Production of Poly(3-Hydroxybutyrate) by Fed-Batch Culture of Filamentation-Suppressed Recombinant Escherichia coli

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Recombinant Escherichia coli XL1-Blue harboring a high-copy-number plasmid containing the Alcaligenes eutrophus polyhydroxyalkanoate synthesis genes could efficiently synthesize poly(3-hydroxybutyrate) (PHB) in a complex medium containing yeast extract and tryptone but not in a defined medium. One of the reasons for the reduced PHB production in a defined medium was thought to be severe filamentation of cells in this medium. By overexpressing an essential cell division protein, FtsZ, in recombinant E. coli producing PHB, filamentation could be suppressed and PHB could be efficiently produced in a defined medium. A high PHB concentration of 149 g/liter, with high productivity of 3.4 g of PHB/liter/h, could be obtained by the pH-stat fed-batch culture of the filamentation-suppressed recombinant E. coli in a defined medium. It was also found that insufficient oxygen supply at a dissolved oxygen concentration (DOC) of 1 to 3% of air saturation during active PHB synthesis phase did not negatively affect PHB production. By growing cells to the concentration of 110 g/liter and then controlling the DOC in the range of 1 to 3% of air saturation, a PHB concentration of 157 g/liter and PHB productivity of 3.2 g of PHB/liter/h were obtained. For the scale-up studies, fed-batch culture was carried out in a 50-liter stirred-tank fermentor, at which the DOC decreased to zero when cell concentration reached 50 g/liter. However, a relatively high PHB concentration of 101 g/liter and PHB productivity of 2.8 g of PHB/liter/h could still be obtained, which demonstrated the possibility of industrial production of PHB in a defined medium by employing the filamentation-suppressed recombinant E. coli.

Biodegradable plastics have been drawing much attention due to the environmental problems caused by the accumulation of nondegradable plastic wastes. Polyhydroxyalkanoates (PHAs), intracellularly accumulated by various microorganisms as carbon and energy storage materials, have been considered to be strong candidates for biodegradable polymer materials (1, 6, 17). A major drawback to the commercialization of PHAs is their much higher production cost compared with petrochemical-based synthetic plastic materials or other biodegradable polymers such as poly(lactide) (7). With the aim of lowering the production cost of PHAs, various fermentation processes employing several different bacteria have been developed to improve the production of PHAs (7, 13). Alcaligenes eutrophus has been the most widely employed bacterium for the production of PHAs since it can accumulate a large amount of PHAs in a simple medium (4). Poly(3-hydroxybutyrate) (PHB) is a homopolymer of 3-hydroxybutyrate and is the most widespread and best-characterized member of the PHAs. In A. eutrophus PHB is synthesized from acetyl coenzyme A (acetyl-CoA) by three sequential enzymatic reactions (1). Two acetyl-CoA moieties are condensed to acetoacetyl-CoA by β-ketothiolase. An NADPH-dependent reductase converts acetoacetyl-CoA to β-(−)-3-hydroxybutyryl-CoA, which is subsequently added to the growing chain of PHB by the PHA synthase. The genes coding for these three enzymes were cloned in Escherichia coli and shown to form an operon (16, 18, 19). We constructed the stable high-copy-number plasmid pSYL104 containing the A. eutrophus PHA synthesis genes (6, 14). A high concentration of PHB (81 g/liter) could be produced with a high productivity of 2.1 g of PHB/liter/h by the pH-stat fed-batch culture of recombinant E. coli XL1-Blue harboring pSYL104 in a complex medium supplemented with a large amount of yeast extract and tryptone (14). However, it is not desirable to use a large amount of expensive yeast extract and tryptone for the production of PHB, which is to be used as an inexpensive bulk product. With the aim of lowering the production cost of PHB, fed-batch cultures were carried out in a chemically defined medium. Unfortunately, only less than 25 g of PHB/liter could be produced by fed-batch culture of XL1-Blue (pSYL104) in a defined medium (11). One of the reasons for the reduced accumulation of PHB in a defined medium was filamentation of cells accumulating PHB (15). It was observed that cells became considerably elongated (up to 150 μm in length) in flask and fed-batch cultures (11, 15), and the extent of filamentation was much more severe in a defined medium (5). Cell filamentation was due to the inactivation of an essential cell division protein, FtsZ, and could be suppressed by amplifying the activity of FtsZ (5). More interestingly, in flask culture, recombinant E. coli with amplified FtsZ activity could more efficiently synthesize PHB in a defined medium compared with cells that underwent filamentation (5). In this study fed-batch cultures of filamentation-suppressed recombinant E. coli were carried out for the production of PHB in a defined medium. A cultivation strategy that allowed a high PHB productivity of greater than 3 g of PHB/liter/h was developed. It was also demonstrated that PHB could be efficiently produced in a pilot-scale fermentor by using this strategy.

