PRODUCTION OF POLY- β -HYDROXYBUTYRATE(PHB) BY FED-BATCH FERMENTATION USING HOLLOW FIBER MEMBRANE SYSTEM

JOON TAEK OH**

Engineering Research Institute, Yonsei University, Seoul, 120-749, Korea

WOO SIK KIM

Department of Chemical Engineering, College of Engineering, and Bioproducts Research Center, Yonsei University, Seoul, 120-749, Korea

Keywords: Poly- β -Hydroxybutyrate(PHB), Hollow Fiber Membrane, *A. Eutrophus*, By-Product

Introduction

Poly- β -hydroxybutyrate(PHB) is an intracellular storage polymer whose function is to provide a reserve of carbon and energy (Dawes and Senior, 1973). PHB is a biodegradable, biocompatible, and thermoplastic polymer made by microorganisms. This material can be formed into films, fibers and sheets that can be molded into shapes and bottles (Byrom, 1987). The combination of properties indicates that PHB can be used as absorbable prosthetic devices, surgical sutures, food packing, controlled release of drugs, piezoelectric sensors, mulch films and shielding materials in electronic circuits and so forth (Holmes, 1985; Howells, 1982; King, 1982). PHB accumulation rate can be promoted under unbalanced growth conditions. Unbalanced growth conditions occur in otherwise adequate media in which a particular element or microelement is totally lacking or present insuboptimal conditions.

The study of Schlegel and Vollbrecht (1980) showed that *Alcaligenes eutrophus* was strict aerobe and under conditions of restricted oxygen supply,

byproducts such as lactate, butandiol, and ethanol which inhibit cell growth were produced. In a previous study (Oh *et al.*, 1994) the effects of the byproducts were quantitatively verified. To get higher PHB production, the byproducts concentration in the culture broth should be maintained as low as possible. Therefore, the batch and fed-batch fermentation method usually used to produce PHB are not effective methods. In this study, to improve the biomass and PHB concentration, fed-batch fermentation using hollow fiber membrane was introduced and its results were compared with those of conventional batch and fed-batch fermentation.

1. Experimental

1.1 Fermentation and membrane system

Alcaligenes eutrophus NCIB 11599 was used in this study. Inocula was grown at 34°C on a reciprocal shaker in a 250 mL flask containing 100 mL of nutrient-rich medium (Doi *et al.* 1987). The cell was harvested after 24 h, and transferred into a culture medium. Each liter of the culture medium contained 6.7 g of Na₂HPO₄·H₂O, 1.5 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 60 mg of ferrous ammonium citrate, 10 mg of CaCl₂·2H₂O, 1 g of yeast extract, and 1 mL of trace-element solution (Ramsay *et al.*, 1990).

* Received on April 25, 1996. Correspondence concerning this article should be addressed to J. T. Oh.

** Present Address: Dept. of Chem. Eng., Univ. of Delaware, Newark, Delaware 19716, U.S.A.

Glucose and ammonium phosphate were used as a carbon and ammonium source, and their initial concentrations were 30–50 g/L and 2 g/L respectively. A 2.5 L jar fermentor was used with a 1.0 L of working volume. The temperature was kept at 34°C, and the pH was maintained at 6.9 (± 0.1) by adding 2 N NaOH. The culture was agitated at 500 rpm, and the air was supplied at a flow rate of 1 L/min.

The hollow fiber membrane module which was used for feeding of fresh medium and removal of byproducts was manufactured for blood dialysis by Baxter (Model No: Ca-90, USA). The membrane was made of cellulose acetate. The surface area was 0.9 m², and wall thickness was 15 μ m. Pure water flux was 4.3 mL/h·mmHg. The hollow fiber membrane module was sterilized by circulation of 100 ppm NaOCl solution for 10 hours, and washed by sterilized water. Fermentation broth and fresh medium was pumped respectively shell and tube side countercurrently.

1.2 Analytical procedures

The intracellular PHB concentration was determined by using a modified method of Riis and Mai where benzoic acid ethylester is used as an internal standard. The analytical procedures of GC are the same as the previous study (Yoo and Kim, 1994). Glucose in the supernatant broth was estimated by the enzymatic (Christian, 1986) and DNS (denitrosalicylic acid) methods. The commercial glucose-E kit (Yeongdong Pharmarm. Corp., Korea) was used for enzymatic glucose analysis. The glucose was reacted with a mixture of the enzymes glucose oxidase and peroxidase, and the produced hydrogen peroxide formed a red color which was detected by spectrophotometry (Shimadzu double beam spectrometer UV-200S). Ammonium phosphate in the supernatant broth was measured by the Berthelot reaction using spectrophotometry. Ethanol concentration in the supernatant was determined by GC where butanol was used as an internal standard. The flame ionization detector was employed.

2. Results and Discussions

2.1 Batch fermentation

The initial batch culture medium contained 50 g/L of glucose and 2 g/L of (NH₄)₂HPO₄, and the working volume was 1 L. As shown in Fig. 1, after a lag time of 5 or 6 h, the biomass and PHB content were both increased. PHB accumulation increased continuously to the exponential and stationary phase, and attained 4.5 g/L at 18 h, and its ratio to biomass was 56%. Biomass yield to glucose, $Y_{X/G}$ was 0.49.

