Production of poly(3-hydroxybutyrate) by high cell density cultivation of recombinant *Escherichia coli* from whey

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1. Introduction

Whey is the major by-product in the manufacture of cheese or casein from bovine milk, representing 80-90% of the volume of milk transformed. It contains approximately 4.5% (w/v) lactose, 0.8% (w/v) protein, 1.0 % (w/v) salts, and 0.1%-0.8% (w/v) lactic acid.[1]Only the half of the whey produced annually in the USA is recycled into useful products such as food ingredients and animal feed, and the rest is regarded as a pollutant due to high biological oxygen demand. Disposal of whey is being managed at considerable costs.

Polyhydroxyalkanoates (PHAs) are polyesters, which are accumulated as energy and/or carbon storage materials by numerous microorganisms usually when a nutritional component such as nitrogen, phosphorus, sulfur, oxygen, or magnesium is limited in the presence of excess carbon source.[2] PHAs have been considered to be good substitutes for petroleum-derived synthetic plastics because of their similar material properties to synthetic polymers and complete biodegradability after disposal. A major problem in commercializing PHAs is their high production cost. Much effort has been devoted to lower the production cost of PHA by developing better bacterial strains and efficient strategies for fermentation and recovery of PHAs. Economic evaluation of the process for the production of PHB suggested that the major contributor to the overall PHB production cost was substrate cost (up to 50%).[3] Therefore, it is desirable to produce PHB from cheap carbon source such as whey using recombinant *E. coli*.

In this study, we report the fermentation strategies for the production of PHB from whey in recombinant *E. coli* without removing culture broth during fermentation. A highly concentrated whey solution was successfully employed for the efficient production of PHB from whey to a high concentration by recombinant *E. coli* and successfully produced higher cell concentration and PHB productivity.

2. Materials and methods

Bacterial strain and plasmid. *E. coli* CGSC 4401 (*E. coli* Genetic Stock Center, New Haven, Conn., USA) was used in this study. The plasmid pJC4 containing the *A. latus* PHA biosynthesis genes has been described previously.[4] *E. coli* strains were transformed with pJC4 by electroporation. Cells were maintained as a 15% (v/v) glycerol stock at –75 °C after growing in LB medium (pH 6.7) or chemically defined MR medium (see below) containing 20 g/L lactose.

Pre-treatment of whey solution. Bovine whey powder was purchased from SamIk dairy

industry (Samlk Co., Seoul, Korea). Crude whey solution was prepared by dissolving 700 g whey powder in 1 L distilled water. To remove the excessive proteins in whey solution, the pH of the whey solution was adjusted to 4.5 by the addition of 37 wt% HCl [19]. The solution was autoclaved at 121 °C for 15 min and centrifuged at 11,000 ×g in a sterilized bottle for 15 min to remove aggregates. By adding the diatomaceous earth (Sigma Co., St. Louis, MO, USA) to 2% (w/v), small protein particles were removed by filtration (Watman No. 3 filter paper). The pH of filtered solution was adjusted to 6.5 with 12 N NaOH.

Culture condition The MR medium (pH 6.9) contains per liter: 6.67 g KH_2PO_4, 4 g $(NH_4)_2HPO_4$, 0.8 g $MgSO_47H_2O$, 0.8 g citric acid, and 5 mL trace metal solution. The trace metal solution contains per liter of 5 M HCl: 10 g $FeSO_47H_2O$, 2 g $CaCl_2$, 2.2 g $ZnSO_47H_2O$, 0.5 g $MnSO_44H_2O$, 1 g $CuSO_45H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24}4H_2O$, and 0.02 g $Na_2B_4O_710H_2O$. Two different feeding solutions were used for the fed-batch cultures. In fermentation A, whey solution containing 210 g/L lactose plus 4.5 g/L $MgSO_47H_2O$ was used. In fermentation B, whey solution containing 280 g/L lactose plus 6 g/L $MgSO_47H_2O$ was used. Feeding solutions were prepared as described above.

Using the recombinant *E. coli* CGSC 4401 (pJC4), fed-batch cultures were carried out at 30 °C in a 6.6 L jar fermentor (Bioflo 3000, New Brunswick Scientific Co., Edison, NJ, USA) containing 1.3 L of MR medium plus pretreated whey solution equivalent to 20 g/L lactose. Seed cultures (130 mL) were prepared in the same medium. The culture pH was controlled at 6.95 by the automatic addition of 28% (v/v) NH_4OH. The level of dissolved oxygen concentration (DOC) which desired was controlled by automatically increasing the agitation speed up to 1000 rpm and varying pure oxygen percentage.

Nutrient feeding solution was added by the pH-stat feeding strategy. When the pH rose to a value greater than its set point (6.95) by 0.1 due to the depletion of lactose, an appropriate volume of feeding solution was automatically added to increase the lactose concentration in the culture broth to 20 g/L. The feeding volume was calculated on-line using the fermentation software AFS3.42 (New Brunswick Scientific Co.).

Foam formation was suppressed by adding Antifoam 289 (Sigma Chemical Co., St. Louis, MO, USA) during the initial stage of fed-batch cultures.

Analytical procedures. PHB concentration was determined by gas chromatography (Donam Co., Seoul, Korea) equipped with a fused silica capillary column (Supelco SPB™-5, 30 m × 0.32 mm ID 0.25 μm film, Bellefonte, PA, USA) using benzoic acid as an internal standard. Cell concentration, defined as cell dry weight per liter of culture broth, was determined as previously described. The residual cell concentration was defined as the cell concentration minus PHB concentration. The PHB content (wt%) was defined as the percentage of the ratio of PHB concentration to cell concentration. The concentrations of lactose, galactose and glucose were measured by HPLC (Hitachi L-4200 UV-Vis detector, L-600 pump, D-2500 chromato-integrator, Tokyo, Japan) equipped with an ion exchange column (Aminex HPX-87H, 300 mm

× 7.8 mm, Hercules, CA, USA) using 0.01 N H_2SO_4 as a mobile phase.

