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Optimization of Fed-Batch Fermentation for Production of Poly-β-Hydroxybutyrate in *Alcaligenes eutrophus*

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Production of poly-β-hydroxybutyrate (PHB) in fed-batch fermentation was studied. Utilization of carbon for PHB biosynthesis was investigated by using feeding solutions with different ratios of carbon to nitrogen (C/N). It was observed that at a high C/N ratio carbon source was more preferably utilized for PHB accumulation while its consumption for cellular metabolism appeared to be more favored at a low C/N value. A high cell concentration (184 g/l) was achieved when ammonium hydroxide solution was fed to control the pH, which was also utilized as the sole nitrogen source. For the mass production of PHB, two-stage fed-batch operations were carried out where PHB accumulation was observed to be stimulated by switching the ammonium feeding mode to the nitrogen limiting condition. A large amount of PHB (108 g/l) was obtained with cellular content of 80% within 50 hrs of operation.

Poly-β-hydroxybutyrate (PHB) is synthesized by many bacteria as an energy reserve material (9). Recently, PHB is getting a great deal of attention as a biodegradable thermoplastic polyester with several properties similar to conventional synthetic plastics (3). It is well known that, in most bacteria synthesizing PHB, the accumulation of the polymer is stimulated by limiting noncarboneous nutrients such as nitrogen, phosphorous, magnesium, sulphur, or potassium (7, 8). Of these nitrogen limitation renders the easiest measure for the PHB accumulation due to its convenience for bioprocess control.

In our previous study (5), the effects of molar ratio of carbon to nitrogen (C/N ratio) on cell growth and PHB synthesis were investigated in batch cultivation. It was observed that at low C/N ratios, cell growth was favored while PHB accumulation increased with high C/N values. It was also noted that the PHB yield was greatly affected by initial C/N ratios of the culture medium. This observation indicated that C/N ratios could directly influence PHB yields as well as carbon conversion efficiency of the cells. However, with the batch experiments, the effect of C/N ratios was not quite apparent since nitrogen or carbon sources were depleted as the fermentation proceeded.

In this study, therefore, a series of fed-batch fermentations were conducted with continuous feeding of nutrients at different C/N ratios. The carbon conversion efficiencies on cell growth and PHB accumulation were examined and PHB production with a high yield was attempted by optimizing the strategy of the fed-batch operation.

MATERIALS AND METHODS

Bacterial Strain and Cuture Conditions

Alcaligenes eutrophus NCIMB 11599, one of the glucose utilizing mutants derived from *A. eutrophus* H-16, was used throughout the experiments.

The fermentation medium contains (per liter): 20 g glucose, 3.0 g $(NH_4)_2SO_4$, 3.32 g $Na_2HPO_4 \cdot 12H_2O$, 0.83 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 10 mg $CaCl_2$, and 1 ml of a trace element solution (0.3 g H_3BO_3, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnSO_4 \cdot 7H_2O$, 30 mg $MnCl_2 \cdot 4H_2O$, 30 mg $NaMoO_4 \cdot 2H_2O$, 20 mg $NiCl_2 \cdot 6H_2O$, 10 mg $CuSO_4 \cdot 5H_2O$ per liter of 0.1 N HCl). The feed solution for the fed-batch operation was prepared by dissolving 500 g glucose, 16.6 g $Na_2HPO_4 \cdot 12H_2O$, 4.15 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 100 mg $FeSO_4 \cdot 7H_2O$, 50 mg $CaCl_2$, 2.0 g Na-citrate, 5 ml of trace element

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solution, and a predetermined amount of $(NH_4)_2SO_4$ in 1 liter of 0.05 N HCl. The carbon/nitrogen (C/N) ratio was calculated on the molar basis of ammonium and glucose by varing ammonium sulfate concentrations at a fixed glucose concentration, 500 g/*l*. Ammonium hydroxide solution (28%) was used for pH control in the two-stage fed-batch operation. The feed solution described above was fed to the first stage without any nitrogen source, and only the glucose solution to the second stage. Therefore, the only nitrogen source available in the two-stage fed-batch operation was ammonium from NH_4OH added for pH control.

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 m*l* seed culture, cultivated at 30°C for 24 hr in shake flasks, was transferred to a fermentor containing 2.8 liter of the fermentation medium. The pH was controlled at 7.0. The air flow rate and agitation speed were controlled in the range of 0.5~2.0 vvm and 300~1000 rpm, respectively.

Analytical Methods

Cell growth was monitored by measuring 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 a glucose analyzer (YSI,Ohio,USA). Phosphate was measured according to the procedure of Bartlett (1) and ammonia by the indophenol method (10). PHB concentration was determined by gas chromatography (Hewlett Packard, Avondale, USA) with benzoic acid as an internal standard (2). For the determination of enzyme activity the cells were prepared as described in the previous report (5). The protein was determined by the Lowry method (6) and the enzyme activity of β-ketothiolase (KT) by using the thiolysis reaction (4).

