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Maximization of Poly-β-Hydroxybutyrate Accumulation by Potassium Limitation in *Methylobacterium organophilum* and Its Related Metabolic Analysis

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Abstract When methanol was the sole carbon source, *Methylobacterium organophilum* NCIB 11278, a facultative methylotroph, accumulated poly-β-hydroxybutyrate (PHB) as 59% (w/w) of dry cell weight under potassium limitation, 37% under sulfate limitation, and 33% under nitrogen limitation. Based on a stoichiometric analysis of PHB synthesis from methanol, it was suspected that PHB synthesis is accompanied by the overproduction of energy, either 6–10 ATP and 1 $FADH_2$, or 6 ATP and 3 NADPH to balance the NADH requirement, per PHB monomer. This was confirmed by observation of increased intracellular ATP levels during PHB accumulation. The intracellular ATP with limited potassium, sulfate, and ammonium increased to 0.185, 0.452, and 0.390 μmoles ATP/g Xr (residual cell mass) during PHB accumulation, respectively. The intracellular ATP level under potassium limitation was similar to that when there was no nutrient limitation and no PHB accumulation, 0.152–0.186 μmoles ATP/g Xr. We propose that the maximum PHB accumulation observed when potassium was limited is a result of the energy balance during PHB accumulation. Microorganisms have high energy requirements under potassium limitation. Enhanced PHB accumulation, in ammonium and sulfate limited conditions with the addition of 2,4-dinitrophenol, which dissipates surplus energy, proves this assumption. With the addition of 1 mM of 2,4-dinitrophenol, the PHB content increased from 32.4% to 58.5% of dry cell weight when nitrogen limited and from 15.1% to 31.0% of dry cell weight when sulfate limited.

Key words: Poly-β-hydroxybutyrate, *Methylobacterium organophilum*, potassium limitation, surplus energy, intracellular ATP level

Poly-β-hydroxybutyrate (PHB) is a polymeric ester that acts as an energy and carbon reserve in prokaryotic cells [1, 27]. Recently, there has been an increasing interest in a variety of 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 [14]. This microbial polyester is usually formed as intracellular inclusions during unbalanced growth, in the presence of an excess of carbon or energy on the one hand and a limiting nutrient or growth factor on the other [4]. Therefore, it is important to identify the limiting nutrients that promote PHB production in any given microorganism. Nitrogen limitation has generally been applied to PHB production. There are, however, exceptions. In *Alcaligenes latus*, a mutant *Azotobacter vinellandii* UWD and a recombinant *E. coli*, nutrient limitation is not required for PHB production [5, 15, 22]. In *Azotobacter* sp., PHB production is favored when oxygen is limited [24]. We observed that PHB production in *M. organophilum*, a facultative methylotroph, is maximized under potassium limitation [16]. Potassium is an essential nutrient for microbial growth and the cytoplasmic potassium concentration is surprisingly high in gram-negative bacteria, about 50–100 mM [12]. A large amount of energy is required to maintain high intracytoplasmic potassium concentrations in a potassium-limited environment [7, 28, 30]. Maximized PHB production with potassium limitation is unusual and is unlikely to occur in most microorganisms because there is insufficient energy for PHB synthesis. PHB production with potassium limitation is also observed in *Bacillus thuringiensis* and *Alcaligenes eutrophus*, but the amount produced is much less than that produced in these species when nitrogen is limited [26, 34]. In this paper, we investigate the maximum PHB production by *M. organophilum* when potassium is limited, focusing on the energy balance in cells during PHB synthesis.

Materials and Methods

Microorganism and Growth Medium

The microorganism studied, *M. organophilum* NCIB 11278, is a pink-pigmented, facultative methylotroph.

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Minimal growth medium was supplemented with methanol, 1% (w/v). Each liter of the medium contained (per liter): $(NH_4)_2SO_4$, 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; H_3BO_3, 30 μg.