MATERIALS AND METHODS

Microorganism and plasmid. The E. coli strain used in this study was XL1-Blue (recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac[proAB, lacF7ΔM15]

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contained the following per liter: glucose, 700 g; MgSO4 (0.02% [vol/vol]) (antifoam 289; Sigma Chemical Co., St. Louis, Mo.) was added to agitation speed (up to 1,000 rpm) and the pure oxygen percentage. Antifoam 6.9 by the addition of 28% (vol/vol) ammonia-water. The dissolved oxygen concentration (DOC) could not be maintained above 20% of air saturation, oxygen-enriched air (83% O2) generated by an oxygen generator (Korea Energy Industry Co., Edison, N.J.) containing 1.2 liters of MR medium. Culture pH was controlled at 30°C in a jar fermentor (6.6 liters) (Bioflo 3000; New Brunswick Scientific Co., Edison, N.J.) containing 12 liters of MR medium. The culture conditions were similar to those previously described R medium (9). The MR medium (pH 6.9) contained the following per liter of 5 M HCl: FeSO4 3H2O, 0.5 g; MgSO4 7H2O, 10.0 g; CaCl2, 2.0 g; ZnSO4 4H2O, 0.7 g; citric acid, 0.8 g; trace metal solution, 5 ml. The trace metal solution contained the following per liter of 5 M HCl: FeSO4 3H2O, 7H2O, 10.0 g; CuSO4 5H2O, 1.0 g; (NH4)6Mo7O24 4H2O, 0.1 g; Na2B4O7 10H2O, 0.02 g. Separately sterilized glucose and thiamine were supplemented the medium to final concentrations of 20 g/liter and 10 mg/liter, respectively.

Seed cultures were prepared in flasks and incubated in a rotary shaker overnight at 30°C and 250 rpm. Laboratory-scale fed-batch culture was carried out at 30°C in a jar fermentor (6.6 liters) (Bioflo 3000; New Brunswick Scientific Co., Edison, N.J.) containing 1.2 liters of MR medium. Culture pH was controlled at 6.9 by the addition of 28% (vol/vol) ammonia-water. The dissolved oxygen concentration (DOC) was controlled as desired by automatic control of the agitation speed (up to 1,000 rpm) and the pure oxygen percentage. Antifoam (0.02% [vol/vol]) (antifoam 289; Sigma Chemical Co., St. Louis, Mo.) was added at the onset of cultivation. The feeding solution used for the fed-batch culture contained the following per liter: glucose, 700 g; MgSO4 7H2O, 15 g; thiamine, 250 mg. The pH-stat feeding strategy, which was based on the sharp pH rise upon carbon source depletion, was employed in fed-batch cultures. When the pH rose to a value greater than its set point (6.9) by 0.1, an appropriate volume of feeding solution was automatically added to increase the glucose concentration in the culture medium to 20 g/liter. The feeding volume was calculated on-line by using fermentation software (AFS3.42; New Brunswick Scientific Co.).

