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PHB Accumulation Stimulated by Ammonium Ions in Potassium-limited Cultures of *Methylobacterium organophilum*

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Abstract *Methylobacterium organophilum* can use nitrogen in the form of ammonium ions ($(NH_4)_2SO_4$ and NH_4Cl) and from nonammonium sources such as glycine, alanine, peptone, and yeast extract. When potassium was limited, significantly more PHB was produced when the ammonium ion was the nitrogen source rather than a nonammonium form. With ammonium, the amount of PHB produced was 0.50~0.53 g PHB/l or 52.0~53.2% of the dry cell weight. If nitrogen was from a nonammonium source, the respective values were 0.04~0.06 g PHB/l or 8.1~11.3% of dry cell weight. When ammonium sulfate was the sole source of nitrogen under potassium-limited conditions, cell growth and PHB accumulation increased as the pH increased from 6.0 to 7.5. Cell growth and PHB amount at pH 7.5 were 2.50 g dry cell weight/l and 1.40 g PHB/l, respectively.

Key words: *Methylobacterium organophilum*, PHB, futile cycling, ammonium ions, potassium limitation

Poly-β-hydroxybutyrate (PHB) is a polymeric ester that acts as an energy and carbon reserve in prokaryotic cells [1, 12, 16]. Recently, there has been increasing interest in industrial applications for PHB since its physical and chemical properties are similar to those of polypropylene and polyethylene, and it is a safe and biodegradable thermoplastic [7]. This microbial polyester (PHB) is usually formed as intracellular inclusions during unbalanced growth when there is an excess of either carbon or energy on the one hand, and a limiting nutrient supply or growth factor on the other [2, 11]. Therefore, it is important to identify the limiting nutrients that promote PHB production in any given microorganism. Nitrogen limitation has generally been used to promote PHB production. There are, however, exceptions. Nutrient limitation is not required for PHB production in either *Alcaligenes latus*, a mutant *Azotobacter vinellandii* UWD, or a recombinant *E. coli*, [3, 8, 14]. In *Azotobacter* sp., PHB production is favored when oxygen is limited [10, 15].

We observed that PHB production in *Methylobacterium organophilum*, a facultative methylotroph, is maximized under potassium limitation [9]. Potassium is an important nutrient that is found inside cells in an unmodified and largely unbound state [17]. A high affinity potassium uptake system (the active transporter) is induced under potassium-limited conditions, while a low affinity potassium uptake system (the facilitated diffusion transporter) acts constitutively (Fig. 1) [13]. The high

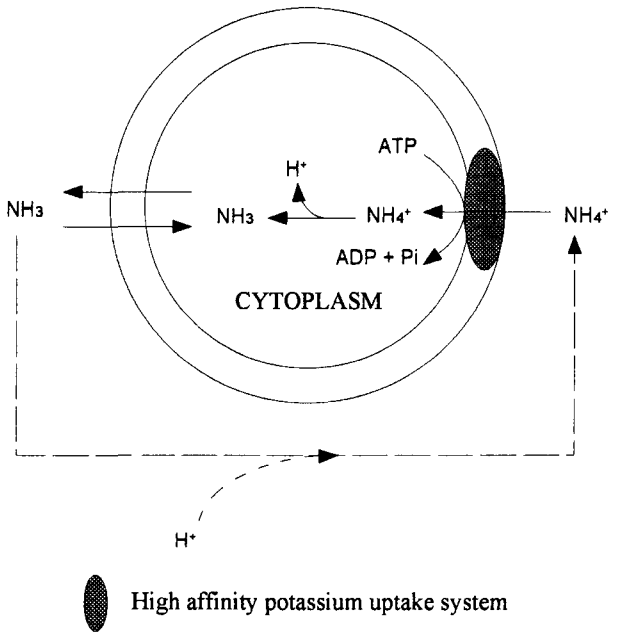

Fig. 1. Schematic representation of futile cycling of ammonium ions under potassium-limited condition.

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affinity uptake system cannot discriminate between potassium and ammonium ions because they are similar to each other in both size and charge. When microorganisms are in potassium-limited environments, ammonium ion is imported into the cytoplasm by the high affinity uptake system at the cost of ATP. However, ammonium ion is subsequently converted to ammonia and is released from cells by diffusion. The continual cycling of ammonium ions under potassium-limited conditions is futile and wastes energy in the cell [6].

