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PRODUCTION OF POLY-β-HYDROXYBUTYRATE BY FED-BATCH CULTURE OF RECOMBINANT *ESCHERICHIA COLI*

Beom Soo Kim, Sang Yup Lee, and Ho Nam Chang*

BioProcess Engineering Research Center and Department of Chemical Engineering, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

SUMMARY

A recombinant *Escherichia coli* strain harboring the PHB biosynthesis genes from *Alcaligenes eutrophus* was used to produce poly-β-hydroxybutyrate (PHB) by pH-stat fed-batch culture. Initial glucose concentration for optimal growth was found to be 20 g/L from a series of flask cultures. A final PHB concentration of 88.8 g/L could be obtained after 42 hrs of cultivation.

INTRODUCTION

Poly-β-hydroxybutyrate (PHB) is a biodegradable thermoplastic produced by many microorganisms as a storage material upon environmental stress. It has recently drawn much attention as an environmentally harmless alternative to petrochemical derived plastics (Byrom, 1987). All bacteria known to date accumulate PHB when an essential nutrient such as nitrogen or phosphate is limited providing that an excess of carbon source is present (Dawes and Senior, 1973).

The PHB biosynthetic pathway has been studied extensively in *Alcaligenes eutrophus* and the operon responsible for PHB production has recently been cloned and expressed in *Escherichia coli* (Schubert et al., 1988; Slater et al., 1988; Peoples and Sinskey, 1989). Since the growth of *E. coli* is fast and the level of PHB accumulation approaches ca. 90 % (Slater et al., 1988), use of a recombinant *E. coli* harboring the PHB biosynthesis genes seems to be economically attractive.

One of the most popular methods to achieve high cell density, which is often necessary for high yield and productivity of the desired product, is fed-batch culture by controlling the nutrient feeding by pH, DO, or specific growth rate (Yamane and Shimizu, 1984). There have been a numerous reports of employing fed-batch culture of wild type and recombinant *E.coli* strains harboring genes for the production of various bioproducts (Pan et al., 1987; Li et al., 1990; Riesenberg et al., 1990).

We carried out a series of flask cultures of a recombinant *E. coli* strain harboring the PHB synthesis operon from *A. eutrophus* to find an optimal glucose concentration. We also evaluated several different growth media for the fed-batch culture. And we report high PHB production by the pH-stat fed-batch culture of a recominant *E. coli* strain.

MATERIALS AND METHODS

Organism and culture condition

A recombinant *E. coli* XL1-Blue (pBluescript KS$^-$::SE52) harboring *A. eutrophus* PHB biosynthesis genes (Schubert et al., 1988) was used. Cells were routinely grown in Luria-Bertani (LB) medium (Sambrook et al., 1989). For flask cultures, LB medium supplemented with various amount of glucose: 0, 5, 10, 20, and 30 g/L. Ampicillin was added at a concentration of 100 μg/mL. For fed-batch culture, the 50 mL seed culture was prepared in 250 mL flask and grown in a rotary shaker overnight at 37 °C. Fed-batch culture was carried out in a jar fermentor (2.5 L, Korea Fermentor Company, Korea). The initial culture volume was 0.9 L of LB supplemented with 20 g/L glucose. To maintain the dissolved oxygen level above 10 %, the agitation speed was adjusted up to 900 rpm. Pure oxygen was also used when required. The concentrations of glucose, yeast extract, and tryptone in feeding nutrient solution were 400, 100, and 100 g/L, respectively. Culture pH was controlled at 6.8 by the addition of 28 % ammonia water. Ampicillin was added only at the beginning of the fed-batch operation.

Feeding by pH-stat during the fed-batch operation

Substrate feeding strategy was the pH-stat with high limit suggested by Suzuki et al. (1990). When glucose becomes exhausted, pH rises rapidly. When the pH becomes higher than 7.1, 50 mL of feeding nutrient solution, which corresponds to 20 g glucose + 5 g yeast extract + 5 g tryptone, was added for a definite on-time.

