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Effects of Nitrogen and Oxygen Supply on Production of Poly-β-Hydroxybutyrate in *Azotobacter chroococcum*

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Production of poly-β-hydroxybutyrate (PHB) in a strain of *Azotobacter chroococcum*, a nitrogen-fixing bacteria, was investigated at various levels of nitrogen and oxygen. Feeding nitrogen source increased both cell growth and PHB accumulation. Oxygen supply appeared to be one of the most important operating parameters for PHB production. Both cell growth and PHB accumulation increased with the sufficient supply of air in the fed-batch fermentation of the strain. However, it was also noted that keeping the oxygen level under limited condition was critical to achieve high PHB productivity. A high titer of PHB (52 g/l) with a high cellular content (60%) was obtained after 48 hr of fed-batch operation by controlling the oxygen supply. Dual limitation of nitrogen and oxygen did not further increase the PHB accumulation probably due to the greater demand for reducing power and ATP for nitrogen fixation.

Poly-β-hydroxybutyrate (PHB) is a biodegradable polyester synthesized and accumulated in the form of intracellular granule in many bacterial species. It is well known that, in most bacteria synthesizing PHB, the accumulation of the polymer is stimulated by limiting noncarboneous nutrients such as nitrogen, oxygen, phosphorous, magnesium, sulphur, or potassium (10, 13). Of these nitrogen limitation is known as one of the most useful measures for PHB accumulation due to its convenience for bioprocess control in the cultivations of *Alcaligenes* spp.(5, 7, 9), *Pseudomonas* spp. (12, 17), and methylotrophic bacteria (1, 18, 19).

Strains of *Azotobacter* sp. have some distinguished characteristics from those strains because of their nitrogen-fixing capability. The nitrogen-fixation system of the strain is operated via the metabolic pathway involving nitrogenase, and most active under nitrogen-deficient condition. It requires large amount of ATP and reducing power such as NAD(P)H, which are also needed for the PHB synthesis. Consequently, as reported by Page and Knosp (11), the PHB yield from a *Azotobacter* sp. significantly decreased under nitrogen-fixing conditions mainly because of the intracellular shortage of reducing power. In accordance with this, it was suggested that oxygen limitation could be an effective measure to stimulate the PHB accumulation in *Azotobacter* spp. (15, 20). It was also noted that the conversion efficiency of glucose to PHB was enhanced under oxygen-limited condition (3, 11). However, oxygen-limitation could cause an adverse effect, reduced cell growth. Hine and Lees (8) observed that the growth of *Azotobacter chroococcum* was significantly enhanced with intensive aeration. This indicates that there exists an optimal condition for supplies of nitrogen and oxygen in this culture system for production of PHB. However, little has been reported on the control strategy for fed-batch operation for PHB production, which is considered of importance for industrial application.

In this study, effects of nitrogen source and aeration on cell growth and PHB accumulation were investigated with a strain of *Azotobacter chroococcum* in order to achieve high productivity of PHB in fed-batch cultivations.

MATERIALS AND METHODS

Bacterial Strain and Cuture Conditions

A strain of *Azotobacter chroococcum* 23 obtained from Latvian University, Riga, Latvia was used in this study. The basic fermentation medium contained (per liter): 30 g glucose, 3.0 g NH_4NO_3, 0.64 g K_2HPO_4, 0.2 g KH_2PO_4, 0.41 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 50 mg $FeSO_4$

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· $7H_2O$, 0.5 g Na-citrate, and 6 mg Na_2MoO_4 · $2H_2O$. Glucose was supplemented into the culture media when required. Flask experiments were carried out in 500 ml baffled flasks at 30°C and 150 rpm on a rotary shaker. Any changes of culture conditions, if necessary, are described separately. The medium for fed-batch fermentation was the same as above except that 2.5 g $(NH_4)_2SO_4$ was used instead of NH_4NO_3. Feed solution for fed-batch operation was prepared by dissolving 700 g glucose, 12.8 g K_2HPO_4, 4.0 g KH_2PO_4, 6.0 g $MgSO_4$ · $7H_2O$, 1.0 g $CaCl_2$ · $2H_2O$, 0.4 g $FeSO_4$ · $7H_2O$, 2.5 g Na-citrate, 60 mg Na_2MoO_4 · $2H_2O$, and 20 ml of trace element solution (which contains (per liter): 0.3 g H_3BO_3, 0.2 g $CoCl_2$ · $6H_2O$, 0.1 g $ZnSO_4$ · $7H_2O$, 30 mg $MnCl_2$ · $4H_2O$, 20 mg $NiCl_2$ · $6H_2O$, 10 mg $CuSO_4$ · $5H_2O$) in 1 liter of 0.05 N HCl. Ammonia water (28%) was supplied as the nitrogen source at the amount determined necessary for pH control.

