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# Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of methylotroph

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**Summary.** Fifty-one methylotrophs were checked with respect to their ability of poly-β-hydroxybutyric acid (PHB) production from methanol. One of them, *Pseudomonas* sp. K, was chosen from its good growth on a minimum synthetic medium. Optimal temperature and pH for its growth were 30°C and 7.0, respectively. Concentrations of $PO_4^{3-}$ and $NH_4^+$ in the medium should be kept at low levels. PHB formation was stimulated by deficiency of nutrient such as $NH_4^+$, $SO_4^{2-}$, $Mg^{2+}$, $Fe^{2+}$ or $Mn^{2+}$. Among them, nitrogen deficiency was chosen from its effectiveness and easiness for PHB accumulation.

The microorganism was cultivated to produce a large amount of poly-β-hydroxybutyric acid (PHB) from methanol by means of microcomputer-aided fully automatic fed-batch culture technique. During the cultivation, temperature, dissolved oxygen concentration (DO), and methanol concentration in the culture broth were maintained at 30°C, 2.5 ± 0.5 ppm and 0.5 ± 0.2 g/l, respectively. Other nutrients, nitrogen source and mineral ions, were also controlled to maintain their initial concentrations in the medium during cell growth phase. When the high cell concentration was achieved (160 g/l), feedings of ammonia and minerals were stopped and only methanol was supplied successively to accumulate PHB. At 175 h, high concentration of PHB (136 g/l) was obtained and total cell concentration became 206 g/l. DO must be maintained above the critical level during the PHB formation phase, too. PHB yield from methanol (g PHB/g methanol) was 0.18 and the maximum PHB content reached 66% of dry weight. Solid PHB produced by the strain had the melting point of 176°C and the average molecular weight of $3.0 \times 10^5$.

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## Introduction

Poly-β-hydroxy butyricacid (PHB) is a carbon-energy storage material accumulated inside a variety of microorganisms under appropriate conditions such as limitation of nitrogen (Dawes and Senior 1973; Emeruwa and Hawirko 1973; Schlegel et al. 1961) and/or oxygen (Ward et al. 1977). Some microorganisms can accumulate this polymer up to more than 80% of the total mass (Lafferty and Heinzle 1977). PHB can be produced from relatively cheaper substrates such as carbon dioxide (Schlegel et al. 1961), methanol (Braunegg et al. 1978) or ethanol (Taylor and Anthony 1976). Some biotechnological, biochemical and physiological studies on PHB biosynthesis have been carried out (Senior and Dawes 1973; Repaske and Mayer 1976; Ward et al. 1977; Heinzle and Lafferty 1980).

Solid PHB is a biodegradable thermoplastic polyester with very interesting characteristics and is utilizable in various ways similar to many conventional synthetic plastics now in use (Lafferty et al. 1977). For instance, it can be moulded, reinforced with inorganic fillers, spun into a fiber or formed into a film with excellent gas barrier properties. It is also possible to use PHB for making copolymers with other substances which may not be biodegraded.

Although a paper has been published (Braunegg et al. 1978) and a patent developed (Pawell et al. 1981) on PHB production by methylotrophs, little is known about distribution of methylotrophs having ability of producing PHB and

about physiological conditions for its maximum accumulation. Therefore, we first have screened a number of methylotrophs with respect to PHB production and have examined cultural conditions of an excellent PHB producer, *Pseudomonas* sp. K by carrying out batch culture. Then, to achieve high PHB productivity we have attempted to produce massive PHB from methanol by carrying out fully automatic fed-batch cultures of *Pseudomonas* sp. K, while maintaining the culture optimal for cell growth and/or metabolite formation. All the relevant techniques developed in our laboratory have been employed in the fed-batch cultures, such as a DO-stat for the control of dissolved oxygen concentration (DO) (Yano et al. 1981a and 1981b), a microporous Teflon tubing sensor combined with a flame ionization detector and controller for volatile carbon source feeding (Yamane et al. 1980), pH-stat for ammonia feeding and a control strategy of mineral ions supplementations based on stoichiometrical estimation (Suzuki et al. 1985).