Analytical procedure

Growth was monitored by measuring optical density at 600 nm. Cell mass concentration was determined by weighing dry cells obtained as follows. A 2 mL of culture broth was collected in a pre-weighed tube, pelleted by centrifugation, and dried in a vacuum oven. The PHB concentration was determined by gas chromatography (Varian 3300, U.S.A.) with n-butyric acid as an internal standard (Braunegg et al., 1978). Glucose and acetic acid concentrations were determined by liquid chromatography with RI detector (Hitachi L-6000, Japan). P/X (%) was defined as the percentage of the ratio of PHB to dry cell weight.

RESULTS AND DISCUSSION

Flask culture

Our initial attempts to produce PHB using a recombinant *E. coli* strain harboring the PHB biosynthesis genes were devoted to growth medium development. LB medium supplemented with various amount of glucose was investigated first. As shown in Table 1, relatively good growth and PHB production were observed. PHB accumulation in LB without glucose was not detectable. Among the media tested, LB supplemented with 20 g/L glucose gave the best results (Table 1). The profiles of cell growth and PHB production in LB + glucose 20 g/L are shown in Fig. 1. No considerable PHB production was observed during the exponential growth phase. PHB started to accumulate significantly when cells go into the stationary growth phase. The final PHB content was 79 %. It is noteworthy that PHB content increased linearly until the end of the culture (Fig. 1). Between 14.2 and 57.5 hrs, the amounts of PHB and cell mass increased were 4.1 and 3.68 g, respectively. This indicates that PHB accumulation becomes more significant as cell growth slows down. The PHB synthesis enzymes seem to be expressed constitutively from its own promoter in *E. coli* as discussed in Peoples and Sinskey (1989).

Several minimal media including M9 (Sambrook et al., 1989) and Harrison's (Harrison and Pirt, 1967) with or without minor modification were investigated next. However, in spite of repeated attempts, we could not find any benefit since cell mass and PHB production were inefficient compared to those complex media described above.

Table 1. Final OD_{600}, cell mass and PHB concentrations, and P/X in flask cultures.

LB + glucose (g/L)	0	5	10	20	30
OD_{600}	4.3	9.8	13.9	28.7	8.7
Cell mass (g/L)	1.1	2.5	3.6	7.5	2.3
PHB (g/L)	nd[a]	1.1	2.2	5.9	1.8
P/X (%)	-	44	61	79	57

[a]nd: not detectable.

Fed-batch culture

A pH-stat fed-batch culture was carried out to achieve high cell mass and high PHB production using LB medium supplemented with glucose. The results are shown in Fig. 2. The pH-stat feeding strategy was used because preliminary studies showed that pH rose

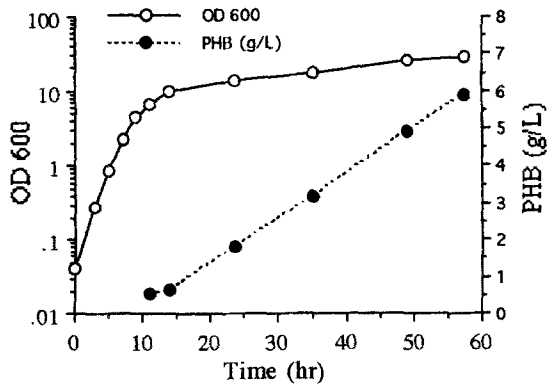

Figure 1. Cell growth and PHB production in flask culture using LB + 20 g/L glucose.

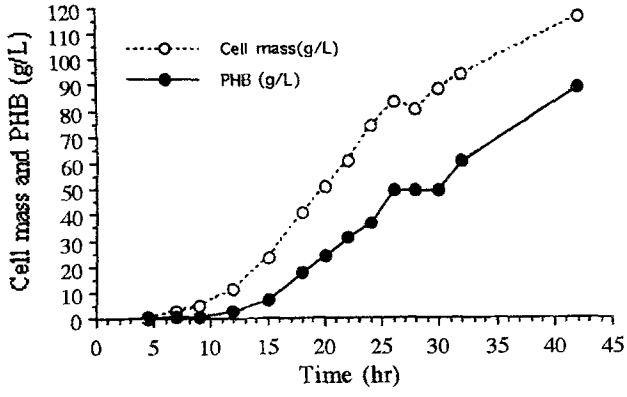

Figure 2. Cell mass and PHB concentrations in the fed-batch culture.