Fed-batch fermentation was carried out in a 5-liter jar fermentor (Korea Fermentor Co., Inchon,Korea) equipped with a DO analyzer and a pH controller. 200 ml seed culture, cultivated at 30°C for 36 hrs in shake flasks, was transferred to the fermentor containing 1.8 liter of the fermentation medium. Feed solution was supplied in such a manner that the glucose level in the culture broth was not limiting. The pH and the temperature were controlled at 7.2 and 30°C, respectively. Air flow rate and agitation speed were controlled in the range of 0.5~2.5 vvm and 300~1200 rpm, respectively.

Analytical Methods

Cell growth was monitored by measuring the optical density of the culture broth at 600 nm. The cell concentration was also determined by measuring the dry cell weight. Glucose concentration was determined by using a glucose analyzer (YSI, Ohio, USA). Phosphate was measured according to the reaction of amidol reagent (6) and ammonia by the indophenol method (16). PHB concentration was determined by using a gas chromatograph (Hewlett Packard, Avondale, USA) with benzoic acid as an internal standard (2).

RESULTS

Effects of Nitrogen Source and Oxygen Level in Flask Culture

In our preliminary experiment, NH_4NO_3 was found to be the most suitable nitrogen source for the cultivation of *Azotobacter chroococcum* mainly because of its pH stabilizing capability. Accumulation of PHB and cell growth were examined under the nitrogen deficient and sufficient conditions. Flask experiments were carried out to investigate the effect of nitrogen limitation on PHB accumulation by transferring the cells grown in ammonium-containing medium to nitrogen-free one. Table 1 shows that both cell growth and PHB accumulation significantly increased with supply of the nitrogen source, and also indicated that nitrogen limitation could result in a significantly reduced PHB accumulation.

Table 1. Comparison of cell growth and PHB accumulation under culture conditions of nitrogen source addition and nitrogen fixation[a]

Culture conditions	Culture volume (ml)	Cell conc. (g/l)	PHB conc. (g/l)	PHB content (wt %)
NH_4NO_3 addition	100	16.0	10.8	67.5
	200	4.2	3.1	73.8
Nitrogen limitation	100	2.5	1.0	40.0
	200	2.1	1.4	66.7
NH_4NO_3 addition→	100	13.5	10.6	78.5
Nitrogen limitation[b]	200	3.9	2.4	61.5

[a] Flask cultures were carried out in 500 ml baffled flask at 30°C and 150 rpm for 45 hrs. [b] After cultivated in the medium containing NH_4NO_3 for 30 h, cells were harvested and transferred into nitrogen-free medium.

Another interesting thing to note is that cell concentration and PHB accumulation are greatly influenced by the culture volume which gives different levels of dissolved oxygen. In ammonium-containing medium, PHB accumulation from the smaller culture volume (100 ml) was 3.5 times higher than that from the larger one (200 ml), while a reverse trend was observed in nitrogen-limiting conditions. Flask culture experiments were carried out extensively with various volumes of culture media to examine the effect of oxygen levels. As shown in Fig. 1-a, smaller culture volumes resulted in higher cell growth, indicating that oxygen could be a limiting factor for the growth of the microorganism. However, PHB accumulation at various culture volumes showed different pattern during the cultivation (Fig. 1b). At 34 h cultivation, PHB accumulation was the highest with the 100 ml of culture volume, while after 45 h cultivation, the smaller culture volume yielded a higher titer. PHB content at various culture volumes demonstrated distinctly time-dependent pattern of PHB accumulation (Fig. 1c). At an earlier culture time PHB content increased with the culture volume, indicating that the oxygen-limiting condition occurred earlier in the larger culture volume. However, this pattern appeared to be reversed as cultivation proceeded. It is therefore believed that PHB accumulation is favored under the oxygen-limiting environment. All these observations strongly suggest that a sufficient aeration rate but oxygen-limiting condition is necessary to achieve high PHB productivity in a jar fermentor operation.