In a previous report, we suggested that high levels of PHB accumulate in potassium-limited cells in order to maintain an overall balance between the energy requirements under potassium limitation and the surplus energy produced when PHB is synthesized from methanol, a high energy substrate (S. W. Kim *et al.* 1995. Abstr. 8^{th} *Int. Symp. on Microb. Growth on* C_1*-compds.*, San Diego, U.S.A., p. 82). Herein, we report the effect of ammonium ion under potassium limitation with a viewpoint of energy balance on PHB synthesis.

MATERIALS AND METHODS

Microorganism and Growth Medium

Methylobacterium organophilum NCIB 11278 is a pink-pigmented, facultative methylotroph. It was grown in minimal growth medium supplemented with methanol, 0.5% (w/v). Each liter of the medium contained: $(NH_4)_2SO_4$, 1.2 g; KH_2PO_4, 1.305 g; $Na_2HPO_4 \cdot 12H_2O$, 2.13 g; $MgSO_4 \cdot 7H_2O$, 0.45 g; $CaCl_2 \cdot 2H_2O$, 3.3 mg; $FeSO_4 \cdot 7H_2O$, 1.3 mg; $MnSO_4 \cdot 4H_2O$, 130 μg; $ZnSO_4 \cdot 5H_2O$, 40 μg; $CuSO_4 \cdot 5H_2O$, 40 μg; $Na_2MoO_4 \cdot 2H_2O$, 40 μg; $CoCl_2 \cdot 6H_2O$, 40 μg; and H_3BO_3, 30 μg.

Culture Conditions

The effect of the nitrogen source on PHB accumulation was examined using a two-stage culture technique. First stage cultures were grown in 250-ml Erlenmeyer flasks with 50 ml of growth medium containing 0.5% methanol (w/v). In the second stage, cells harvested from the first stage were washed with sterile saline and aseptically transferred to a potassium-deficient growth medium containing $(NH_4)_2SO_4$, NH_4Cl, glycine, alanine, peptone, or yeast extract as the sole source of nitrogen. Methanol feeding of the second stage culture was divided to avoid cell inhibition. The initial concentration of methanol was 0.5% (w/v). After 28 h of cultivation additional methanol at a concentration of 0.5% (w/v) was added. The second stage culture was maintained for 48 h in a rotary shaking incubator (Inova 4330, NBS Co., U.S.A.) at 30°C and 250 rpm. The pH of the culture was monitored with a portable pH meter (pH Boy-C1, FESTA, Japan) and maintained at 7.0 ± 0.5 by addition of 3 N NaOH.

Cultures used to investigate the effect of pH on PHB accumulation were also conducted using the two-stage culture technique described above. The pH of these cultures was maintained at different levels between 6.0 and 8.0 by addition of either 3 N HCl or 3 N NaOH. The initial methanol concentration of the culture medium was 0.5% (w/v). Methanol, 0.5 % (w/v), was added once to cultures at pH 6.0 and 6.5, and twice to cultures at pH 7.0, 7.5, and 8.0 to prevent its exhaustion with cell growth.

Analytical Methods

The maximum specific growth rate of cells was measured at 600 nm with a Bio-SCR (Lab System, Sweden), an automated O.D. measuring machine. The dry cell weight was determined gravimetrically after the culture broth had been centrifuged, washed with distilled water, and dried at 105°C. The residual cell mass was calculated by subtracting the weight of PHB from the dry cell weight. PHB samples were prepared according to a modified method of Braunegg *et al.* [4]. The amount of ethyl esters produced from PHB monomers was quantified using a gas chromatograph (model GC-8A, Shimazu Co., Japan) with a capillary column (CBP1, Shimazu Co., Japan), and a chromatopac integrator (model C-R6A, Shimazu Co., Japan) in isothermal mode (injector/detector temp., 280°C, column temp., 110°C). The internal and external standards used for this analysis were benzoic acid and PHB, respectively, and were obtained from Aldrich Co.

RESULTS AND DISCUSSION

Effect of Nitrogen Sources on PHB Accumulation

We measured the maximum specific growth rate of *M. organophilum* in growth media supplemented with various forms of nitrogen to determine which form was most easily assimilated (Fig. 2). *M. organophilum* was able to use all of the nitrogen sources tested and there were no significant differences between the maximum specific growth rates with each source. The maximum specific growth rate was highest with ammonium sulfate, 0.250 h^{-1}, and lowest with alanine, 0.183 h^{-1}.