For the scale-up studies in a 50-liter stirred tank fermentor (Korea Fermentor Company, Inchon, Korea), seed culture (4 liters) was prepared in a 6.6-liter fermentor by batch culture. When glucose was depleted, as indicated by a sharp increase of the pH, cell broth was immediately transferred to a 50-liter fermentor containing 12 liters of MR medium. The culture conditions were similar to those described above except for some modifications in the feeding strategy and the DOC control scheme to be suitable for the 50-liter fermentor. Since the 50-liter fermentor was not interfaced to a computer, a fixed feeding volume (0.5 liters per pulse) was added upon the increase of the pH. The DOC was maintained above 20% of air saturation during the first 15 h by varying the air flow rate at the fixed agitation speed (500 rpm, the maximum agitation speed of this fermentor). When the DOC could not be maintained above 20% of air saturation, oxygen-enriched air (83% O2) generated by an oxygen generator (Korea Energy Industry Co., Uiwang, Korea) was provided. When the DOC could not be maintained above 20% of air saturation even after the oxygen-enriched air supply was added, cultivation was simply continued at this maximum level of oxygen supply.

Analytical procedures. Growth was monitored by measuring the optical density at 600 nm. Cell concentration, defined as the amount (dry weight) of cells per liter of culture broth, was determined by weighing dry cells as described previously (14). PHB concentration was determined by gas chromatography (HP5890; Hewlett-Packard, Wilmington, Del.) with n-butyllic acid as an internal standard and the DOC was maintained above 7% of air saturation during the active growth phase.

RESULTS

Fed-batch culture in a 6.6-liter fermentor. The pH-stat fed-batch culture of XL1-Blue(pSYL107) was first carried out in a laboratory-scale fermentor. The time profiles of cell growth, PHB accumulation, and DOC are shown in Fig. 1. The final cell concentration, PHB concentration, and DOC content obtained in 44 h were 206 g/liter, 149.7 g/liter, and 73%, respectively, which resulted in a PHB productivity of 3.4 g of PHB/liter/h. For convenience, the fermentation process could be divided into two phases, an active growth phase and an active PHB synthesis phase. Before cell concentration reached 60.2 g/liter at 15 h, the PHB content was kept nearly constant at ca. 16% (active cell growth phase). Afterwards, it steadily increased to 73% (active polymer synthesis phase). The DOC could not be maintained above 12% of air saturation when the cell concentration reached 97 g/liter at 18 h. From 18 h to the end of the cultivation (44 h), the DOC fluctuated in the range of 7 to 12% of air saturation. At the end of cultivation, a total of 4.9 liters of cell broth was harvested. A total amount of 734 g of PHB (without considering the PHB in samples) was produced from 2,692 g of glucose (including the glucose in the initial medium and seed culture), resulting in a yield of 0.27 g of PHB/g of glucose.

Effect of insufficient oxygen supply on PHB synthesis. The effect of insufficient oxygen supply on PHB production was investigated in fed-batch cultures. Oxygen limitation of maintaining the DOC at 1 to 3% of air saturation was applied during two different phases of the fermentation as described earlier. First, oxygen limitation was applied at a low cell concentration of 8.3 g/liter (active cell growth phase). As shown in Fig. 2, cell growth stopped after 1.5 h of oxygen limitation. PHB concentration and PHB content increased only from 1.3 to 3.0 g/liter and from 15 to 28%, respectively. Therefore, it was concluded that oxygen limitation during the active growth phase.

FIG. 1. Time profiles of cell concentration, PHB concentration, residual cell concentration, PHB content, and DOC during the fed-batch culture of recombinant E. coli XL1-Blue(pSYL107) in a chemically defined medium. The DOC was maintained above 7% of air saturation.