Culture Conditions

The effect of nutrient limitation on PHB accumulation was examined by using a two-stage culture technique. The first culture was performed in 250-ml Erlenmeyer flasks with 50 ml of growth medium containing 1% (w/v) methanol. In the second stage, cells harvested from the first stage were washed with sterile saline and aseptically transferred to a specific nutrient-limited media in which either NH_4^+, K^+, Mg^{2+}, SO_4^{2-}, or PO_4^{3-} was absent from the growth medium. Methanol feeding in the second stage culture was divided to avoid cell inhibition. The initial methanol concentration was 0.5% (w/v). When K^+, Mg^{2+}, SO_4^{2-}, or PO_4^{3-}, was limited, 0.5% (w/v) methanol, was added to the second stage culture three more times to prevent its exhaustion as cells grew. The second stage culture was maintained for 40 to 60 h, until cell growth stopped completely as the result of nutrient limitation. The flask cultures were kept in a rotary shaking incubator (Inova 4330, NBS Co.) at 30°C and 250 rpm shaking speed. pH was monitored with a portable pH meter (pH Boy-C1, FESTA) and maintained at 7.0±0.5 by the addition of 3 N NaOH. Cultures used to determine intracellular ATP content during PHB accumulation were also set up using the two-stage culture technique described above. The second stage culture was maintained for 13 h. A two-stage culture was also used for the experiment using the energy uncoupler, 2,4-dinitrophenol, but the second stage was maintained for 16 h. 2,4-Dinitrophenol was added to the second stage culture at concentrations of 0.1, 0.5, and 1.0 mM.

Analytical Methods

Cell growth was measured by optical density at 570 nm. Dry cell weight was determined gravimetrically after the culture broth was centrifuged, washed with distilled water, and dried at 105°C. Residual cell mass was calculated by subtracting the weight of PHB from the dry cell weight. PHB samples were prepared according to the modified method of Braunegg *et al.* [6]. The resulting ethyl esters produced from PHB monomers were quantified using a gas chromatograph (model GC-8A, Shimazu Co.) with a capillary column (CBP1, Shimazu Co.) and a chromatopac integrator (model C-R6A, Shimazu Co.) in isothermal mode (injector/detector temp. 280°C, column temp. 110°C). The internal and external standards used in this analysis were benzoic acid and PHB, respectively, and were obtained from Aldrich Co.

Intracellular ATP levels were determined by using firefly luciferase [13, 18]. One volume of bacterial culture was extracted for 90 sec with nine volumes of boiling Tris-EDTA buffer (20 mM Tris, 2 mM EDTA, pH 7.75). This extract was assayed for ATP by using the ATP bioluminescent assay kit (FL-AA, Sigma) and the light produced was measured with a Spectrofluorophotometer RF-5000 (Shimazu Co.). Intracellular polyphosphate was analyzed by the method of Doi *et al.* [11]. The cell suspensions for ^{31}P NMR analysis were placed in 10-mm NMR sample tubes and kept at 4°C. ^{31}P NMR spectra of cell suspensions were recorded at 243-MHz on a BRUKER DMX-600 spectrometer in the Fourier transform mode. The sample tubes were kept at 5°C during NMR measurement. The spectra were obtained with a repetition rate of 0.7 sec, 32,000 datum points, and 10,000 accumulations. ^{31}P NMR chemical shifts were referenced to an external of 85% phosphoric acid.

Stoichiometric Analysis of PHB Synthesis from Methanol

It should be noted that when methylotrophs use methanol, all cellular reactions are initiated by the oxidation of methanol to formaldehyde. This is catalyzed by NAD^+-independent methanol dehydrogenase which provides ATP but no NAD(P)H and so markedly influences the balance of ATP and NADH in methylotrophs [2]. At present, the way in which formaldehyde is oxidized is unclear. There are two possible routes for the oxidation of formaldehyde to formate (Fig. 1); one involves the presence of formaldehyde dehydrogenase [3] and the other 5,10-methylene tetrahydrofolate (THF) dehydrogenase [9]. We stoichiometrically analyzed PHB synthesis in *M. organophilum*.