In this study, ethanol was selected for the representative byproduct, and concentrations of other byproducts were considered to be proportional to ethanol concentration. Ethanol produced according

Fig. 1 Time courses of dry cell weight, PHB, and substrate in batch fermentation of *A. eutrophus*

Fig. 2 Time courses of dry cell weight, PHB, and substrate in fed-batch fermentation of *A. eutrophus*

to the biomass and its specific growth rate, and its concentration profile was similar to that of the biomass. The final concentration of ethanol was 0.4 g/L.

2.2 Fed-batch fermentation

Fed-batch fermentation was carried out to get the high concentration of PHB. The initial glucose and (NH₄)₂HPO₄ were 30 g/L and 2 g/L. The initial working volume was 1 L. The supplied medium contained as much as the initial medium concen-

Fig. 3 Overall mass transfer coefficient of substrate according to tube side (a) and shell side flow rate in hollow fiber membrane (b)

tration, yeast extract 10 g/L and glucose 300 g/L. The feeding of medium was started at 14 h when the biomass concentration in the broth was 10 g/L, and the feeding rate was 10 mL/h. $(\text{NH}_4)_2\text{HPO}_4$ concentration was fed intermittently and maintained its concentration between 0.5 and 1.3 g/L in the medium. At 29 h $(\text{NH}_4)_2\text{HPO}_4$ supply was stopped to promote PHB accumulation. The results are shown in Fig. 2. The cell and PHB concentration reached 47.6 g/L and 28.0 g/L respectively at 50 h. Although the biomass and PHB concentration were much higher than that of batch fermentation, the fermentation time was longer. The $Y_{P/X}$ and $Y_{X/G}$ were 0.588 and 0.264 respectively. At 50 h, the volume in fermentor had increased to 1.58 L by feeding medium and NaOH solution for pH adjustment. The volume increase inhibited the high concentration of biomass and PHB in the culture medium. The ethanol concentration, byproduct, was 1.79 g/L at 50 h, and it was much greater than that of batch fermentation. According to previous results (Kim *et al.*, 1994), when the culture medium contains ethanol 1.8 g/L, the specific growth rate of *A. eutrophus* had decreased about 20%.

2.3 The mass transfer coefficient of the hollow fiber membrane

To estimate the mass transfer coefficient of the hollow fiber membrane system, the medium containing glucose 50 g/L and $(\text{NH}_4)_2\text{HPO}_4$ 2 g/L was forced to flow tube side, and pure water was forced to flow shell side. At steady state the glucose and $(\text{NH}_4)_2\text{HPO}_4$ concentration of shell side was measured to estimate the mass transfer coefficient. The mass transfer coefficient was estimated by the following equation.

$$J_s = K_D(C_{b_i} - C_{b_s})$$

where K_D is the membrane dialysis coefficient. C_{b_i} and C_{b_s} stands for the bulk solute concentration of tube and shell side respectively. As shown in Fig. 3, the mass transfer coefficient was affected by the flow rate of both sides of the membrane. The mass transfer coefficient was increased by the flow rate of tube side or shell side whereas the tube velocity higher than 0.6 cm/s did not affect the mass transfer coefficient. The transfer rate of $(\text{NH}_4)_2\text{HPO}_4$ was greater than that of glucose.

2.4 One-stage fed-batch fermentation using a hollow fiber membrane

To reduce the byproduct effect and working volume increase, the feeding medium was supplied by a hydrophilic hollow fiber membrane. The feeding medium and fermentation broth was respectively pumped tube side and shell side in a counter flow. They contacted on the membrane and the nutrient medium including glucose was transported to the broth by concentration gradient. The byproduct including ethanol was transported in the opposite direction. The superficial velocity of the feeding medium and fermentation broth were 0.5 cm/s. The initial glucose and $(\text{NH}_4)_2\text{HPO}_4$ concentration were 30 g/L and 2 g/L respectively. The feeding medium contained twice as much as the initial medium concentration, glucose 90 g/L, and yeast extract 5 g/L. The $(\text{NH}_4)_2\text{HPO}_4$ was supplied intermittently to maintain its concentration between 0.5 and 1.0 g/L in the broth. To improve the PHB content, $(\text{NH}_4)_2\text{HPO}_4$ supplement was stopped at 20 h. As shown in Fig. 4, the medium feeding started at 8 h, and the

Fig. 4 Time courses of dry cell weight, PHB, and substrate in one-stage fed-batch fermentation of *A. eutrophus* using hollow fiber membrane system

glucose concentration in broth was increased to 34 g/L at 21 h. Until 35 h, the glucose concentration could be maintained between 35 and 10 g/L, and after that time the cell growth rate decreased rapidly. The biomass and PHB concentration were 68.4 g/L and 43 g/L respectively at 50 h, and were greater than those of batch or fed-batch fermentation. The $Y_{P/X}$ was 0.629, and $Y_{X/G}$ was 0.539. The ethanol concentration increased continuously, but its increasing rate was decreased. Biomass was greater than that of fed-batch fermentation, but the final ethanol concentration was 1.2 g/L which was lower than that of fed-batch fermentation. The result shows that the removal of byproducts can enhance the biomass and PHB content by providing better condition. The working volume was maintained at 1 L during the fermentation time which was the same as the initial volume.