PHB Production in a Fed-batch Cultivation

From the preliminary study with flask cultures, it was

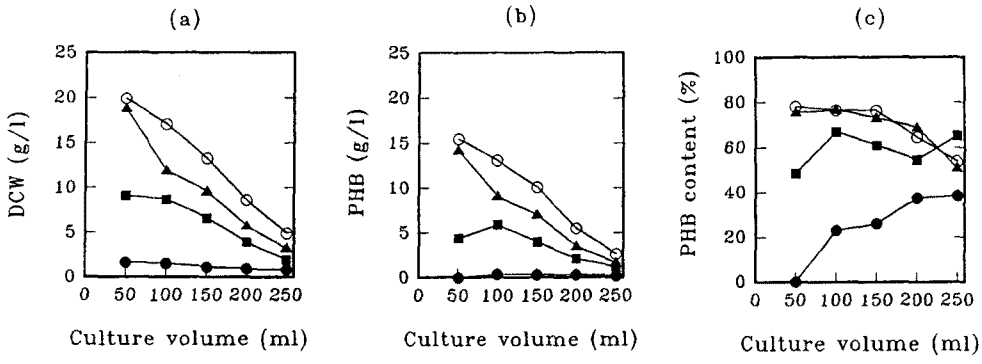

Fig. 1. Effect of aeration rate on cell growth and PHB accumulation.
(a) Cell growth, (b) PHB accumulation, (c) PHB content, Symbols: culture time; (●) 20 h, (■) 34 h, (▲) 45 h, (○) 60 h

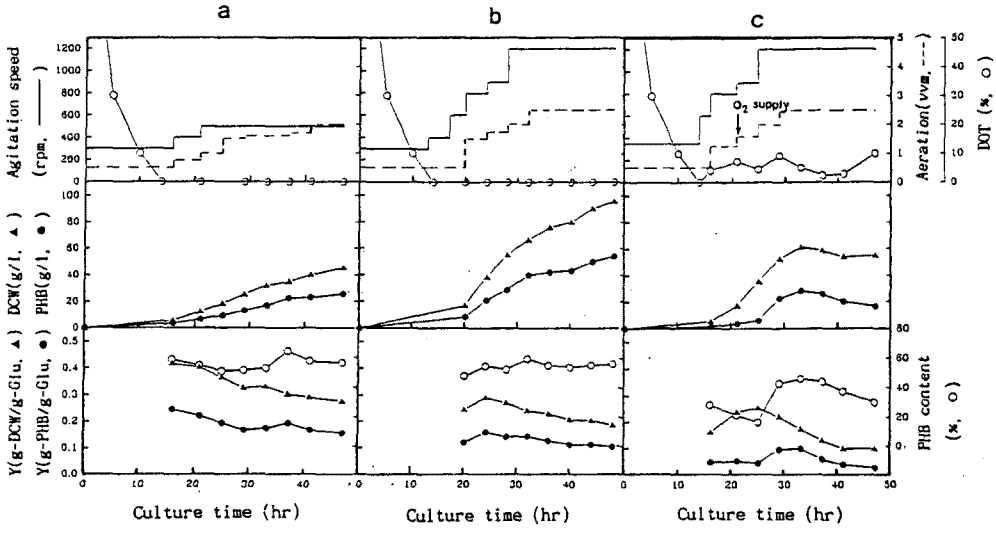

Fig. 2 Time courses of cell growth, PHB accumulation, cell yield, PHB yield, and PHB content at three different air-supply conditions.
Changes of agitation speed and aeration rate at the cases are drawn in the top of the figure. In case of (c), pure oxygen was supplied together with the air to maintain the dissolved oxygen tension around 5% saturation after 21 hr cultivation.

found that the aeration rate could significantly influence both cell growth and PHB accumulation. Fed-batch fermentation, which is considered important for industrial application, was therefore designed to investigate aeration effect in more detail. Fig. 2 shows time courses of the yields of cell mass and PHB, and the cellular PHB content obtained at three different air-supply conditions.

Aeration rates were increased stepwisely from 0.5 to 2.5 vvm at three cases. In case of (b) and (c), a relatively higher agitation speed was applied to give higher oxygen transfer rate to culture media. Moreover, in case of (c), pure oxygen was supplied together with the air to maintain the dissolved oxygen tension (DOT) around 5% saturation. Cell growth and PHB accumulation were enhanced at a higher level of agitation speed in the oxygen-limiting conditions (Fig. 2a and b). But an additional supply of pure oxygen, preventing oxygen limitation, reduced both the cell growth and PHB accumulation (Fig. 2c). It is worthwhile to note that there exists an optimal supply condition of oxygen for production of PHB in this culture system. A high level of PHB accumulation (52 g/l) with PHB content of 60% could be obtained when cells were cultivated at a higher air supply (Fig. 2b). However, keeping the oxygen level under limited condition was found to be critical to achieve this high productivity. It was also observed that cell yield and PHB yield from glucose decreased as oxygen supply increased, indicating that glucose was metabolized inefficiently at a higher oxygen level.

