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COMPARISON AND OPTIMIZATION OF POLY(3-HYDROXYBUTYRATE) RECOVERY FROM *Alcaligenes eutrophus* AND RECOMBINANT *Escherichia coli*

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Abstract – The recovery of poly(3-hydroxybutyrate) [PHB] from *Alcaligenes eutrophus* and a recombinant *Escherichia coli* strain harboring the *A. eutrophus* poly(3-hydroxyalkanoates) biosynthesis genes was studied. When PHB was recovered using sodium hypochlorite or sodium dodecyl sulfate (SDS), non-PHB cell materials (NPCM) of the recombinant *E. coli* seemed to be more easily digested than those of *A. eutrophus*. Furthermore, viscosity increase caused by cell lysis during SDS treatment was negligible for the recombinant *E. coli*, whereas a very viscous suspension was formed for *A. eutrophus*. These results, together with our previous finding that PHB in the recombinant *E. coli* is far less susceptible to molecular degradation by sodium hypochlorite, suggested that the recombinant *E. coli* was more beneficial than *A. eutrophus* in terms of PHB recovery. In order to develop an easy and efficient recovery process, we adopted and optimized the SDS treatment since, with the hypochlorite treatment, we could not handle high biomass concentrations effectively. We could obtain a PHB of 95 % purity with 96 % recovery under the optimal condition of a biomass concentration of 5 %, a ratio of SDS to biomass of 0.6, a treatment time of 60 minutes, and a treatment temperature of 30 °C.

Key words: Poly(3-hydroxybutyrate), Alcaligenes eutrophus, Recombinant Escherichia coli Sodium Hypochlorite, Sodium Dodecyl Sulfate

INTRODUCTION

Recently, there has been an extensive worldwide research effort on biodegradable polymers, as discarded non-biodegradable plastics create serious problems [Swift, 1993]. Among various biodegradable plastics available, there is a growing interest in the group of poly(3-hydroxyalkanoates), PHA [Poirier et al., 1995]. Currently, ZENECA (formerly ICI) in the United Kingdom produces PHA through a bacterial fermentation process employing a glucose utilizing mutant strain of *A. eutrophus* [Holmes, 1985; Byrom, 1987]. The major obstacle to wide usage is their high production cost [Mayer and Kaplan, 1994]. The development of new bacterial strains which produce PHA more efficiently [Yim et al., 1995], and improvements in fermentation [Kim et al., 1994] and PHA recovery processes [Koo et al., 1996] are expected to bring the costs down further [Poirier et al., 1995].

Various PHA recovery processes have been suggested and employed not only for a laboratory-scale but also for an industrial-scale [Lafferty and Heinzle, 1979; Berger et al., 1989; Holmes and Lim, 1990; Hahn et al., 1995]. A major consideration in selecting a PHB recovery process is its ability to provide efficient release of intracellular PHA maintaining its intact properties, especially molecular weights. In general, these methods can be categorized into two classes: solvent extraction of PHA [Lafferty and Heinzle,1979; Hahn et al., 1993] and digestion of non-PHB cellular materials (NPCM) [Ramsay et al., 1990; Page and Cornish, 1993]. Although PHA can be recovered without molecular degradation by using a solvent extraction method, it causes significant raw material costs [Holmes and Lim, 1990]. As an alternative to solvent extraction, an aqueous enzymatic digestion method has been developed by ZENECA. This process consists of thermal treatment of PHA-containing biomass, enzymatic treatment, and washing with an anionic surfactant to dissolve NPCM [Holmes and Lim, 1990].

In this work, we studied PHB recovery from *A. eutrophus* and a recombinant *E. coli* strain harboring the *A. eutrophus* PHA biosynthesis genes (pSYL104). We compared the two microorganisms with respect to recovery efficiency when PHB was recovered with sodium hypochlorite solution or SDS solution. The results suggested that the recombinant *E. coli* was more suitable than *A. eutrophus* for PHB recovery. Optimization was carried out in terms of recovery (%) and purity (%) of recovered PHB for the development of an efficient and economical PHB recovery process from the recombinant *E. coli*.