## Materials and methods

*Screening of PHB producer. Pseudomonas* AM-1, *Pseudomonas* sp. K, *Protaminobacter ruber* NR-1, *Methylomonas clara* and unidentified forty-seven strains isolated from soil, were screened for their ability to accumulate intracellular PHB and for growth rate on methanol which was used as the sole carbon source.

*Optimization of culture medium and conditions.* The effects of concentrations of medium components on cell growth were examined in the strain, *Pseudomonas* sp. K, which showed the best capacity to produce PHB. The starting composition of the medium for this purpose was the same as used for *Protaminobacter ruber* NR-1 in the previous study (Yano et al. 1980). Initial methanol concentration was 1%. The effects of components concentrations on growth were examined by measuring the optical density after 25 h cultivation (0.1 ml of the seed culture was inoculated into 10 ml of each medium). The culture medium optimal for cell growth was determined from the profiles of cell growth vs. concentrations of medium components so that any component did not limit or inhibit the cell growth. The medium is refered to as Medium A in this article.

*Effect of nutrient deficiency on growth and PHB synthesis.* The effect of medium component deficiency on PHB production was examined by eliminating $Fe^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Ca^{2+}$, $Mo^{6+}$ and $Na^{+}$ in turn from Medium A, while $NH_4^+$, $SO_4^{2-}$, and $Mg^{2+}$ were not completely eliminated from Medium A; small concentrations (50 mg/l of $NH_4^+$, 4 mg/l of $SO_4^{2-}$ and 0.8 mg/l of $Mg^{2+}$) were retained to allow growth of the inoculated cells for a while, until their growth was stopped. In all deficient media, initial methanol was 10 g/l and 0.5 g of methanol was added every 2 days into each 100 ml of the culture during cultivation. First, a loop of the organism from the stock culture kept on the agar slant was inoculated into 10 ml of medium and cultivated at 30° C for 40—50 h on a reciprocal shaker. When the optical density at 570 nm of the culture reached about 1.0, the cells were centrifuged aseptically, washed once with sterile water and transferred to 100 ml component-deficient medium in 500-ml flask; this culture was maintained at 30° C for 150—200 h on a reciprocal shaker. The cells were then harvested and their PHB content was determined.

*Initial medium of fed-batch culture.* The composition of the initial medium of the fed-batch culture was the same as the culture medium that was determined as optimal for the cell growth by the batch culture (Medium A). The initical methanol concentration was 0.5 g/l.

*Method of fed-batch culture.* The seed culture was prepared in ten 500-ml flasks on a reciprocal shaker at 30° C for 3 days. Aseptically centrifuged cells of the seed culture were inoculated into a bioreactor (Type MB, Iwashiya Co., Ltd., Tokyo) equipped with a four-bladed disk-turbine impeller ($D_i/D_T=0.5$) and three buffles. The initial volume of the culture was 0.75 l. Temperature and pH were automatically controlled at optimal values (30° C and 7.0).

*Control of methanol feeding.* Concentration of methanol was maintained automatically at $0.5\pm0.2$ g/l by use of a control system composed of a porous Teflon tubing sensor, a flame ionization detector (in a Hitachi gas chromatograph, Model 163) and a microcomputer (Process Controller ST-6001, Sanko Electrical Industries Co., Ltd., Nagoya). A preset PID control program was employed. The parameters of proportional band and differential time constant were determined by material balance and the integral time constant was computed and changed automatically during the fed-batch culture according to the $CO_2$ concentration in the outlet gas which was measured with an infrared $CO_2$ gas analyser. The data of the analyser were transmitted into the microcomputer. A peristaltic pump (Cole-Parmer No. 7017, Cole-Parmer Industrument Co.) connected with a stepping motor (Type 4CPH-004, Oriental Motor Co., Ltd., Tokyo) was used to supply methanol, and the rotation speed of the motor was controlled by the output signal from the microcomputer.