RESULTS AND DISCUSSION

Effects of C/N Ratio on PHB Accumulation and Cell Growth

The cell growth and the PHB accumulation in the fed-batch fermentation of *A. eutrophus* NCIMB 11599 were monitored at two different values of C/N ratios (Fig. 1). The accumulation of PHB was greater (32.0 g/*l*) at a higher C/N ratio, while the cell growth showed a similar pattern irrespective of C/N ratios. The residual cell mass (RCM) at a low C/N ratio was thus higher than that obtained from a higher C/N value. Similar trends were observed at different C/N ratios tested from the previous report (5). This indicates that carbon utilization for cell mass build-up is more faciliated by the presence of available nitrogen (low C/N value) in the culture medium. The yields of PHB and RCM at two different C/N ratios were compared (Fig. 2). At the C/N ratio of 5.0, PHB yield was remarkably increased up to 0.40 after the limitation of nitrogen, while it remained below 0.1 at the lower C/N value. RCM yields from the carbon source showed an opposite trend. Since RCM yields from nitrogen in both cases were almost the same with each other within the range of 5.5~6.0 (g-RCM/g-N), a higher supply of nitrogen (lower C/N value) would result in an enhanced formation of cell material not PHB.

The activity of ketothiolase(KT) as a key enzyme in the PHB synthesis was determined at two different values of C/N ratios. The total activity of KT at C/N ratio

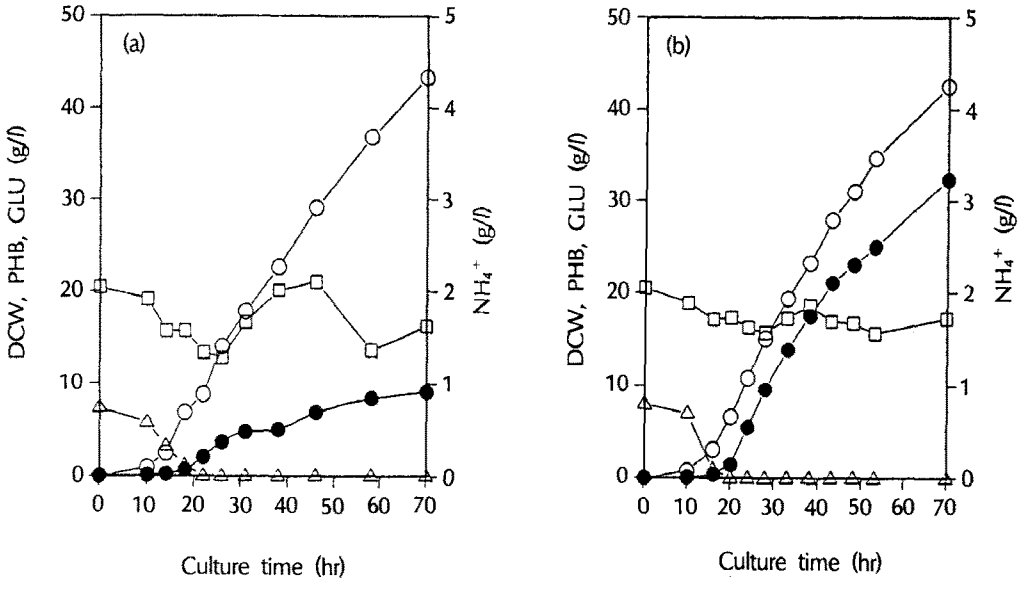

Fig. 1. Effect of molar ratios of carbon to nitrogen on substrate consumption, cell growth, and PHB accumulation. C/N ratios are: (a) 2.0 and (b) 5.0, Symbols: (△) NH_4^+, (●) PHB, (○) DCW, (□) glucose.