MATERIALS AND METHODS

1. Microorganisms and Production of PHB-Containing Biomass

For the production of PHB, we used *A. eutrophus* (NCIMB 11599) and a recombinant *Escherichia coli* strain, XL1-Blue,

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harboring pSYL104 which had been kindly provided by Prof. S. Y. Lee (KAIST, Korea). Fed-batch culture of *A. eutrophus* in a minimal medium was carried out with on-line glucose concentration control as previously described [Kim et al., 1994]. Details of the stable high-copy-number plasmid pSYL104 containing the *A. eutrophus* PHA biosynthesis genes are described elsewhere [Lee et al., 1994a]. Fed-batch culture of XL1-Blue (pSYL104) was carried out in a semi-defined medium employing the pH-stat as described previously [Lee et al., 1994b]. After the fermentation, the cells were harvested by centrifugation at 4,000×g and 25 °C for 15 minutes and washed twice with distilled water. The resulting cell paste was freeze-dried and stored at 4 °C until needed.

2. PHB Recovery by Using Sodium Hypochlorite or Sodium Dodecyl Sulfate

A sodium hypochlorite solution containing 5.68 g active Cl, 7.8 g NaOH, and 32 g Na_2CO_3 in 100 mL was used as the stock solution to make 20 % (w/v) diluted sodium hypochlorite solution. The concentration of PHB-containing cell powder (hereafter biomass) was varied from 1 % (w/v) to 5 % (w/v). After the PHB-containing biomass was treated with hypochlorite solution at 30 °C for 1 hour, PHB granules were separated from the aqueous fraction containing cell debris by centrifugation. The recovered PHB was rinsed with distilled water, centrifuged again, and then rinsed with acetone. Finally, recovered PHB was dried in a vacuum drying oven.

In the case of SDS treatment, the biomass concentration was varied from 4 % (w/v) to 10 % (w/v) and the ratio of SDS to biomass from 0.1 to 0.8. The treatment time was 1 hour at 30 °C, unless otherwise stated. The remaining steps were the same as in the hypochlorite treatment.

3. Analytical Procedures

Purity and recovery (%) of PHB were determined with a gas chromatograph (GC-Varian 3300) by the methanolysis method [Braunegg et al., 1978]. As an internal standard, n-butyric acid was used. The PHB recovery (%) was calculated from the total amount of PHB in the cell powder measured by GC and the amount of PHB recovered. Based on the method of Bradford, protein concentration was determined with a Bio-Rad Protein Assay Kit. A rapid method of ultraviolet absorbance spectrophotometry was used to determine the content of nucleic acids approximately. Isolation of genomic DNA was carried out with a QIAGEN DNA kit.

The molecular weight and its distribution of recovered PHB were analyzed with gel permeation chromatography (GPC). A GPC (Waters 150-CV) equipped with three serially connected gel-permeation columns having pore sizes of 10^3, 10^4, and 10^5 Å was operated at 40 °C for the analysis. Monodisperse polystyrene and chloroform were used as the molecular weight standard and the mobile phase, respectively. One hundred microliters of PHB solution [0.1 % (w/v)] was injected for each analysis.

The crystallinity of PHB in the lyophilized whole cells was estimated from the enthalpy of fusion of PHB, measured with a differential scanning calorimeter (DSC-Dupont 2000). In this procedure, the enthalpy of fusion of the 100 % crystalline (theoretical) sample was assumed to be 146 Joule/gram [Barham et al., 1984].

RESULTS AND DISCUSSION

1. Comparison of *A. eutrophus* and Recombinant *E. coli*

After fermentation, the cellular PHB contents were 62 % for *A. eutrophus* and 59 % for the recombinant *E. coli*, respectively. The results of the fermentation and characterization of PHB in the lyophilized whole cells are summarized in Table 1. Analysis of the freeze-dried cell powders showed that protein contents, DNA contents, and RNA contents were 15 % 1.7 %, and 10.3 % for *A. eutrophus*, and 17 %, 0.4 %, and 9.6 % for recombinant *E. coli*, respectively. There seemed to be no significant difference in their cellular compositions except for a considerable difference in DNA content between the two microorganisms.

As shown in one of our previous reports [Hahn et al., 1995], PHB granules in the lyophilized recombinant *E. coli* had a crystalline morphology, while the major part of PHB in lyophilized *A. eutrophus* was in a mobile amorphous state. According to a recent report [Middelberg et al., 1995], the mean diameter of PHB granules synthesized in a recombinant *E. coli* was as large as 1.13-1.25 μm, whereas that in *A. eutrophus* was only 0.1-0.8 μm. Due to the morphological differences, degradation of PHB to a lower molecular weight was severe for *A. eutrophus*, but it was relatively insignificant for recombinant *E. coli* during PHB recovery by the digestion of NPCM.