*DO control.* DO in the culture broth was controlled in the range of 2—3 ppm with a microcomputer (Yano et al. 1980). The proportion of air and pure oxygen gas flow rates (total flow rate of supplied gas was maintained at 1 l/min) and the agitation speed (in the range of 500—1400 rpm) were automatically controlled according to a preset PID control program similar to that for methanol feeding mentioned above. Details of the control program will be published elsewhere.

*Nitrogen source and mineral ions feeding.* Nitrogen source was supplemented by addition of 33% ammonia water either in such a way as to maintain a constant pH of the culture broth (indirect feedback control by pH-stat) or in coupling with methanol feeding.

The required concentration of each mineral ion in the mineral solution was calculated from the equation proposed by us (Suzuki et al. 1985), using both the elementary composition of dried cells and the values of culture parameters. From the calculation, two kinds of mineral solutions, acid and alkaline solutions, were prepared. The acid solution was supplied in coupling with methanol supply. The alkaline solution was supplied either in coupling with methanol supply when pH of the culture broth was controlled by the addition of ammonia water, or in such a way as to keep pH of the culture broth (indi-

rect feedback control by pH-stat) when the ammonia water was supplied in coupling with methanol feeding. pH-dependent supply of the ammonia water or of the alkaline solution was automatically controlled by a pH-controller (Type PC-5272, Sanko Electrical Industries Co., Ltd., Nagoya) and a peristaltic pump (Type SJ-1211, Atto Co., Ltd., Tokyo). When the supplies of the ammonia water, the acid solution and the alkaline solution were coupled with the methanol supply, the same kinds of stepping motor and pump as those used for the methanol supply were used under the condition that (ml-minerals feed)/(ml-methanol feed)=0.063 and (ml-ammonia feed)/(ml-methanol feed)=0.125. During the exponential growth phase of the fed-batch culture, both the ammonia water and the mineral solution were supplied continuously to maintain the concentrations of nutrient components in the medium at constant levels. At the end of the exponential growth phase where the cell mass concentration reached about 150 g/l, their supply were stopped to let the cells be in a nutrient-deficient environment. Afterwards, only methanol was still supplied continuously. The scheme of the nutrient feeding system of the fully automatic fed-batch culture is shown in Fig. 1.

*Analytical procedure.* Microbial growth was temporarily determined by measuring optical density at 570 nm of the culture broth after dilution with 0.9% NaCl solution. Cell mass concentration was determined by weighing dry matter after 5 ml of the culture broth was centrifuged, the centrifuged cells were washed with distilled water and were freeze-dried in vacuum. PHB was determined by gas chromatographic method (Braunegg et al. 1978) with benzoic acid as internal standard (Hitachi gas chromatograph Model 163).

To obtain a sample of solid PHB, the cells were washed with acetone, dried, and extracted with chloroform at 40°C while applying sonication. The chloroform extract was filtered to remove residual cell materials. To precipitate PHB, 3 volumes of n-hexane were added to one volume of the chloroform extract. The PHB precipitate was washed with ether and dried. The molecular weight distribution of PHB was determined by gel permeation chromatography using polystylene gel (TSK-GEL GMH-6, Toyo Soda Manufacturing Co., Ltd., Tokyo). Eluent solvent was chloroform. Concentrations of P and S as well as ammonium and metal ions were determined according to the method described previously (Suzuki et al. 1985). Chemical composition of dry cells was also determined according to the method described previously (Suzuki et al. 1985).