3. Results

Fed-batch culture with the whey solution containing 210 g/L lactose. Fed-batch culture of *E. coli* CGSC 4401 harboring pJC4 was carried out by pH-stat feeding strategy with the whey solution containing 210 g/L lactose. During the cultivation, the level of DOC was initially maintained at 30%. When the OD_{600} was reached to 180 (cell concentration of ca. 60 g/L), DOC was maintained at 20% to the end of cultivation. The time profiles of cell concentration, PHB concentration, residual cell concentration and PHB content are shown in figure 1. At 49 h, cell and PHB concentration reached 83.1 g/L and 46.8 g/L, respectively, resulting in a PHB content of 56.3 wt% and a productivity of 1.15 g/L-h. Due to the volumetric limitation from the large volume of feeding solution, culture broth was removed to prevent flooding during the cultivation. During the entire cultivation, a total of 7.4 L nutrient feed solution was added to the fermentor and 6.5 L culture broth was removed.

Fed-batch culture with the whey solution containing 280 g/L lactose for achieving high-cell concentration. Fed-batch cultures of *E. coli* CGSC 4401 harboring pJC4 were carried out with the feeding solution containing 280 g/L by the pH-stat fed-batch cultivation. Culture broth removal during fed-batch cultivation could be avoided and higher cell concentration could be achieving by using this highly concentrated whey feeding solution. Initial level of DOC was maintained at 30%. When OD_{600} reached to 240 (cell concentration of ca. 80 g/L), DOC decreased to 20%. The time profiles of fed-batch culture of *E. coli* CGSC 4401 (pJC4) using 280 g/L lactose equivalent as a feeding solution are shown in figure 2. The final cell concentration, PHB concentration and PHB content of 102.9 g/L, 59.6 g/L and 57.9 wt%, respectively, were obtained in 42 h, which resulted in the productivity of 1.42 g PHB/L-h.

4. References

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5. Acknowledgement

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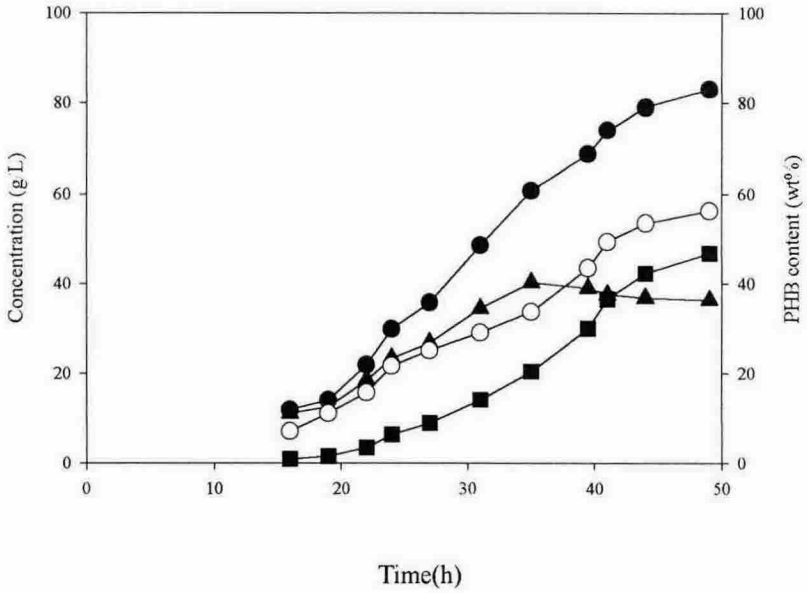

Figure 1. Time profiles of cell concentration (●), PHB concentration (■), residual cell concentration (▲) and PHB content (O) during the fed-batch culture of *E. coli* CGSC 4401 (pJC4) with the feeding solution containing 210 g/L lactose equivalent.

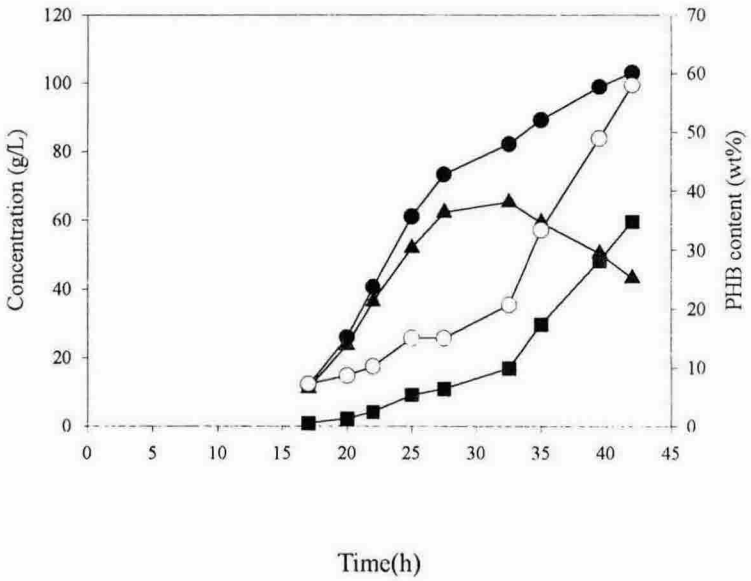

Figure 2. Time profiles of cell concentration (●), PHB concentration (■), residual cell concentration (▲) and PHB content (O) during the fed-batch culture of *E. coli* CGSC 4401 (pJC4) with the feeding solution containing 280 g/L lactose equivalent