2.5 Two-stage fed-batch fermentation using a hollow fiber membrane

To confirm the improvement of one stage fed-batch fermentation using a hollow fiber membrane, the effect of two stage fed-batch fermentation using hollow fiber membrane was studied. At 8 h, a 1-L medium containing glucose 70 g/L, yeast extract 3 g/L and other elements in the same concentration as the initial fermentation medium was supplied by a hollow fiber membrane system. At 8 h in which the glucose concentration in the broth decreased to 20 g/L, the first medium was fed, then the glucose concentration in the broth was increased to 38 g/L at 18 h (Fig. 5). The ethanol concentration had increased to 1.4 g/L

Fig. 5 Time courses of dry cell weight, PHB, and substrate in two-stage fed-batch fermentation of *A. eutrophus* using hollow fiber membrane system

until feeding of the second medium was started. At 40 h the glucose concentration in the broth had fell to 20 g/L, and the second medium containing only glucose 100 g/L and yeast extract 3 g/L was supplied also using the hollow fiber membrane. The volume of the second fresh medium was 2 L. $(\text{NH}_4)_2\text{HPO}_4$ was maintained at 0.5–1.0 g/L by feeding intermittently, and at 40 h its supply was stopped. As the second medium was supplied, the glucose concentration in the culture medium suddenly increase to 65 g/L. However the ethanol concentration was somewhat decreased, and maintained between 0.9 and 1.3 g/L. Only a small volume change was shown during the fermentation time. The fermentation time was 70 h, and the biomass and PHB concentrations were 98 g/L and 58.1 g/L. The $Y_{P/X}$ and $Y_{X/G}$ were 0.593 and 0.45 respectively which were a little lower than those of one stage hollow fiber membrane fed-batch system. However, the biomass and PHB concentrations were much greater than that of one stage fed-batch system using a membrane. With fed-batch fermentation using a membrane system, higher biomass and PHB production could be achieved without a broth volume increase. It was resulted from not only constant volume maintenance but also stable supplement of glucose and other nutrients and removal of byproducts which inhibit cell growth.

Literature Cited

- Byrom, D.; "Polymer Synthesis by Microorganisms: Technology and Economics," *TIBTECH*, 5, 246-250 (1987)
- Christian, G. D.; *Analytical Chemistry*, 4th ed., p. 625–627, John

- Wiley & Sons, New York, USA (1986)
- Dawes, E. A. and P. J. Senior; "The Role and Regulation of Energy Reserve Polymers in Microorganisms," *Adv. Microbial. Physiol.*, **10**, 203-253 (1973)
- Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga; "Biosynthesis of Copolyesters in *Alcaligenes eutrophus* H16 from ¹³C-Labeled Acetate and Propionate," *Macromolecules*, **20**, 2988-2991 (1987)
- Holems, P. A.; "Application of PHB: A Microbially Produced Biodegradable Thermoplastic," *Phys. Technol.*, **16**, 32-36 (1985)
- Howells, E. R.; "Opportunities in Biotechnology for The Chemical Industry," *Chem. Ind.*, **7**, 508-511 (1982)
- Kim, B. S., G. D. Cho, J. T. Oh, and W. S. Kim; "The Effects of Alcohols in Substrate on The Biosynthesis of PHB and P(HB-co-HV)," *Korean J. of Chem. Eng.*, **32**, 441-447 (1994)
- King, P. P.; "Biotechnology: An Industrial View," *J. Chem. Tech. Biotechnol.*, **32**, 2-8 (1982)
- Oh, J. T., D. G. Lee, and W. S. Kim; "The Study on The Optimum Culture Conditions and Effects of By-product of *A. eutrophus* for The Biosynthesis of PHB," *Korean J. Biotechnol. Bioeng.*, **9**, 475-482 (1994)
- Ramsay, B. A., K. Lomaliza, C. Chavarie, B. Dube, P. Bataille, and J. A. Ramsay; "Production of Poly-(β -Hydroxybutyric-Co- β -Hydroxyvaleric) Acid," *Appl. and Environmental Microbiology*, **56**, 2093-2098 (1990)
- Riis, V., and W. Mai; "Gas Chromatographic Determination of Poly- β -Hydroxybutyric acid in Microbial Biomass after Hydrochloric and Propanolysis," *J. Chromatogra.*, **445**, 285-289 (1988)
- Schlegel, H. G., and D. Vollbrecht; "Formation of The Dehydrogenases for Lactate Ethanol, and Butandiol in The Strictly Aerobic Bacterium *Alcaligenes eutrophus*," *J. Gen. Microbiol.*, **117**, 475-481 (1980)
- Yoo, S., and W. S. Kim; "Cybernetic Model for Synthesis of Poly- β -hydroxybutyric Acid in *Alcaligenes eutrophus*," *Biotechnol. Bioeng.*, **43**, 1043-1051 (1994)