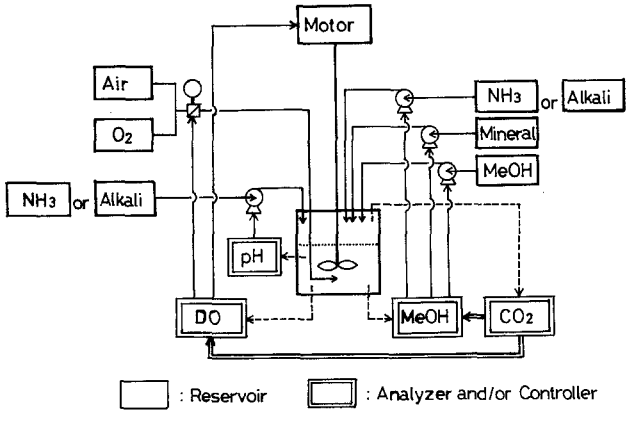


**Fig. 1.** System for computer-aided fed-batch culture

## Results and discussion

### *Screening of PHB producer*

Many microorganism accumulated PHB inside cells, but its contents varied. Three strains produced PHB more than 50% of the dry cell weight. *Pseudomonas* sp. K was adopted for further experiments from its good growth on a minimum synthetic medium.

### *Medium composition and culture conditions of batch culture*

To optimize fed-batch culture, it was necessary to find suitable compositions of initial medium and mineral feed solution as well as cultivation conditions. To this end, many batch cultures were conducted. Maximum specific growth rate was obtained at a temperature of 30°C and a pH of 7.0. The effect of concentrations of various components on growth was examined. As a source of nitrogen, the effect on cell growth of six ammonium compounds was tested; these were: $NH_4Cl$, $NaNH_4HPO_4 \cdot 4H_2O$, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$, $NH_4HCO_3$ and $NH_4NO_3$, used at different concentrations. The effect of all these ammonium compounds was similar: in every case cell growth was optimal at about 0.2 g $NH_4^+ \cdot l^{-1}$, and was severely inhibited when $NH_4^+$ concentration exceeded 1.0 g/l. This suggests that the concentration of $NH_4^+$ should be kept as low as 0.2 g/l. This corresponds to 1 g/l $(NH_4)_2SO_4$ at fed-batch culture for optimal cell growth.

The concentration of $PO_4^{3-}$ was changed by mixing either $K_2HPO_4$ or $Na_2HPO_4 \cdot 12H_2O$ with $KH_2PO_4$. Cell growth did not depend on the kind of cations, $Na^+$ and $K^+$, but was affected considerably by $PO_4^{3-}$ concentration. The optimal $PO_4^{3-}$ concentration was around 2 g/l. Also the maximum growth was observed at 0.1 g/l $MgSO_4 \cdot 7H_2O$. The seven trace mineral ions, $Fe^{2+}$, $Ca^{2+}$, $Zn^{2+}$, $Cu^{2+}$, $Co^{2+}$, $Mn^{2+}$ and $MoO_4^{2-}$ tested at different concentrations were found to have little effect. The presence of these elements was, however, necessary to achieve high concentration of cell mass. In addition, some trace elements may have accelerative effect on PHB accumulation. It is therefore desirable to maintain the concentration of every element at a suitable level at which the cell growth is not limited. The composition of the culture medium Medium A which was finally determined as optimal for cell growth, was (per one liter of distilled wa-

ter): 0.8 g of $KH_2PO_4$, 3.0 g of $Na_2HPO_4 \cdot 12H_2O$, 0.8 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 5 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $CoCl_2 \cdot 6H_2O$, 2 mg of $MnSO_4 \cdot nH_2O$, 5 mg of $ZnSO_4 \cdot 7H_2O$, 0.2 mg of $Na_2MoO_4 \cdot 2H_2O$ and 1 mg of $CuCl_2 \cdot 2H_2O$.