The stoichiometry of PHB synthesis, in the presence of formaldehyde dehydrogenase, consists of the following

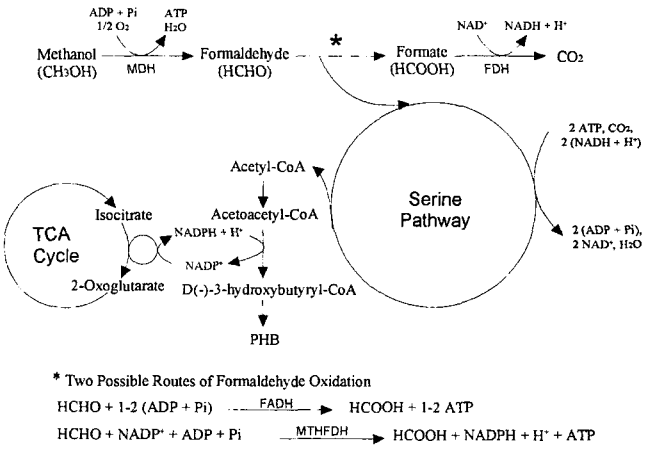

Fig. 1. The pathway of PHB biosynthesis in *M. organophilum* from methanol.
MDH: Methanol dehydrogenase; FADH: Formaldehyde dehydrogenase; MTHFDH: 5,10-methylene THF dehydrogenase; FDH: Formate dehydrogenase.

four steps [33]: (i) formation of acetyl-CoA from methanol via the serine pathway, (ii) influx of acetyl-CoA into the TCA cycle to produce the NADPH necessary for the reduction of acetoacetyl-CoA to D(-)-3-hydroxybutyryl-CoA in PHB biosynthesis, (iii) biosynthesis of PHB from acetyl-CoA, (iv) complete oxidation of methanol to CO_2 to yield the ATP and NADH necessary for the formation of acetyl-CoA. The stoichiometry of these steps in PHB synthesis can be expressed as:

• Formation of acetyl-CoA from methanol via the serine pathway:

$$CH_3OH + 1/2\ O_2 + ATP + 2NADH + 2H^+ + CO_2 \rightarrow \text{Acetyl-CoA} + 2H_2O + 2\ NAD^+ + ADP + Pi \quad (1)$$

• Influx of acetyl-CoA into TCA cycle:

$$\text{Acetyl-CoA} + 2H_2O + NADP^+ + 2NAD^+ + ADP + Pi + FAD \rightarrow NADPH + 2\ NADH + 3\ H^+ + ATP + FADH_2 + 2\ CO_2 \quad (2)$$

• Biosynthesis of PHB from acetyl-CoA ((1/n) PHB refers to a single monomer):

$$2\ \text{Acetyl-CoA} + NADPH + H^+ \rightarrow (1/n)\ PHB + NADP^+ \quad (3)$$

• Oxidation of methanol to CO_2. *M. organophilum* formaldehyde dehydrogenase is a dye-linked type producing 1 or 2 ATP through its coupled electron transport chain [21]

$$CH_3OH + 1/2O_2 + 2\text{-}3\ ADP + 2\text{-}3\ Pi + NAD^+ \rightarrow 2\text{-}3\ ATP + NADH + H^+ + CO_2 + H_2O \quad (4)$$

The net reaction of PHB biosynthesis in *M. organophilum* is expressed by the sum of these four equations: [⑤ =(3× ①) + ② + ③ + (4 × ④)]

$$7\ CH_3OH + 3\ 1/2\ O_2 + 6\text{-}10\ ADP + 6\text{-}10\ Pi + FAD \rightarrow (1/n)\ PHB + 8\ H_2O + 6\text{-}10\ ATP + FADH_2 + 3\ CO_2 \quad (5)$$