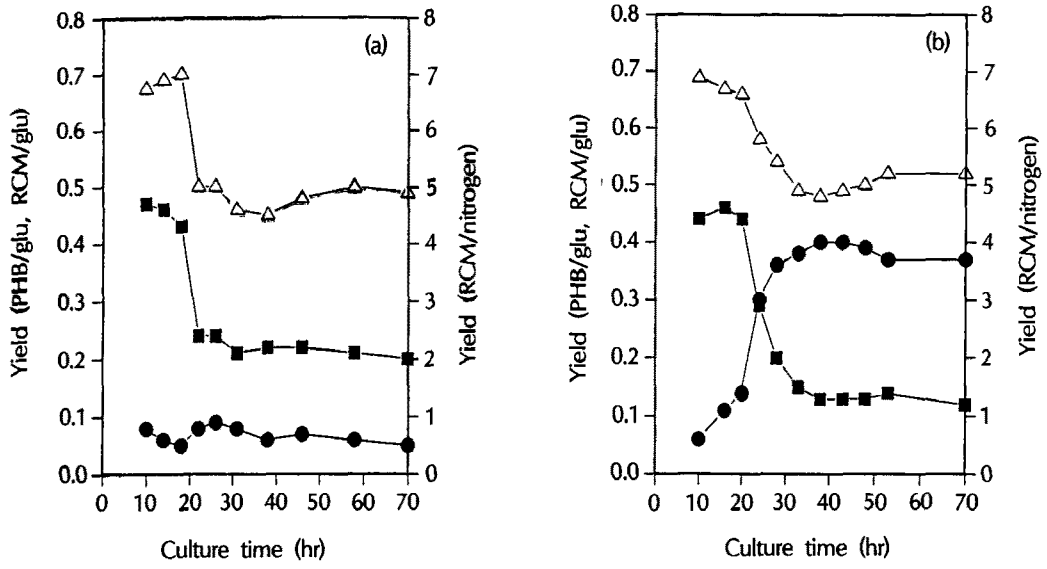

Fig. 2. Effect of molar ratios of carbon to nitrogen on PHB yields from glucose and RCM yields from glucose and nitrogen. C/N ratios are: (a) 2.0 and (b) 5.0, Symbols: (●) PHB yield from glucose, (■) RCM yield from glucose, and (△) RCM yield from nitrogen.

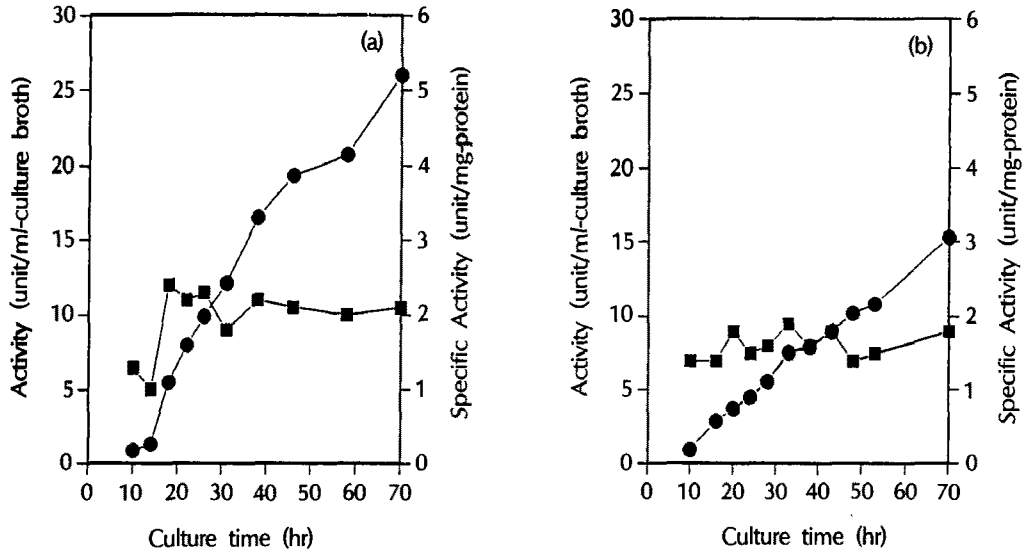

Fig. 3. Effect of molar ratios of carbon to nitrogen on activity of β-ketothiolase. C/N ratios are: (a) 2.0 and (b) 5.0, Symbols: (●) activity and (■) specific activity.

of 2.0 was higher than that at 5.0, while the specific activities were at the same level during the fermentation in both cases of the C/N ratio (Fig. 3). Smaller accumulation of PHB at a low C/N ratio despite the higher activity of KT may indicate that the biosynthesis of PHB is also controlled by other factors, like the presence of cofactors, e.g., CoA or NAD(P)H (9).

Two-Stage Fed-Batch Fermentation

It was noted that the C/N ratio of the feed solution can greatly affect the PHB accumulation and a sufficient supply of nitrogen is required to achieve a high cell concentration. Therefore, a high cell density cultivation was tried by feeding an appropriate nitrogen source to the culture broth. Since the pH of the culture broth decreases, it was necessary to add an alkaline solution during the fermentation. Ammonium hydroxide was found to be the most appropriate for both pH control and a source of nitrogen (data not shown). Under the nitrogen-sufficient condition a large amount of cell mass (184 g/l) but low PHB content (25 wt%) were obtained (Fig. 4). However, when a two-stage fed-batch fermentation was employed, a high cell density in the first stage and high PHB accumulation in the second stage could be obtained. Here, ammonium hydroxide was supplied into the first stage to the amount which was sufficient only for pH control, and in the following stage it was replaced with NaOH to limit nitrogen. Fig. 5 showed the time courses of cell growth, PHB accumulation, and ammonium concentration in the two-stage fed-batch operation. It was observed that PHB accumulation was