### *Influence of nutrient deficiency on growth and PHB synthesis*

*Pseudomonas* sp. K was cultivated batchwise separately on 11 kinds of media in which each of $NH_4^+$, $SO_4^{2-}$, $Mg^{2+}$, $Zn^{2+}$, $Fe^{2+}$, $Mn^{2+}$, $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, $Mo^{6+}$ and $Na^+$ was eliminated or limited from Medium A. The cell growth and the PHB contents are shown in Table 1. In the case of $NH_4^+$, $SO_4^{2-}$, $Mg^{2+}$, $Fe^{2+}$ or $Mn^{2+}$-deficient medium, PHB accumulated a lot, whereas in other element-deficient media, PHB was little synthesized. $Na^+$ or $Ca^{2+}$-deficiency did not accumulate PHB. Under the condition of $Zn^{2+}$, $Co^{2+}$, $Mo^{6+}$, or $Cu^{2+}$-deficiency, cell growth was almost the same as the control. These trace elements might be contained enough in each medium as an impurity of other medium components or might have been transferred together with inoculum, even if it was not added as its salt. In these cases, cells grew well and little PHB accumulated.

### *Determination of compositions of acid and alkaline solutions*

The composition of acid solution that was supplied in the fed-batch culture was determined as follows (per liter of distilled and deionized water): 270 g of $H_3PO_4$, 20 g of $H_2SO_4$, 80 g of $MgSO_4 \cdot 7H_2O$, 2.3 g of $FeSO_4 \cdot 7H_2O$, 0.66 g of $CaCl_2 \cdot 2H_2O$, 0.29 g of $MnSO_4 \cdot nH_2O$, 0.48 g of $CoCl_2 \cdot 6H_2O$, 0.63 g of $ZnSO_4 \cdot 7H_2O$ and 0.41 g of $CuCl_2 \cdot 2H_2O$. The alkaline solution was 5.2 N-KOH and 0.2 N-NaOH.

### *Optimal temperature, pH and methanol concentration*

Maximum specific growth rate in fed-batch culture at 2.5 g/l of methanol concentration was obtained when temperature was maintained at 30°C and pH at 7.0 (Fig. 2). To examine the inhibitory effect of methanol on cell growth, fed-batch culture with methanol concentration maintained at various levels were carried out. As shown in Fig. 2 the maximum specific growth rate was obtained at about 0.5 g/l of methanol concentration. All subsequent fed-batch cultures were therefore carried out at this methanol concentration.

**Table 1.** Effect of medium component deficiency on PHB production[a]

| Deficient ion | Final cell concentration (g/l) | Final PHB content (%) |
|---|---|---|
| $NH_4^+$[b] | 0.6 | 52—57 |
| $SO_4^{2-}$[b] | 1.3 | 48—53 |
| $Mg^{2+}$[b] | 1.1 | 45—50 |
| $Fe^{2+}$ | 1.6 | 43—48 |
| $Ca^{2+}$ | 0.9 | 0—10 |
| $Zn^{2+}$ | 1.3 | 0—10 |
| $Mn^{2+}$ | 1.9 | 50—55 |
| $Co^{2+}$ | 1.6 | 0—10 |
| $Cu^{2+}$ | 1.7 | 0—10 |
| $Mo^{6+}$ | 1.7 | 0—10 |
| $Na^+$ | 1.2 | 0—10 |
| Control | 1.7 | 0—10 |

[a] The basal medium was Medium A. Initial methanol concentration was 10 g/l and 0.5 g of methanol was added into each 100 ml of the culture every 2 days during cultivation

[b] These ions were not completely eliminated from the initial medium (see text for further details)

### *PHB accumulation by nitrogen-limited fed-batch culture*

Among the five cases where PHB accumulated significantly, that is, N-, S-, Mn-, Mg- and Fe-de-