In addition to the stability against molecular degradation, the NPCM of the recombinant *E. coli* seemed to be more easily digested than those of *A. eutrophus* by the hypochlo-

Table 1. Molecular compositions of *Alcaligenes eutrophus* and recombinant *E. coli* and the characterization of PHB in the lyophilized whole cells

Microorganisms	Dry cell mass (g/L)	PHB (g/L)	PHB content (%)	Crystallinity (%)	M_n (10^4)	PI
A. eutrophus	137	85	62	16	121	1.8
Recombinant *E. coli*	108	64	59	60	154	2.0

Microorganisms	Macromolecular compositions of microorganisms (%)				
	PHB	Proteins	RNA	DNA	Others
A. eutrophus	62	15	10.3	1.7	11
Recombinant *E. coli*	59	17	9.8	0.4	14

where PI is polydispersity index.

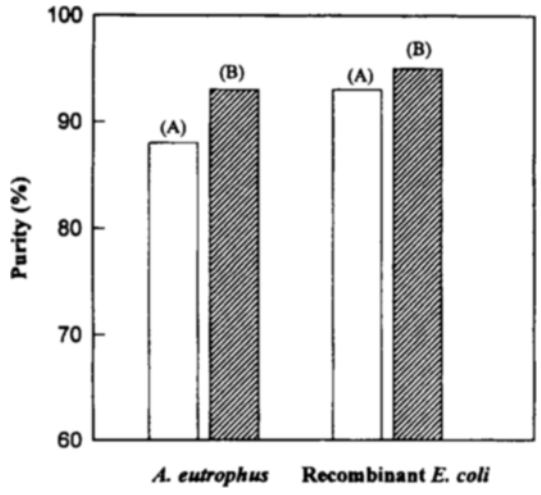

Fig. 1. Comparison of the purity of recovered PHB from *Alcaligenes eutrophus* and recombinant *E. coli*.
(A) PHB recovered with 1 % (w/v) biomass concentration and 20 % (w/v) sodium hypochlorite solution; (B) PHB recovered with 5 % (w/v) biomass concentration and 3 % (w/v) SDS solution after autoclave for 15 minutes at 110 °C.

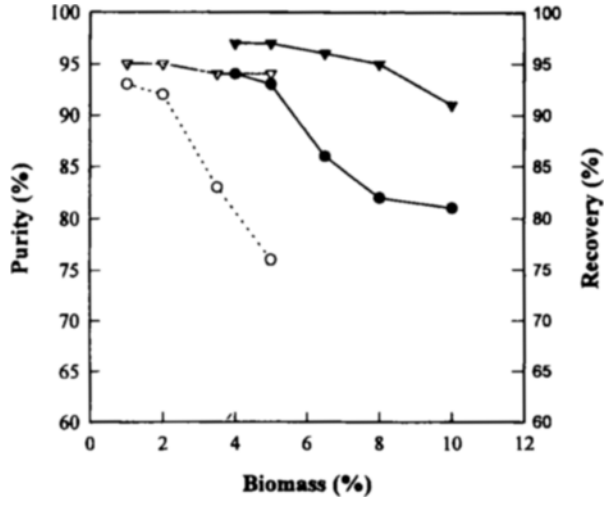

Fig. 2. Comparison of the dispersion method and SDS treatment method.
Dispersion method: PHB recovered with 20 % (w/v) sodium hypochlorite: ▽: recovery (%), ○: purity (%). SDS method: PHB recovered with 2 % (w/v) SDS solution: ▼: recovery (%), ●: purity (%).

rite treatment. This was considered to be one of the reasons that we could obtain purer PHB from the recombinant *E. coli* than *A. eutrophus*. When PHB was recovered with 20 % sodium hypochlorite solution, the purity of PHB recovered from *A. eutrophus* was only 88 %, whereas that from the recombinant *E. coli* was 93 % as specified in Fig. 1. As reported in the literature [Lee et al., 1994a], another advantage of PHB in a recombinant *E. coli* is that it is not hydrolyzed during or after fermentation since *E. coli* has no intracellular PHB depolymerase systems.