Cell growth and PHB accumulation in the strain of *Azotobacter chroococcum* were further investigated under dual nutrient-limiting condition, nitrogen and oxygen. Under the same fermentation conditions employed for PHB accumulation described in Fig. 2b, fed-batch culture

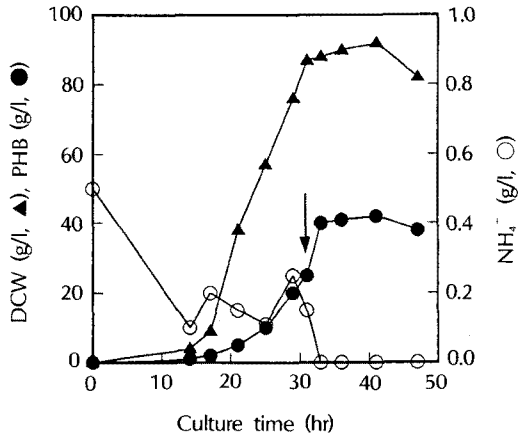

Fig. 3 Effects of nitrogen-fixing condition on cell growth and PHB accumulation. Arrow indicates the timing of switching mode to nitrogen fixation from ammonium supply.

was carried out except that ammonium hydroxide was replaced with NaOH to yield nitrogen limitation. As ammonium was exhausted in the medium, no further increase of both cell growth and PHB accumulation was observed (Fig. 3).

DISCUSSION

Ward *et al.* (20) reported that a strain of *Azotobacter beijerinkii* could accumulate PHB to at least 70% of its dry weight under nitrogen-fixing condition or when supplied with ammonium as the nitrogen source. This work also showed that cellular PHB content in *A.chroococcum* was more than 60% in either condition. However, it was also noted that the cell growth with the supply of nitrogen source was much higher than that under nitrogen-limiting conditions (Table 1). In addition, it was found that switching the culture mode to nitrogen-limiting from nitrogen-rich condition in both flask culture and jar fermentation could not result in further increases of cell growth and PHB accumulation (Table 1 and Fig. 3). As Senior *et al.* (14) suggested, this indicates that cellular reducing power, such as NAD(P)H, is mainly utilized under nitrogen limiting conditions for nitrogen fixation rather than PHB synthesis. Thus, limiting a nitrogen source would not give any advantage for the PHB synthesis in the *Azotobacter* strain.

It was also observed that oxygen is a major growth-limiting factor, which in turn promotes PHB accumulation (Fig. 1). Thus oxygen supply is an important operating parameter for both cell growth and PHB accumulation in this strain. It is worthwhile to note that both cell growth and PHB accumulation can be significantly improved if oxygen is supplied in a carefully controlled manner, that is, to maintain an optimal oxygen level. Higher oxygen supply (oxygen-sufficient condition) resulted in reduced cell growth and lower PHB accumulation. That is probably due to the respiratory protection, that is, lowering growth-inhibitory effects of oxygen on the *Azotobacter* strain by increasing its oxidative activity (4). It was also interesting to note that, even in the non-limiting condition of oxygen, PHB could be accumulated to some level along with a change of cellular PHB content. In this case, it is likely that the initial decrease in PHB content resulted from the unfavorable utilization of NAD(P)H for the respiratory protection mechanism to endure high dissolved oxygen tension. Subsequent increase of PHB content can then be attributed to the enhanced generation of NADPH through increased enzyme activities in the tricaboxylic acid cycle. PHB biosynthesis can therefore be further facilitated by higher availability of such cofactors.

The results obtained from the present study are comparable to that by Page and Knosp (11). They reported a hyperproducton of PHB (2.26 g/l) in a flask culture by *Azotobacter vinelandii* UWD strain defective in the respiratory oxidation of NADH with cellular content of 65 %. In our flask culture, by using a strain of *A.chroococcum*, significant accumulation of PHB (15.5 g/l) with a cellular content of 78% was obtained after 60 hr cultivation. A higher accumulation of PHB (52 g/l) could be achieved within 48 hr with the strain by fed-batch cultivation under controlled oxygen supply. This is the highest titer of PHB ever reported with the *Azotobacter* sp. It is believed that a further development of the fermentation strategy will allow one to use this strain for the industrial production of PHB.

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