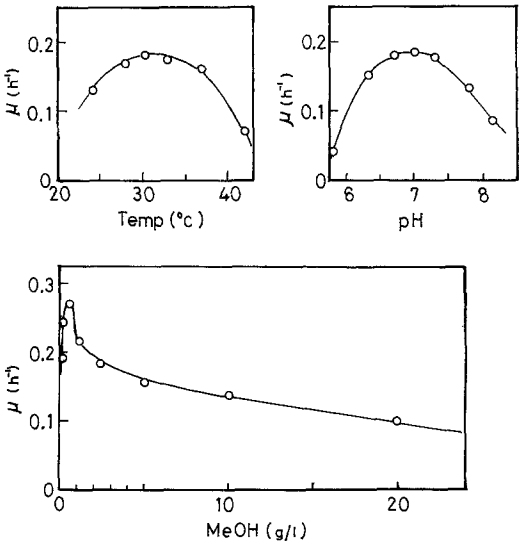


**Fig. 2.** Effects of temperature, pH, and methanol concentration on the specific growth rate of *Pseudomonas* sp. K grown on Medium C

ficient media, N-limited fed-batch culture was chosen in the subsequent experiments for the following reasons. It is easier to make the culture N-limited than to make it other element-limited because the growth of microorganisms is more dependent on nitrogen than on other mineral ions and the nitrogen source is assimilated more rapidly than are other mineral ions. When the feedings of ammonium and mineral ions were stopped simultaneously, all the ammonium ions were thus rapidly consumed while the concentrations of other mineral ions scarcely decreased. When ammonia feeding was stopped, PHB accumulated more quickly than when other elements were withheld. The yield of PHB production relative to the amount of methanol consumed was higher in nitrogen-limited fed-batch culture than in cultures in which other elements were limited.

In order to study the effect of ammonium limitation on PHB formation at different culture phases, four fed-batch cultures were carried out, in which ammonia feeding was stopped when the cell mass concentrations reached 25, 60, 100 and 130 g/l. Ammonia water for pH adjustment was substituted by alkaline solution. In these experiments elements other than nitrogen were adequately supplied in coupling with methanol feeding. The results are shown in Fig. 3. In every case, after ammonium ion in the culture liquid had become deficient, the PHB accumulation accelerated while the growth rate gradually decreased. PHB yields based on methanol consumed, $Y_{P/S}$, were calculated from Fig. 3 and summarized in Fig. 4 as a function of PHB content, P/X. The $Y_{P/S}$ values for a, b and c were almost the same, and maximum $Y_{P/S}$ was obtained when PHB content reached about 60%. But in the case d, it was smaller than the others. It was suggested that this might be due to damage to the cells by some inhibitory substances in the culture liquid. To clarify this, the sample taken at 80-h culture time was centrifuged and the supernatant was analysed by gas chromatography. No intercellular accumulation of metabolites was detected. Nutrients other than the ammonium ion were maintained at optimal concentrations. Since ammonia water was supplied to control the pH of the culture broth, the concentration of ammonium ion increased as shown in Fig. 3 and excess ammonium ion seemed to exert a toxic effect on the microorganisms. During 50—80 h of culture time where ammonium ion existed in excess, cell growth stopped and after the supply of ammonia-water had been stopped, the concentration of the ammonium ion began to drop and the cells began to grow a little again until finally the ammonium ions had been completely consumed. It can be seen from Fig. 3 (d) that the ammonium ion concentration should be maintained within a suitable range to obtain a large growth of cells before the PHB production phase. If this is made possible, higher cell mass

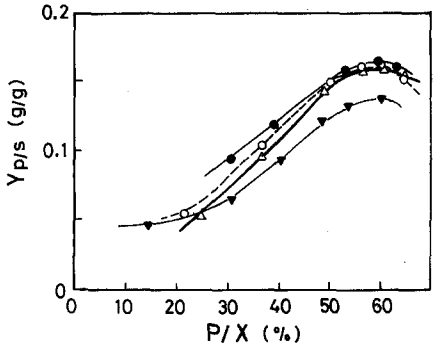


**Fig. 4.** Correlation between the PHB yield based on methanol consumed, $Y_{P/S}$, and PHB content. Ammonia feedings were stopped when the cell mass concentrations reached ●, 25; ○, 60; △, 100; and ▼, 130 g/l