FIG. 2. Time profiles of cell concentration, PHB concentration, residual cell concentration, PHB content, and DOC during the fed-batch culture of recombinant E. coli XL1-Blue(pSYL107) in a chemically defined medium. The DOC was maintained above 7% of air saturation.
PHB production in a pilot-scale fermentor. To examine if the cultivation strategy described above could be applied to the production of PHB in a large-scale fermentor, in which oxygen transfer is generally poor, fed-batch culture of XL1-Blue(pSYL107) was carried out in a 50-liter fermentor (Fig. 4). Oxygen-enriched air generated by the pressure swing adsorption equipment, instead of pure oxygen, was used during the fermentation. In this fermentation, the DOC was not deliberately lowered but rather decreased to zero due to the oxygen transfer limitation of the fermentor itself (maximum agitation speed and oxygen-enriched air flow rate of 500 rpm and 5 liters/min, respectively). During the cultivation the switch from the active growth phase to the active PHB synthesis phase occurred at 12 h (cell concentration of 36.6 g/liter). The DOC dropped to zero when cell concentration reached 54.8 g/liter at 15 h. However, a detrimental effect of oxygen limitation was not observed. Cell and PHB concentrations obtained in 36 h were 153.7 and 101.3 g/liter, respectively, resulting in a PHB productivity of 2.8 g of PHB/liter/h. Therefore, it was concluded that the filamentation-suppressed recombinant E. coli could be employed for the efficient production of PHB in a defined medium in a large-scale fermentor. A total of 19.4 liters of feeding solution was consumed, and 38 liters of cell broth was harvested at the end of cultivation. Therefore, the PHB yield was 0.28 g of PHB/g of glucose (3,849 g of PHB produced from 13,900 g of glucose).

DISCUSSION

Even though PHAs have been considered to be good candidates for completely biodegradable plastic or elastomer material, the high production cost has hampered their use in a wide range of applications. Several fermentation processes employing A. eutrophus, Alcaligenes latus, methylotrophs, and recombinant E. coli have been developed for more economical production of PHB (6, 7, 13). A PHB productivity of 2 to 3 g of PHB/liter/h has been obtained by employing A. eutrophus and recombinant E. coli. Computer-aided process analysis and economic evaluation for PHB production by bacterial fermentation suggested that obtaining high PHB productivity and high PHB content from inexpensive medium was important for reducing the production cost of PHB (3). Among the several bacteria that have been shown to produce PHB efficiently, recombinant E. coli harboring the A. eutrophus PHA biosynthesis genes has been considered to be a strong candidate as a PHB producer due to several advantages such as a wide range of utilisable carbon sources, easy recovery of PHB, and no degradation of PHB during fermentation due to the lack of intracellular depolymerases (12). However, a high concentration of PHB (greater than 80 g/liter) with a high PHB productivity (greater than 2 g of PHB/liter/h) could be obtained only in a complex or semi-defined medium (11, 12, 14). One of the several reasons for the reduced synthesis of PHB in a defined medium was filamentation of cells accumulating PHB (3, 15). We have demonstrated in flask cultures that cell filamentation could be suppressed by overexpressing FtsZ, an essential cell division protein (5). Furthermore, this filamentation-suppressed recombinant E. coli XL1-Blue(pSYL107) could accumulate more PHB in a defined medium in flask cultures (5). Therefore, fed-batch cultures of XL1-Blue(pSYL107) were carried out to examine if PHB could be efficiently produced in a defined medium. As shown in Fig. 1, an even higher concentration of PHB (149 g/liter) and a higher PHB productivity (3.4
g of PHB/liter/h) could be obtained in a defined medium compared with a complex or semi-defined medium. No significant cell filamentation was observed during the entire fermentation. Having achieved the successful production of PHB to a high concentration with high content and high productivity, we next examined the effects of applying oxygen limitation on cell growth and PHB production for two purposes. First, it may be possible to enhance the PHB synthesis rate by increasing the acetyl-CoA flux into the PHB biosynthetic pathway and reducing its flux into the tricarboxylic acid cycle. Second, it is difficult to maintain the DOC above 10% of air saturation at high cell densities without using pure oxygen. Since oxygen transfer is even worse in a large-scale fermentor and the use of a large amount of pure oxygen can be economically unfavorable, it will be beneficial if PHB can be efficiently produced under oxygen-limited conditions.