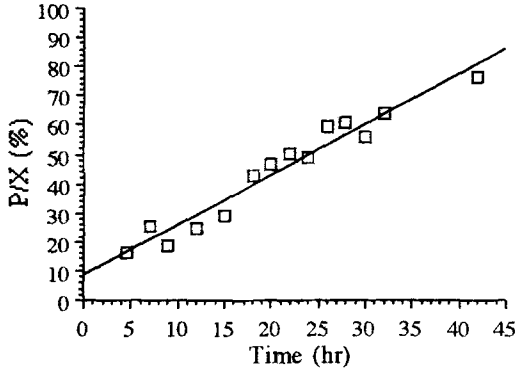

Figure 3. The percentage of PHB to dry cell weight (P/X) in the fed-batch culture.

rapidly when glucose became completely depleted (data not shown). The first feeding occurred at OD_{600} of 21, where the batch part of operation ended. The culture grew, after a short lag phase, exponentially at the specific growth rate of ca. 0.7, which was lower than the value typically obtained using complex medium. This seems to be due to the simultaneous production of PHB during the growth resulting from constitutive expression of the PHB synthesis enzymes. During the later part of the batch culture cell growth slowed down continuously likely due to the inefficient cell division in the presence of PHB granules, even though the size of which was relatively small during this early stage of operation. At the end of the batch part, PHB concentration and P/X were 0.93 g/L and 18.6 %, respectively.

During the fed-batch operation nutrients containing glucose were added intermittently when glucose became depleted, which was indicated by the sharp rise of pH as described earlier. Therefore, glucose concentration during the fed-batch operation was maintained at less than 20 g/L. Cell mass increased linearly during the fed-batch operation until 26 hrs. After 26 hrs cell growth further slowed down but still showed linear pattern. There are at least three possible reasons for the observed linear cell growth. First, cell growth slows down due to the diversion of acetyl-CoA pool from cell growth to PHB production. Second, cell division becomes abnormal as PHB granules are present. Third, culture became oxygen limited even though pure oxygen was used in the later part of culture due to our reactor system configuration.

The concentration of acetic acid, which has been shown as the most deleterious by-product during the high cell density culture of *E. coli*, was continuously monitored during the culture. Acetic acid concentration was maintained at below 6 g/L throughout the culture. This is notable that acetic acid did not accumulate to a growth inhibitory level even though the culture became oxygen limited. This seems to be due to that the constitutively expressed PHB biosynthetic enzymes efficiently utilized acetyl-CoA, which otherwise would form acetic acid. We are now investigating the possibility of producing PHB by switching the culture condition from aerobic to anaerobic to test this hypothesis.

PHB concentration increased monotonically during the whole fed-batch operation as can be seen from Fig. 2. After 42 hrs of culture cell mass and PHB concentrations obtained were 116.6 and 88.8 g/L, respectively. There has been only one report that showed higher PHB concentration than that reported here. PHB concentration of 149 g/L was obtained by fed-batch culture of *Protomonas extorquens* using methanol as a carbon source (Suzuki et al., 1986). However, this value was obtained after 170 hr of operation, which is considerably longer than ours.

During the course of fed-batch operation, the P/X ratio increased linearly to the final value of 76.2 % as shown in Fig. 3. We do not understand the exact reason for this

behavior at this time. Another thing that should be mentioned is that the morphology of *E. coli* cells changed during the culture. As PHB accumulated, cells beame elongated and some cells were significantly longer (data not shown). This observation is not only interesting but also important in a sense that it may allow PHB purification easier.

In this paper we first reported the production of PHB to a high concentration by the pH-stat fed-batch culture of a recombinant *E. coli* strain. The major significance of this report is that we achieved the PHB concentration of 88.8 g/L in 42 hr, which is efficient considering both PHB concentration and operation time. Many possible improvements can be made by modifying the culture conditions. One such way is the optimization of feeding strategy in a way that cells grow more rapidly to high density before significant PHB accumulation occurs. Another possibility is employing an inducible expression system. One can achieve high cell density first and subsequently allow high PHB production by induction. We are currently investigating these possiblities. Considering all these opportunities, it is likely that PHB production by a recombinant *E. coli* may become an economical alternative to those methods currently being used.

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