PHB accumulation under potassium limitation was investigated using nitrogen in either an ammonium form (ammonium sulfate and ammonium chloride) or non-ammonium form (glycine, peptone, and yeast extract) (Table 1). When the nitrogen source was ammonium, the amount of PHB produced and the PHB content were 0.50~0.53 g PHB/l and 52.0~53.2% of dry cell weight, respectively. These values were significantly higher than values for a nonammonium form of nitrogen, which were 0.04~0.06 g PHB/l and 8.1~11.3% of dry cell

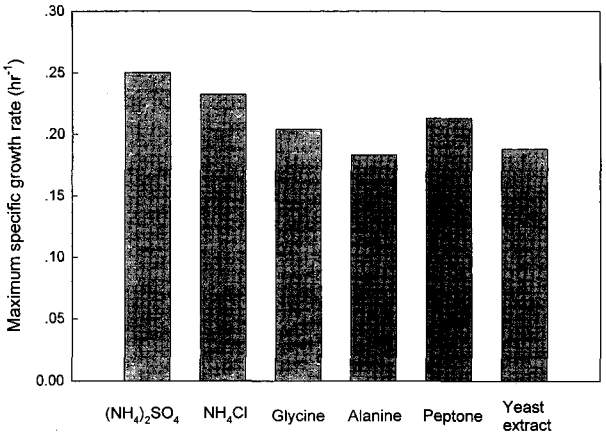

Fig. 2. Effect of nitrogen sources on maximum specific growth rate in normal growth medium.

Table 1. The effect of nitrogen source on cell growth and PHB production.

Nitrogen sources	Dry cell weight (g/l)	PHB (g/l)	Residual cell mass (g/l)	PHB content (%)
$(NH_4)_2SO_4$	1.02	0.53	0.49	52.0
NH_4Cl	0.94	0.50	0.44	53.2
Glycine	0.53	0.06	0.47	11.3
Peptone	0.48	0.04	0.44	8.3
Yeast extract	0.62	0.05	0.57	8.1

a) Initial amount of resuspended cells in the second stage was 0.26 g/l.
b) Residual cell mass is the weight of PHB deducted from the dry cell weight.
c) Equivalents of nitrogen, 0.254 g/l, were added.
d) All experiments were duplicated and relative standard deviations were ±2~4 %.

weight, respectively. Potassium limitation limited increases in the residual biomass to between 0.44 and 0.57 g/l. The nitrogen sources had no significant effect on the increase in residual biomass.

Buurman *et al.* [6] reported that the futile cycling of ammonium ions under potassium limitation significantly increased the energy requirements of *E. coli*. It is described in the Introduction that high levels of PHB accumulate in potassium-limited cells to maintain an overall balance between the energy requirements under potassium limitation and the surplus energy produced when PHB is synthesized from methanol. Considering these facts, a high PHB accumulation rate in the presence of ammonium nitrogen sources can be ascribed to the futile cycling of ammonium ions induced by potassium limitation. Therefore, PHB productions in potassium-limited conditions are strongly affected by the form of nitrogen available.

Effect of Cultivation pH on PHB Accumulation

The concentration of ammonium ions in the culture medium is affected by the pH of the culture because a

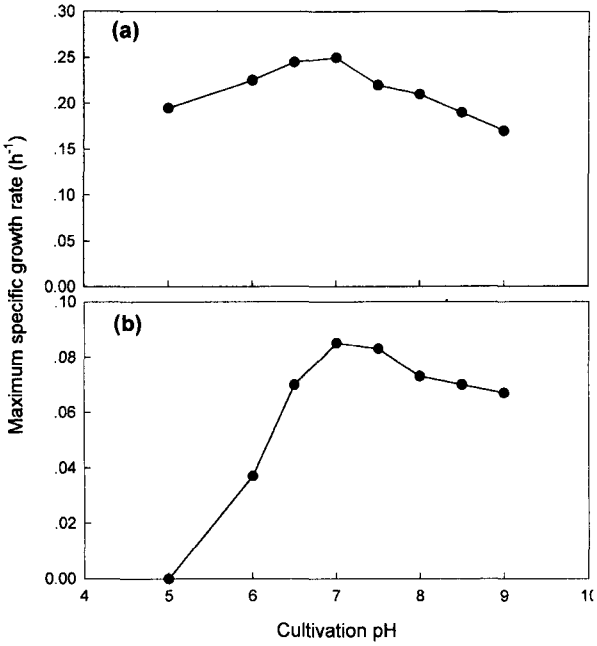

Fig. 3. Effect of cultivation pH on maximum specific growth rate in normal growth medium (a) and potassium-deficient medium (b).