The fed-batch process of fermentation with recombinant E. coli can be conveniently divided into two phases: (i) an active growth phase during which PHB content is kept relatively constant at a low level (15 to 20%) and (ii) an active PHB synthesis phase during which PHB is actively accumulated with a concomitant increase of PHB content. During the active growth phase, cell growth stopped and no apparent enhancement of PHB accumulation was observed (Fig. 2). However, cell growth and PHB production were not hampered when oxygen limitation was applied during the active PHB synthesis phase (the final PHB concentration and PHB productivity were as high as 157.1 g/liter and 3.2 g of PHB/liter/h, respectively). A major reason for this difference may have been related to acetate accumulation. Oxygen limitation applied during the active growth phase resulted in accumulation of acetate to a final concentration of 14 g/liter, which is higher than the critical acetate concentration that shows growth inhibition (8). The acetate concentration was less than 4 g/liter when oxygen limitation was applied during the active PHB synthesis phase. The actual reason for these findings is not clear at this moment. Nevertheless, it is nice from a process point of view to find that oxygen limitation could enhance PHB production by filamentation-suppressed recombinant E. coli in a defined medium. To examine if this strategy could be employed for the production of PHB in a large-scale fermentor, fed-batch culture was carried out in a 50-liter pilot-scale fermentor. This fermentor is a typical stirred tank bioreactor equipped with three bottom-driven Rushton turbine impellers with a maximum agitation speed of 500 rpm. Instead of using pure oxygen, oxygen-enriched air (83% O2) was supplied when required. Although the DOC decreased to zero when cell concentration reached 54.8 g/liter at the maximum gas flow rate and agitation speed (Fig. 4), a high concentration of PHB (101.3 g/liter) was still obtained in 36 h, resulting in a relatively high PHB productivity of 2.8 g of PHB/liter/h. Since an oxygen-limited condition is likely to be encountered in a large-scale fermentor without using pure oxygen, it is encouraging to find that PHB could be efficiently produced under this condition.

We next examined whether the enhanced production of PHB in a defined medium under oxygen limitation was due to the increased PHB synthesis rate. The maximum PHB synthesis rates during the fermentation under oxygen-sufficient (Fig. 1) and oxygen-limited (Fig. 3) conditions were 0.11 and 0.12 g of PHB/g of residual cell/h, respectively. Therefore, oxygen limitation apparently did not enhance the PHB synthesis rate. However, the maximum PHB synthesis rate obtained in a defined medium with or without oxygen limitation was lower than that obtained in a complex or semi-defined medium (0.15 to 0.20 g of PHB/g of residual cell/h) (11, 14, 15). Comparison of the time profiles of the residual cell concentrations in defined (Fig. 1 and 3), complex (11), and semi-defined (10) media suggests that the higher PHB concentration and productivity obtained in a defined medium were due to the higher residual cell concentration before cells entered the active PHB synthesis phase. In a complex or semi-defined medium, the typical residual cell concentration at the switch from active growth phase to active PHB synthesis phase was about 30 g/liter (10, 11), while that in a defined medium was greater than 60 g/liter (Fig. 1 and 3). Since PHB is accumulated in the cytoplasm, the residual cell concentration determines how much PHA can potentially be accumulated. However, we cannot increase the residual cell concentration before the active PHA synthesis phase to too high a value because, as was shown previously, such a concentration results in incomplete PHB synthesis and, subsequently, a low PHB content (7). The residual cell concentration of 60 g/liter was found to be optimal for the production of PHB by this recombinant E. coli strain in a defined medium.

Even though recombinant E. coli has been suggested to have several advantages as a PHB producer, there were two problems to be solved: the requirement for complex nitrogen sources and a high oxygen demand. In this study we demonstrated that PHB could be efficiently produced in a defined medium by the filamentation-suppressed recombinant E. coli strain. We also solved the problem of high oxygen demand by demonstrating that PHB could be produced to a high concentration with high productivity by applying oxygen limitation during the active PHA synthesis phase. However, there is one new problem observed, i.e., a slightly lower PHB yield on glucose in a defined medium. The PHB yield obtained in this study was 0.27 to 0.28 g of PHB/g of glucose, which is lower than that obtained in a complex medium (0.37 g of PHB/g of glucose) (12). It seems that more glucose was wasted to carbon dioxide in a defined medium. To investigate the possibility of improving the PHB yield, we are now carrying out fed-batch cultures under various conditions while monitoring the carbon dioxide evolution rate by using an on-line mass spectrometer.

ACKNOWLEDGMENTS

We thank the BioProcess Engineering Research Center for the partial support of Fulai Wang. This work was also supported by the Ministry of Science and Technology and by LG Chemicals, Ltd.

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