In order to recover PHB from *A. eutrophus* using SDS treatment, the suspension should be heated to at least 80 °C prior to or during the digestion, since, in the absence of such a heating step, a very viscous suspension was formed by the nucleic acids released from the cells. Without the heat-treatment, a separation process for PHB recovery was practically impossible since severe aggregation of the lysate occurred. However, in the case of the recombinant *E. coli*, no heat treatment was necessary allowing a slightly higher purity of recovered PHB (Fig. 1). These phenomena might be caused by the difference in DNA content. Some cells of the recombinant *E. coli* accumulated PHB to an extent large enough to cause distortion of cell envelopes, and there existed a number of cells that had protruding PHB granules, as reported previously [Lee et al., 1995]. Although it is not yet certain, the cell wall of the recombinant *E. coli* seemed to be weakened with PHB accumulation. These results suggested that recombinant *E. coli* was more beneficial than *A. eutrophus* in terms of ease and efficiency of the PHB recovery process.

2. Optimization of SDS Treatment for Recombinant *E. coli*

In order to develop an efficient and economic recovery process for the recombinant *E. coli*, the sodium hypochlorite treatment was considered at first. The recovery (%) was ca. 95 % over the range of biomass concentration (1-5 %) treated, as shown in Fig. 2. If we consider treatment capacity, the higher the biomass concentration we adopt, the more desirable it is. However, there must be a limitation since the purity decreases as the biomass concentration increases. In the present study, we assumed that the purity should be at least 80 %. Based on this criterion, when PHB was recovered with 20 % (w/v) sodium hypochlorite solution, 3.5 % (w/v) of biomass concentration was the maximum allowable among that tested, for which PHB with a purity of 83 % was produced (Fig. 2). However, this was considered not to be satisfactory owing to the high recovery cost caused by a low treatment capacity. From the results, we concluded that the sodium hypochlorite treatment was not quite satisfactory for PHB recovery in quantity.

As an alternative to the hypochlorite treatment, the SDS treatment was tried for PHB recovery. According to the results from a preliminary study, this method seemed to be a simple, effective, and economic process for PHB recovery, and its optimization was carried out in terms of recovery (%) and purity (%) of recovered PHB. The details are given below.

With only 2 % (w/v) of SDS concentration, it was possible to obtain PHB with a purity higher than 80 % all over the biomass concentration range, from 4 % (w/v) to 10 % (w/v), as shown in Fig. 2. In the case of 5 % (w/v) biomass concentration, the purity of PHB recovered was as high as 93 %. A slightly higher purity could be obtained using 4 % (w/v) biomass concentration. However, it seemed reasonable to adopt a higher biomass concentration of 5 % (w/v), since the recovery results showed no significant difference between 4 % (w/v) and 5 % (w/v) biomass concentrations.

The recovery (%) decreased with increasing biomass concentration due to the increased foaming of suspension and the increased viscosity caused by a larger amount of nucleic acids released. It was much more serious in the case of 10 % (w/v) biomass concentration. In terms of the recovery (%), it seemed that a biomass concentration higher than 8 % (w/v)

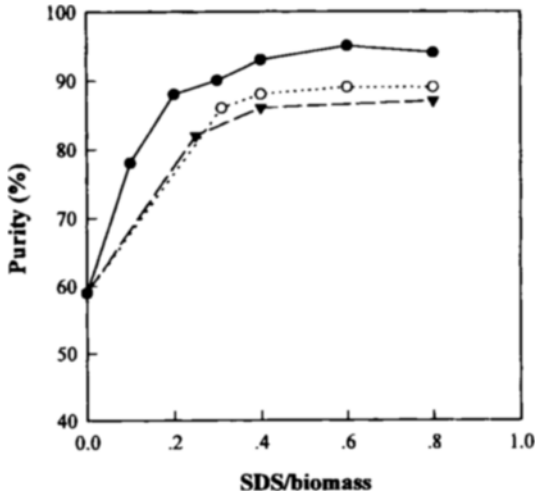

Fig. 3. Effect of the ratio of SDS to biomass on the purity of PHB for different biomass concentrations.
Biomass: ●: 5 % (w/v), ○: 6.5 % (w/v), ▼: 8 % (w/v).