pH change can shift the equilibrium between ammonium ions and ammonia. It has been reported that energy consumption due to the futile cycling of ammonium ions is proportional to the ammonium concentration [5]. Therefore, we investigated the effect of culture pH on PHB accumulation. The maximum specific growth rate was measured in both a normal growth medium and a potassium-deficient medium at a pH range from 5.0 to 9.0 (Fig. 3). In the normal growth medium, pH had no significant effect on the maximum specific growth rate over the range of pH tested, although the lowest growth rates were under alkaline conditions. The highest maximum specific growth rate of 0.250 h^{-1} was measured at pH 7.0, while the lowest rate of 0.175 h^{-1} occurred at pH 9.0. In the potassium-deficient medium, however, there was no growth at pH 5.0 and the maximum specific growth rates were relatively higher under alkaline pH conditions. This result is different from the observations in the normal growth medium. Decreased growth under acidic conditions was attributed to a shift in equilibrium between ammonium ions and ammonia. Under acidic conditions, the equilibrium favors ammonium ions and the resulting increased need to dissipate energy increases the futile cycling of ammonium ions. Buurman *et al.* [5] also reported that cell growth in *Bacillus stearothermophilus* and *Klebsiella pneumoniae* cultures was strongly dependent on pH when ammonium ions were used as the nitrogen source under potassium-limited conditions.

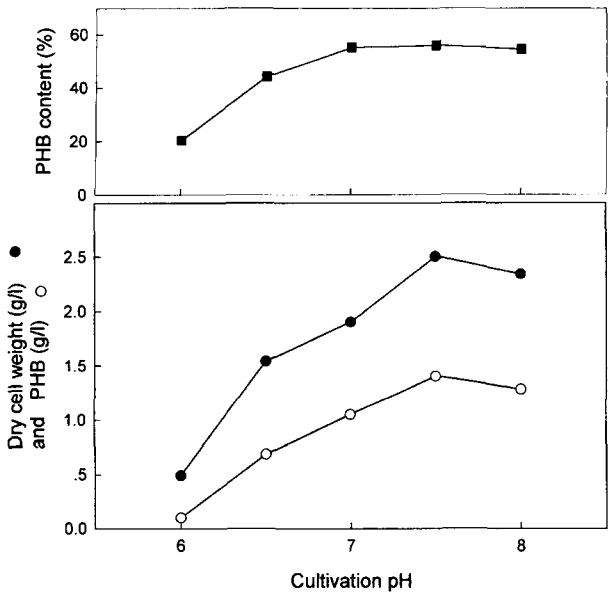

Fig. 4. Effect of cultivation pH on PHB accumulation and cell growth. Initial amount of resuspended cells in the second stage was 0.46 g/l.

The effect of the futile cycling rate on PHB accumulation was observed in the pH range from 6 to 8 (Fig. 4). Cell growth and the PHB amount produced increased significantly as the culture pH increased from 6.0 to 7.5. At pH 7.5 the respective values were 2.50 g dry cell weight/l and 1.40 g PHB/l, while at pH 6.0 these values were 0.49 g dry cell weight/l and 0.10 g PHB/l. There was a slight decrease in the amount of cell growth and PHB produced at pH 8.0, leading us to suspect that *M. organophilum* favors a neutral pH, as shown in Fig. 3a. PHB content was between 44.2 and 56.0% of dry cell weight, except at pH 6.0 when the value was 20.4% of dry cell weight.

The futile cycling of ammonium ions inhibited both cell growth and PHB accumulation under acidic conditions, although it stimulated PHB synthesis under weakly alkaline conditions. Based on these results, ammonium ions should be used as the nitrogen source and cultures should be kept at pH 7.5 to maximize PHB production under potassium-limited conditions.

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