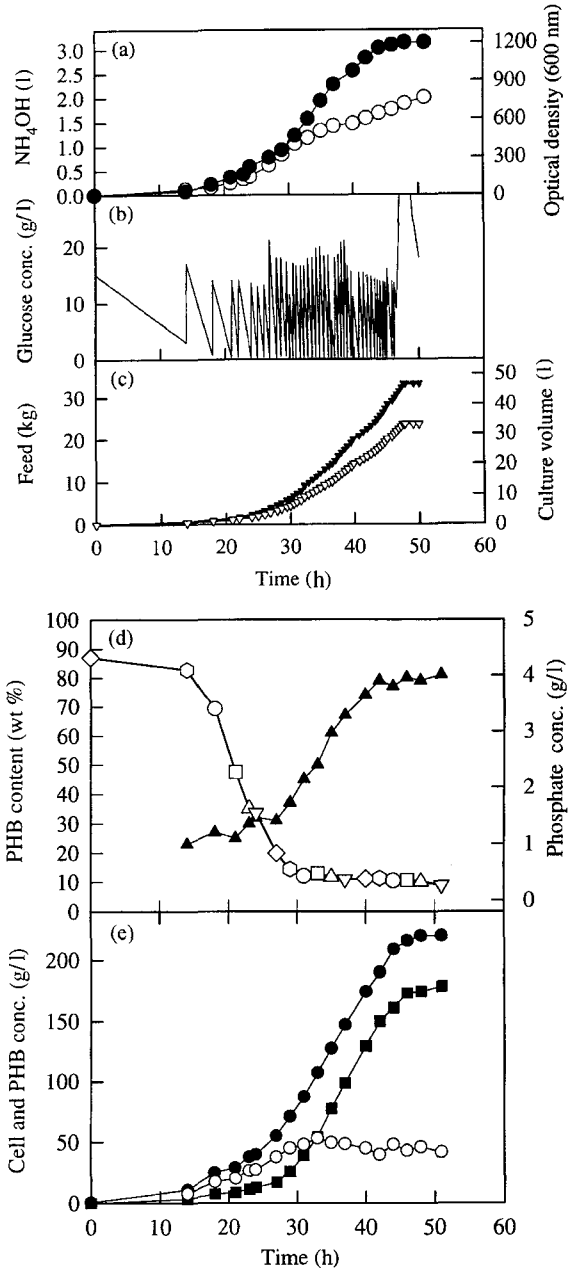

Fig. 4. DO-stat fed-batch culture of *R. eutropha* with phosphate limitation in 60-l fermentor.

The initial phosphate concentration was 4.3 g/l. NH_4OH, ○ ; OD, ● . (b) glucose concentration. (c) total amount of glucose added, ▼ ; culture volume, ▽ . (d) PHB content, ▲ ; phosphate concentration, ◇. (e) cell concentration, ● ; PHB concentration, ■ ; residual biomass concentration, ○.

DISCUSSION

In this study, high cell density cultures of *R. eutropha* by two different fed-batch culture techniques of nitrogen limitation and phosphate limitation were carried out in 2.5-l and 60-l fermentors for mass production of PHB. In a nitrogen-limited fed-batch culture, glucose feeding was manipulated by incorporating both an on-line glucose monitoring and control system. For the fed-batch cultures with phosphate limitation, a DO-stat glucose feeding strategy was employed.

Our previous works [12, 13] successfully applied for the production of PHB homopolymer and P(3HB-co-3HV) copolymer in a small-scale fermentor of 2.5-l. Cell and PHB concentrations, PHB content, and productivity obtained were in the ranges of 93-164 g/l, 71-121 g/l, 74-76%, and 1.51-2.42 g/l/h, respectively, depending upon cell concentration level at the beginning of the nitrogen starvation procedure (30-70 g/l) [12]. In the 60-l fermentor, the PHB concentration reached 60 g/l in 54 h when nitrogen was limited at a low cell concentration of 25 g/l (Fig. 1). This was indeed much lower than that (71 g/l) obtained by inducing nitrogen limitation at a similar cell concentration (30 g/l) in the 2.5-l fermentor. The PHB productivity (1.1 g/l/h) and PHB content (70%) were also quite lower than those in the 2.5-l fermentor [12]. When nitrogen limitation was introduced at a higher cell concentration of 50 g/l in the 60-l fermentor, the result turned out to be quite unsatisfactory (Fig. 2). There are several possible reasons for this unsatisfaction: They include cell lysis and a relatively large fluctuation of glucose concentration (5-30 g/l) observed at higher cell concentration levels during the latter part of the culture. One of the most probable reasons for the cell lysis was the toxicity of NaOH, which adversely affected cell activities such as cell growth and PHB. In the PHB accumulation period, the NH_4OH solution cannot be used for the pH control and, thus, inhibition of the cell activity was unavoidable by NaOH. The metabolic activity of cells under a total nitrogen-limited condition can be severely suppressed. As an example, PHB synthesis was stimulated when a small amount of ammonia was fed during the PHB accumulation period [32]. It suggested that it was essential

to maintain the nitrogen concentration level at a certain critical range to support the cellular metabolic activity and PHB accumulation, making the fermentor operation very difficult. Although most studies conducted for the PHA production were performed under nitrogen limitation and mass production of PHB under such a condition has been reported [6, 7, 9, 12, 13, 20, 30-32], no commercial processes using nitrogen limitation have been reported thus far. This suggested that commercial mass production of PHB might be difficult with limited nitrogen. To avoid NaOH toxicity, the limitation of other nutrients (P, K, O_2, S, and Mg) for PHA accumulation rather than nitrogen should be seriously considered. Another problem encountered with the glucose feeding by on-line glucose monitoring was poor control of glucose concentration at high cell concentrations. With increasing cell concentration, the regulation of glucose concentration at a desired level became very difficult due to plugging of the ceramic membrane modules used to filter the culture broth for glucose analysis. In order to overcome such a problem, some other alternative feeding method such as a carbon dioxide evolution rate-based method and pH- or DO-stat method could be considered.

When the DO-stat fed-batch culture with phosphate limitation was employed instead of the nitrogen limitation with an on-line glucose monitoring and control, high cell concentrations could be obtained in the 60-l and 2.5-l fermentors (Figs. 3 and 4). It was discovered that *R. eutropha* accumulated a high concentration of PHB very rapidly under the phosphate limitation condition. The final PHB contents were 81-83%. In the 2.5-l fermentor, the final PHB concentration (175 g/l) and productivity (3.02 g/l/h) (Fig. 3) were much higher than those obtained with nitrogen-limitation [12]. The theoretical yield of PHB from glucose *via* the Entner-Duodoroff pathway is known to be 0.48 g PHB/g glucose [36]. The yield obtained with phosphate limitation in this study was 0.38 g PHB/g glucose, which was lower than the theoretical value, but quite higher than that obtained with nitrogen limitation (0.3 g PHB/g glucose) [12].

Monsanto (U.S.A.) previously produced P(3HB-co-3HV) by using a fed-batch culture of *R. eutropha* under phosphate limitation on a fairly large-scale [4]. However, they did not report their results in detail, such as the final concentrations of cells and PHA obtained. Only the total fermentation time was reported to be 100 to 120 h.

One of the major advantages of a phosphate-limited fed-batch culture was an easy realization of a nutrient-limited condition. When a required amount of phosphate was provided to support a desired amount of the cell growth, it was initially added to the fermentor, and phosphate automatically became a limiting factor to induce PHB accumulation after a certain length of time. This was also the case when magnesium or potassium was used as the limiting nutrient [2, 15].

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