Alternatives to these equations result from slightly different assumptions. When the oxidation of formaldehyde is mediated by 5,10-methylene THF dehydrogenase instead of formaldehyde dehydrogenase, then a different stoichiometry must be derived. Since NADPH and ATP are produced by the oxidation of formaldehyde, it may be assumed that there is no need for the metabolism of acetyl-CoA in the TCA cycle in order to provide the NADPH required for the reduction of acetoacetyl-CoA. Thus PHB synthesis would consist of 3 steps (complete oxidation of methanol, formation of acetyl-CoA, and biosynthesis of PHB). The stoichiometry of the complete oxidation of methanol to CO_2 changes in the following manner:

$$CH_3OH + 1/2\ O_2 + 2\ ADP + 2\ Pi + NAD^+ + NADP^+ \rightarrow 2\ ATP + NADH + NADPH + 2\ H^+ + CO_2 \quad (6)$$

When formaldehyde oxidation is mediated by 5,10-methylene THF dehydrogenase, the net reaction of PHB biosynthesis is [⑦ =(2 × ①) + ③ + (4× ⑥)]

$$6\ CH_3OH + 3\ O_2 + 6\ ADP + 6\ Pi + 3\ NADP^+ \rightarrow (1/n)\ PHB + 4\ H_2O + 6\ ATP + 3\ NADPH + 3\ H^+ + 2CO_2 \quad (7)$$

RESULTS

Effect of Limiting Nutrients on Cell Growth and PHB Accumulation

A two-stage flask culture was performed. Cell growth occurred in the first stage with no nutrient limitation, while PHB formation occurred with nutrient limitation in the second stage (Table 1). Dry cell weights obtained when sulfate, phosphate, magnesium, and potassium were limited were 3.17-4.20 g/l. These were comparable to the control value of 3.83 g/l. The increased dry cell weight with nutrient limitation in the second stage culture was attributed to increases in both PHB and residual cell mass, whereas the increased dry cell weight of the control resulted solely from an increase in residual cell mass. Dry cell weights obtained when ammonium and dissolved oxygen were limited were 1.48 g/l and 1.32 g/l, respectively. These were not significantly different from the initial dry cell weight (1.1 g/l) at the start of the second stage. In these cases, there were no increase in residual cell mass and only a small amount of PHB accumulation. This indicates that cellular activity, including PHB synthesis, is low when ammonium and dissolved oxygen are limited. When potassium was limited, 2.45 g/l of PHB were produced. This represented 59% of the dry cell weight, the highest amount for the six limiting nutrients tested. For the other

Table 1. The effect of nutrient limitation on cell growth and PHB production.[a]

Limiting nutrients	Dry cell weight[b] (g/l)	PHB (g/l)	Residual cell mass[c] (g/l)	PHB content (%)
Control[d]	3.83	0.22	3.61	6
NH_4^+	1.48	0.50	0.98	33
SO_4^{-2}	3.71	1.38	2.33	37
PO_4^{-3}	4.20	1.38	2.82	33
Mg^{+2}	4.05	1.30	2.75	32
K^+	4.17	2.45	1.72	59
Dissolved oxygen[e]	1.32	0.27	1.05	20

[a]All experiments were duplicated and relative standard deviations were ±2~4%.
[b]Initial amount of resuspended cells in the second stage was 1.1 g/l.
[c]Residual cell mass is the weight of PHB deducted from the dry cell weight.
[d]Control, no limiting nutrients.
[e]Dissolved oxygen limitation was carried out with 150 ml medium in 250 ml flask at 100 rpm.

nutrients, 0.27- 1.38 g/l of PHB were produced, representing 20- 37% of dry cell weight, which are significantly lower values. These results are very interesting, because maximum PHB accumulation with potassium limitation has not previously been observed in microorganisms.