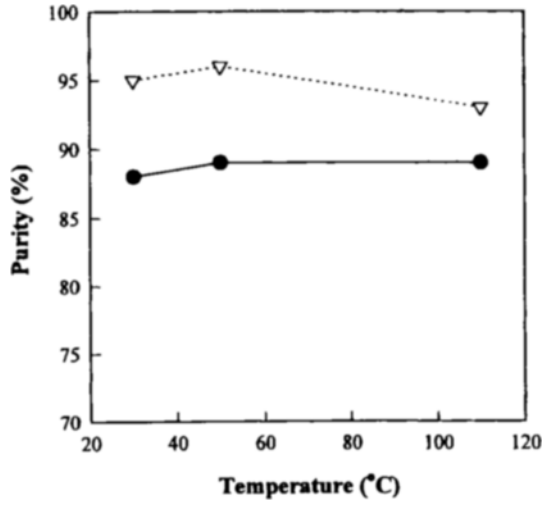

Fig. 5. Effect of treatment temperature on the purity of PHB recovered with 5 % (w/v) biomass concentration.
Ratios of SDS to biomass: ●: 0.2 % (w/w), ▽: 0.6 % (w/w).

was not practical for SDS treatment. The results suggested that the optimal biomass concentration for PHB recovery using SDS treatment ranged from 5 % to 8 % (w/v).

Several values of SDS to biomass ratio were tested to determine the optimal ratio for an efficient PHB recovery. The zero data (no SDS treatment) in Fig. 3 implies that the PHB content in the untreated cell powder was about 59 %. As a whole, the purity of recovered PHB increased with increasing ratio of SDS. In the case of 5 % (w/v) biomass concentration, the purity went up as high as 95 % when the ratio was 0.6. For 6.5 % and 8 % (w/v) biomass concentrations, however, only small increases resulted, in spite of using a higher ratio of SDS. The purity approached, as the ratio increased, to ca. 89 % for 6.5 % (w/v) biomass concentration and ca. 87 % for 8 % (w/v) biomass concentration, respectively. Considering the purity of recovered PHB, we concluded that the optimal ratio of SDS to biomass was 0.6 with a biomass concentration of 5 % (w/v).

For a biomass concentration of 5 % and a ratio of SDS to biomass of 0.6, treatment time was optimized in view of the purity of PHB recovered. As shown in Fig. 4, the purity increased as the treatment time increased. For sufficient digestion of NPCM, a treatment time longer than 30 minutes was required. From the results, we concluded that the optimal treatment time was ca. 60 minutes.

For a biomass concentration of 5 % and a treatment time of 60 minutes, the effect of treatment temperature was investigated for ratios of SDS to biomass of 0.2 and 0.6. It could be said from Fig. 5 that a higher treatment temperature is not crucial to improve the purity of recovered PHB. Treatment at 50 °C brought about a purity increase of only 1 %, compared with the case of at 30 °C. It was interesting that the purity of PHB recovered with heating (autoclave) at 110 °C was rather lower than that of 30 °C. The results seemed to be caused by the fact that there was no mixing during the SDS treatment in the autoclave.

In conclusion, we could recover a PHB of 95 % purity from the recombinant *E. coli* with 96 % recovery under the optimal conditions of a biomass concentration of 5 %, a ratio of SDS to biomass of 0.6, a treatment time of 60 minutes, and a treatment temperature of 30 °C.

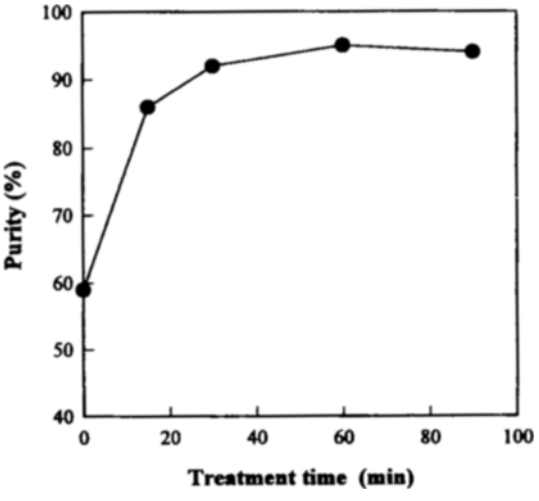

Fig. 4. Effect of treatment time on the purity of PHB recovered with 5 % (w/v) biomass concentration and a ratio of SDS to biomass of 0.6.

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