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NOTE

A Simple Method for Recovery of Microbial Poly-β-hydroxybutyrate by Alkaline Solution Treatment

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A novel and simple purification method for microbial poly-β-hydroxybutyrate (PHB) was developed. Sodium hydroxide was found to be efficient for digesting cell materials. Initial biomass concentration, NaOH concentation, digestion time, and incubation temperature were optimized. When 40 g/l of biomass was incubated in 0.1 N NaOH at 30°C for 1 h, PHB purity of 88.4% with a weight average molecular weight (M_w) of 770,000 and a polydispersity index (PI) of 2.4 was recovered with a yield of 90.8 % from the biomass which initially contained PHB of a M_w of 780,000 and a PI of 2.3.

Poly-β-hydroxybutyrate (PHB) is a reserve polyester material accumulated as intracellular granule in a variety of bacteria (1). As an intracellular product, its separation from the cell is prerequisite to obtaining an appropriate quality for its application. Although a number of recovery processes (2-4, 7, 8, 10, 11) have been developed, their commercial application has not been fully exploited because of high recovery costs.

The majority of recovery processes involve the extraction of PHB with organic solvents (2, 3, 11). While the solvent extractions are generally used to recover PHB with a high purity, these methods require large quantities of toxic and/or volatile solvents. These processes also require pretreatment of cells such as milling and spray drying to improve the solvent extractability. Consequently, multiple treatment and subsequent solvent recovery make the process costly one. A novel alternative, developed by ZENECA (formerly ICI), involves thermal treatment of PHB-containing biomass followed by enzymatic digestion and washing with an anionic surfactant to dissolve non-PHB cell material (NPCM) (8). Although it is reported quite efficient, it also requires additional digestion or solvent extraction steps to increase purity of the product, resulting in an increased recovery cost. Another procedure reported is the use of a differential digestion method employing sodium hypochlorite (4, 10). Although simple and effective, at the same time, it caused degradation of PHB, which resulted in a lower molecular weight and a higher polydispersity index (PI) compared to those of intact cellular PHB. Recently, Hahn *et al.* (7) reported that the use of a dispersion made of hypochlorite solution and chloroform enabled one to recover the cellular PHB with a high purity while preventing severe degradation of PHB. However, much amount of hypochlorite and chloroform required to treat the biomass containing PHB and subsequent separation of chloroform after PHB extraction seems to be burdensome to enable exploitation this method for industrial purposes.

In this study, therefore, we have attempted to develop a novel and simple recovery method for cellular PHB, being economically more profitable. Various acids and alkalies were tested to examine the digestability of NPCM. Optimization was carried out to recover PHB with a high purity while ensuring that initial molecular weight was not significantly affected.

Alcaligenes eutrophus NCIMB 11599 was used for the production of microbial PHB. A two-stage fed-batch technique was empolyed to accumulate the PHB under nitrogen limited condition. The composition of culture medium and the culture method were described in our previous paper (9). The biomass harvested by centrifugation from the culture broth was stored at 4°C. Cells containing PHB were treated with NaOH solution. PHB recovered by NaOH treatment was further purified to determine molecular weight. Lyophilized biomass (100 mg) was mixed with 10 ml of chloroform at 40°C for 1 h. Residual biomass was removed by filtration. 5 volumes of

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Table 1. Effect of acid and alkali on PHB purification.

acid/alkali	purity (%)	recovery (%)
NaOH	98.0	85.0
KOH	94.7	85.7
NH_4OH	81.1	92.3
HCl	80.3	92.8
H_2SO_4	72.7	92.2
H_3PO_4	76.9	92.1

405 mg of cells with PHB content of 67.2% was treated with 10 ml of 0.1 N either acidic or alkaline solution. Reactions were carried out at 80°C for 1 h. Data are the mean of three independent experiments, standard deviations in all the values are less than 10%.

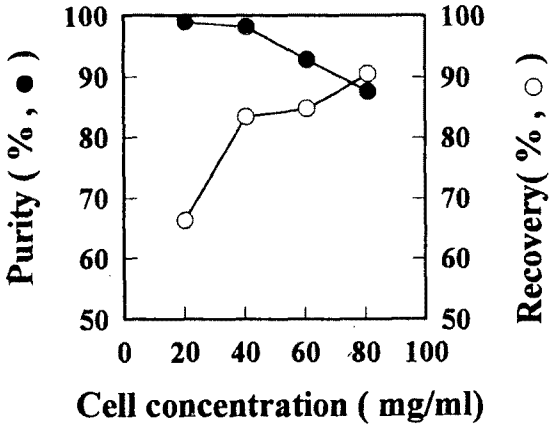

Fig. 1. Effect of cell concentration on PHB recovery.
Various concentrations of cells with PHB content of 67.2% were treated with 10 ml of 0.1 N NaOH solution. Reactions were carried out at 100°C for 1 h.

cold methanol was added to the PHB solution. The precipitate was then pressed to remove methanol and dried under ambient conditions for 2 days. The PHB concentration was determined by using gas chromatograph (Hewlett Parkard, Avondale, USA) as described by Braunegg *et al.* (5). Molecular weight and its distribution of PHB recovered were analyzed by using gel permeation chromatograph (Waters 150-C models). The purity of PHB was determined from a known mass of the sample by using gas chromatography. From a known amount of PHB in the biomass, the percent PHB recovered was calculated based on the purity of the total mass of the sample recovered from a given separation process.