The stoichiometry of PHB synthesis from methanol was presented in the Materials and Methods section. As shown in equations (5) and (7), a theoretical excess of 6- 10 ATP and $FADH_2$, or 6 ATP and 3 NADPH is produced per PHB monomer. Since maximum PHB accumulation was observed under potassium limitation, when microorganisms have high energy requirements, it is thought that the surplus energy would be an obstacle to PHB accumulation. According to the mass action law, over-accumulation of the end product (surplus energy) in a reaction slows the reaction rate (PHB synthesis rate), ultimately stopping the reaction.

Change of Intracellular ATP Contents during the PHB Accumulation Stage

Since $FADH_2$ and NADPH can be converted to ATP, it is assumed that intracellular ATP accumulates during PHB synthesis. Intracellular ATP levels were analyzed during PHB accumulation under NH_4^+, K^+, and SO_4^{2-}-limited conditions. When sulfate was limited, the intracellular ATP levels increased from 0.174 to 0.452 μmoles ATP/g Xr (residual cell mass) as the PHB content increased from 7.4% to 17.6% of dry cell weight. When ammonium was limited, the intracellular ATP level and PHB content increased to 0.390 μmoles ATP/g Xr and 17.0% of dry cell weight, respectively, after 8 h of growth. After 13 h, the rate of PHB production slowed and the intracellular ATP level fell to 0.332 μmoles ATP/g Xr. When potassium was limited, the intracellular ATP level increased from 0.092 to 0.185 μmoles ATP/g Xr as the PHB content increased from 9.9% to 28.5% of dry cell weight. In control with no nutrient limitation, no significant changes in either intracellular ATP level or PHB content were observed. In the control, the intracellular ATP level and PHB content were 0.152- 0.186 μmoles ATP/g Xr and 5.4% to 7.0% of dry cell weight, respectively. This confirms our hypothesis, based on stoichiometric analysis, that intracellular ATP levels increase as PHB accumulates. Increases in intracellular ATP when ammonium and sulfate were limited were twice that of the control, whereas when potassium was limited the intracellular ATP levels were similar to those of the control. This implies that the excess ATP formed during PHB synthesis is consumed when potassium is limited and, consequently, does not accumulate in cells. As mentioned in the Introduction, microorganisms require large amounts of ATP to maintain a high intracytoplasmic potassium concentration in a potassium-limited environment. It was therefore assumed that the net energy overproduction in PHB synthesis and the high energy demand in a potassium-limited environment cause the high levels of PHB accumulation in cells observed under these conditions. To prove this assumption, the surplus energy produced during PHB accumulation was artificially dissipated by using an energy uncoupler when ammonium and sulfate were limited.

Effect of an Energy Uncoupler, 2,4-Dinitrophenol, on PHB Accumulation

2,4-Dinitrophenol is an energy uncoupler which reduces intracellular ATP formation by uncoupling the link between the proton motive force (PMF)-generating electron transport and the PMF-utilizing proton-driven ATPase [19, 25]. 2,4-Dinitrophenol was added to the second stage culture media at concentrations of 0.1 mM, 0.5 mM, and 1 mM (Table 2). These second stage cultures were maintained for 16 h, so the amount of PHB produced differed from the amounts obtained in the respective nutrient-limited experiments. Since the sulfate culture required a longer time for complete exhaustion of the limited nutrient than potassium or ammonium, PHB yield with sulfate limitation was especially low. When 1 mM of 2,4-dinitrophenol was added, with limited ammonium and sulfate, PHB content increased to 58.5% and 31.0%, respectively, twice the value without 2,4-dinitrophenol. When potassium-limited, a maximum PHB content of 53.6% was obtained with 0.5 mM of 2,4-dinitrophenol. This is not significantly greater than the PHB yield without 2,4-dinitrophenol (44.3% of dry cell weight). The effect of 2,4-dinitrophenol in enhancing PHB accumulation was only significant when ammonium and sulfate were limited. In these cases, there is an energy unbalance; an abnormal increase in intracellular ATP level is observed with PHB accumulation. This means that the surplus energy produced in PHB synthesis is an obstacle to the favorable accumulation of PHB. In the control, PHB contents were from 6.5% to 12.7% of dry cell weight. These were very low, and unchanged by the addition of 2,4-dinitrophenol, because PHB synthesis is not initiated when no nutrients are limited. Thus, we conclude that favorable PHB accumulation with potassium limitation results from the dissipation of surplus ATP formed during PHB synthesis.