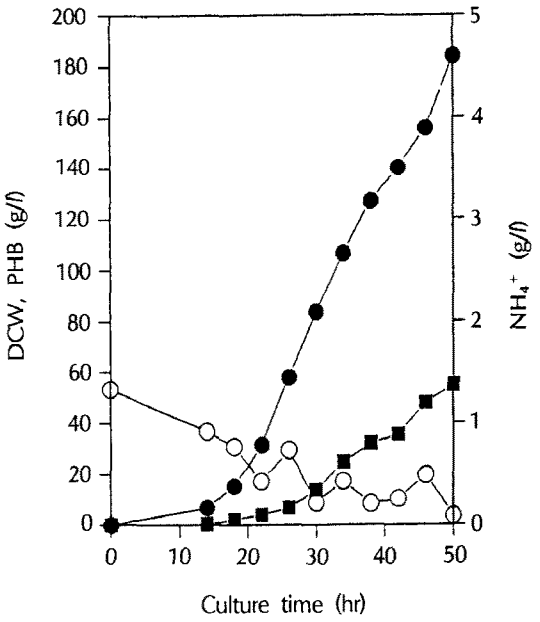

Fig. 4. Time courses of cell growth, PHB accumulation, and ammonium concentration under nitrogen non-limiting condition.
Symbols : (○) NH_4^+, (■) PHB, and (●) DCW.

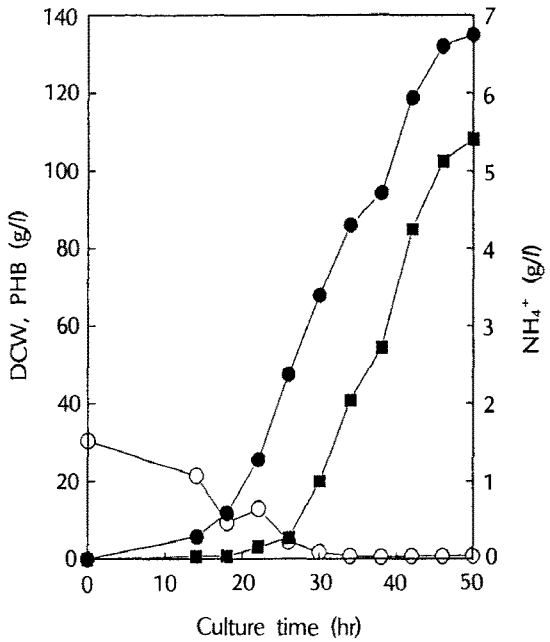

Fig. 5. Time courses of cell growth, PHB accumulation, and ammonium concentration in a two-stage fed-batch cultivation.
Symbols: (○) NH_4^+, (■) PHB, and (●) DCW.

significantly stimulated by the limitation of nitrogen, yielding a high concentration of PHB (108 g/l) after 50 hrs of operation.

Optimal timing of nitrogen limitation for PHB accumulation in the two-stage fed-batch cutivation was investigated by limiting the nitrogen source at various cell concentrations (Table 1). It was observed that the productivity of PHB was the highest when nitrogen was limited at the 68 g/l of cell concentration. In addition, yields of PHB were within the range of 24~29 wt% (g-PHB/g-glu) in case of nitrogen limitation, which corresponds to 50~60% of the theoretical maximum conversion yield from glucose.

In conclusion, fed-batch fermentation for production of PHB could be successfully carried out by using a feeding solution with high C/N ratio, and it was found that high PHB productivty was achieved by a two-stage fed-batch operation with an appropriate nitrogen feeding technique; in this study, nitrogen feeding determined by pH control was found to be the most successful.

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Table 1. Effect of nitrogen-limiting timing on PHB accumulation[a]

DCW before limitation (g/l)	Final concentration DCW (g/l)	PHB (g/l)	PHB content (wt%)	PHB yield (wt%)	Productivity (g/l/hr)	$Qp_{,max}$ (g/g/hr)
Non-limitation	184.2	54.6	25.0	8.0	1.09	0.04
34.5	100.4	76.0	75.7	29.0	1.52	0.10
56.0	111.2	86.8	78.1	24.0	1.74	0.12
68.0	135.0	108.0	80.0	27.0	2.16	0.16
92.8	143.2	94.5	66.0	25.0	1.89	0.12

[a]Timing of nitrogen limitation was described as the various cell concentrations obtained at different culture times in the first stage of cultivation.

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