The cell concentration obtained after fermentation was 110 g/l and the amount of PHB accumulated was 67.2%(w/w) of the total cell mass. First, to examine the digestability of cell material, various acids and alkalies were tested. Among the chemicals tested, NaOH was found to be the most efficient one to recover cellular PHB with a purity higher than 90% (Table 1). Optimization was therefore carried out by

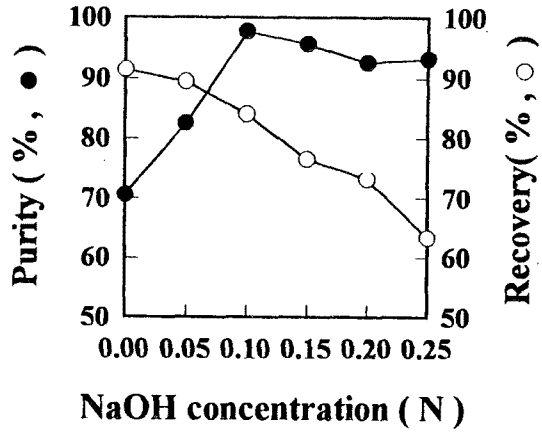

Fig. 2. Effect of NaOH concentration on PHB recovery.
405 mg of cells with PHB content of 67.2% were treated with 10 ml of NaOH solution. Reactions were carried out at 100°C for 1 h.

using NaOH. Various cell concentrations were applied to NaOH solution. It was observed that as the cell concentration increased, PHB recovery increased while its purity decreased (Fig. 1). However, even at higher cell concentrations (up to 80 mg/ml), the purity was higher than 85%. It was also noted that PHB could be degraded to some extent by NaOH. Effect of NaOH concentration on PHB recovery and purity was thus examined. Cellular PHB with a purity higher than 90% could be obtained at NaOH concentrations higher than 0.1 N, while PHB yield decreased as NaOH concentration increased (Fig. 2). From these observations, the optimal NaOH concentration was found to be 0.1 N, yielding 80% recovery yield with 90% purity at the cell concentration of about 4%(w/v).

Optimal incubation time and temperature for the PHB recovery were further investigated at fixed concentrations of cell and NaOH. In the presence of NaOH, the purity increased but recovery yield decreased as the incubation temperature increased, while in the absence of NaOH the purity of PHB was hardly improved even at 100°C (Fig. 3(a) and (b)). This indicates that a higher incubation temperature accelerated digestion of biomass in the presence of NaOH. As shown in Fig. 3(c), the pH of the solution decreased as digestion proceeded. This was probably due to the cellular nucleic acid excreted (8). Fig. 3(d) shows the weight average molecular weight (M_w) of PHB with the incubation time and temperature. The initial M_w of PHB from *A. eutrophus* determined in this study was found to be 780,000, while others reported higher molecular weights (for example, 1,200,000 by Berger *et al.* (4)). As Daniel *et al.* (6) and Yeom and Yoo (12) reported, the discrepancy on

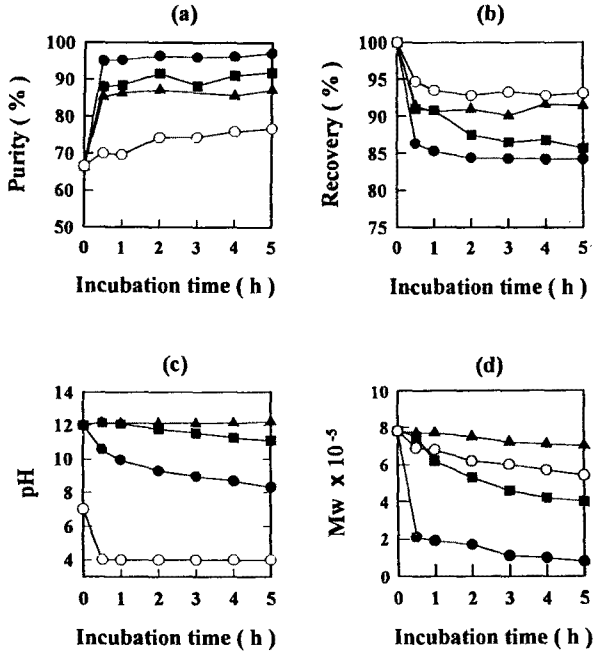

Fig. 3. Effect of incubation time and temperature on the PHB recovery from cells with PHB content of 67.2% by treatment of 0.1 N NaOH.

Cell concentration was 4.05%(w/v). Symbols; (●), 100°C; (■), 60°C; (▲), 30°C; (○), 100°C-no treatment of NaOH.

the M_w of PHB was probably due to their culture conditions. Degradation of PHB was also greatly affected by its incubation temperature. Approximately 73% of reduction in M_w was observed in half an hour at 100°C, while only a slight reduction at 30°C. It was thus concluded that all the digestion reactions should be completed within 1 hr. On the other hand, the polydispersity index (PI; Mw/Mn) representing the molecular weight distribution was maintained within the range of 2.3-2.6 when cells were incubated at 30°C. It was noteworthy that the very small PI value of PHB obtained by this method can allow broader applicability compared with the higher PI values (more than 4.0) by hypochlorite treatment (4). Under the specified condition (30°C, 1 h), the present method resulted in a recovery yield of 90.8% from the biomass containing PHB (M_w 780,000 and PI 2.3). The purity of the recovered PHB (M_w 770,000 and PI 2.4) was 88.4%.

It should be mentioned that under the conditions that the cellular PHB was not severely degraded, the purity of PHB recoverd was not higher than that (above 95%) obtained by hypochlorite (4, 7). However, compared to the processes reported so far, which mostly involve pretreatment of biomass, through cell harvest, washing, drying, and/or boiling, our method enables the recovery of the PHB by direct treatment with NaOH solution. It will also offer a significant economic merit from process point of view that only a small amount (0.4%, w/v) of NaOH was sufficient to recover the microbial polyester. In addition, it is believed that this very simple and cheap method can make further purification processes easier with a lower cost; a smaller amount of organic solvent would significantly enhance the purity of PHB.

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