Table 2. The effect of the energy uncoupler, 2,4-dinitrophenol, on PHB accumulation.[a]

2,4-Dinitrophenol (mM)	PHB content (%) in			
	Control[b]	NH_4^+-limitation	K^+-limitation	SO_4^{2-}-limitation
0.0	8.7	32.4	44.3	15.1
0.1	6.5	49.5	49.2	25.4
0.5	12.7	54.4	53.6	28.7
1.0	10.7	58.5	50.1	31.0

[a]Initial amount of resuspended cells in the second stage was 0.2 g/l. 2,4-Dinitrophenol was added at the start of the second stage. All measurements were made after 16 h of growth. All experiments were performed in triplicate. The relative standard deviations were ±5~7%.

[b]Control, growth medium with no limiting nutrients.

DISCUSSION

Nitrogen limitation has been widely used to promote PHB production in many microorganisms because of the ease with which it can be limited. Nitrogen limitation is believed to be superior to other forms of nutrient limitation in PHB production. Therefore, studies have focused on the optimization of culture conditions in nitrogen limited PHB production rather than on the effects of limitation of other nutrients. The optimal limiting nutrient in PHB production should depend on the metabolic characteristics of the microorganism used. With methylotrophs, the greatest amount of PHB accumulation in *Pseudomonas* sp. K and *Pseudomonas* 135 was observed with nitrogen limitation [10, 29], while it occurs with potassium limitation in *M. organophilum* NCIB 11278. Powell and Collinson [23] also studied PHB production in *M. organophilum* (NCIB 11482 to 11488) with nitrogen limitation. Maximum PHB contents obtained from each strain ranged from 29 to 41% of the dry cell weight. This is significantly lower than our result (59% of dry cell weight), obtained when potassium was limited. The maximum PHB content of their strains of *M. organophilum* (NCIB 11482 to 11488) should increase with potassium limitation. When potassium is limited, a large amount of energy is required to maintain a high cytoplasmic potassium concentration against the low potassium concentration of the potassium-limited environment. Energy dissipation is a major characteristic of potassium limitation [20]. Stoichiometric analysis shows that the formation of surplus energy is a characteristic of PHB synthesis in *M. organophilum* as shown in Fig. 2.

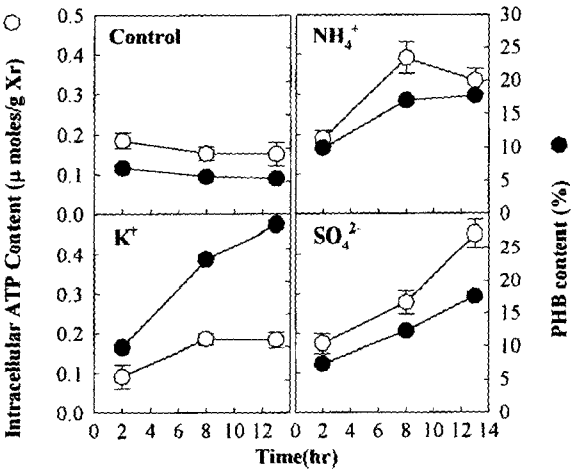

Fig. 2. The effect of limiting nutrients on intracellular ATP levels and PHB content during PHB accumulation: (○) intracellular ATP level; (●) PHB content.
K^+-, NH_4^+-, or SO_4^{2-}-limitations were applied to the second stage. The control was a culture with no imposed nutrient limitation. Error bars represent maximum deviations from means of triplicate analyses of ATP in each duplicate pair of cultures. Xr is the residual cell mass, the dry cell weight less the amount of PHB.

Considering the mass action law, it is reasonable to expect enhanced PHB accumulation, in *M. organophilum*, when potassium is limited. In *M. organophilum*, PHB accumulation increased two-fold with the addition of 1 mM 2,4-dinitrophenol when ammonium and sulfate were limited. Tempest and Wouters [31] also reported that the physiological response of microorganisms under ammonium-limited conditions became similar to that of potassium limitation through the addition of 2,4-dinitrophenol. We conclude that PHB accumulation in *M. organophilum* is stimulated by dissipating the surplus energy formed during PHB synthesis from methanol. This conclusion is strongly supported by Linton's report [17] that the overproduction of ATP associated with the production of exopolysaccharide caused a marked reduction in the polysaccharide production rate. Linton suggested that ATP dissipation enhanced the production process. Doi *et al.* [11] reported the intracellular accumulation of polyphosphate during PHB accumulation in *Alcaligenes eutrophus*. Polyphosphate is synthesized by the action of polyphosphate kinase, which catalyzes the transfer of the terminal phosphoryl group of ATP to polyphosphate. Polyphosphate synthesis could be a sink for surplus energy (ATP). Therefore, intracellular polyphosphate accumulation in *M. organophilum* was

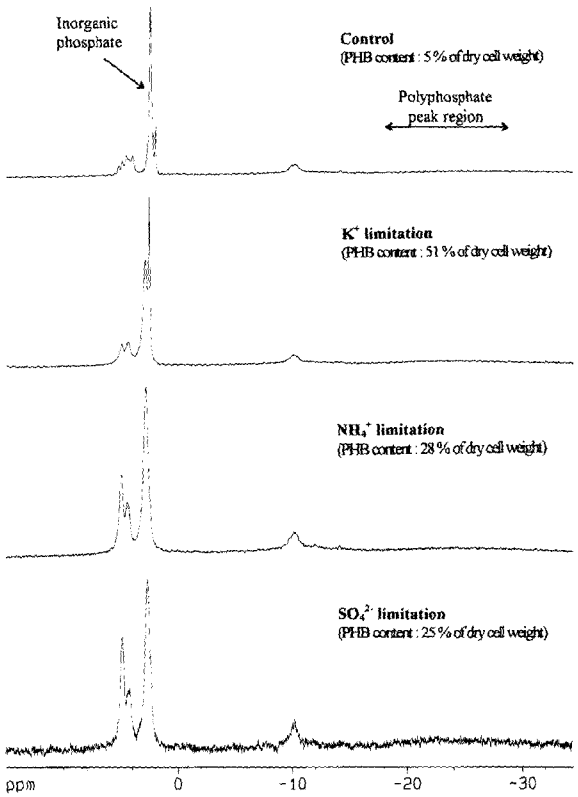

Fig. 3. 243-MHz ^{31}P NMR spectra of *M. organophilum* cells in suspension at 5°C.
Controls have no limiting nutrients. Chemical shifts are in parts per million from an external of 85% phosphoric acid.

investigated in PHB containing cells when ammonium, sulfate, and potassium were limited. The broad ^{31}P resonance at -23 ppm characteristic of polyphosphate was not observed, neither in cells with low PHB content under no limitation (control), nor in cells with high PHB contents under nutrient-limited conditions, as shown in Fig. 3. *M. organophilum* is known to be unable to synthesize polyphosphate, and so cannot dissipate the surplus energy from PHB synthesis by accumulating polyphosphate in cells.

The amount of potassium in microorganisms (g/g organisms) is as great or greater than the amount of phosphorous. Potassium-limited cultures are easy to make, so potassium limitation as a means of initiating PHB production is as easily applied as nitrogen limitation. Therefore, potassium limitation of *M. organophilum* could be used for the industrial production of PHB from methanol, an inexpensive substrate.

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