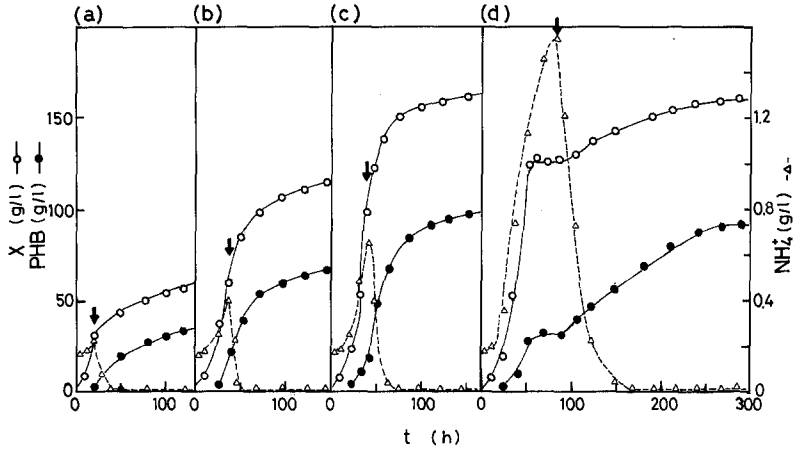


**Fig. 3.** Effect of ammonium deficiency at various culture phases on PHB formation. Ammonia feedings were stopped when the cell mass concentrations reached (a) 25, (b) 60, (c) 100, and (d) 130 g/l, as indicated by arrows

concentration will be obtained until other growth-limiting factors such as DO deficiency prevails.

### *Mass production of PHB by fully automatic fed-batch culture*

In many microbial cultivations ammonia water is used not only to control pH of the cultures but also as a supplementary source of nitrogen. In this study, however, this method failed when high cell concentration was required. Ammonia water should be added so as to keep the ammonium concentration in the culture liquid at a low level, preferably around 0.2 g/l during the exponential growth phase. This may be possible if the ammonia water is supplied in coupling with carbon source feeding (Suzuki et al. 1985). The quantity required to be fed can be estimated by the same equation as the mineral solution described above. In this method pH control was separated from N-feeding and was effected with alkaline solution. The culture result is shown in Fig. 5. The ammonium concentration could be kept at a low level throughout the growth phase and cell growth was not depressed, although the specific growth rate ($0.17\ h^{-1}$) was a little less than the maximum value. At 72 h culture time, DO was no longer able to be maintained at 2—3 ppm, owing to the limitation of oxygen transfer rate of the bioreactor used (the agitation speed and the flow rate of pure oxygen gas became 1,400 rpm and 1 l/min, respectively). The cell mass concentration reached 160 g/l and the culture broth volume 1.2 l. At this time feeding of ammonia water and mineral solution were stopped. The intracellular PHB content then began to increase due to the decrease in the ammonium concentration in the culture liquid. It took about 100 h from the time of N-feed stopping to reach the maximum concentration of PHB, 136 g/l and the cell mass concentration, 206 g/l. This means that the maximum PHB content was 66%. The culture broth increased up to 1.7 l. Figure 6 shows changes in the course of time of PHB yield from methanol ($Y_{P/S}$), yield of cell constituent other than PHB from methanol ($Y_{Xr/S}$) and PHB content in the cell (P/X) with elapsed time. In calculating these variables the loss of methanol due to evaporation was assumed to be negligible. In the preceding exponential growth phase, $Y_{Xr/S}$ was as high as about 0.33 (g/g), $Y_{P/S}$ was about 0.04 (g/g), P/X was kept almost constant at 18%. After nitrogen in culture liquid made deficient, $Y_{Xr/S}$ decreased soon to zero, $Y_{P/S}$ increased over 0.2 (g/g) and then decreased gradually to 0.17 (g/g) at the final stage. Overall $Y_{P/S}$ was about 0.18 (g/g).

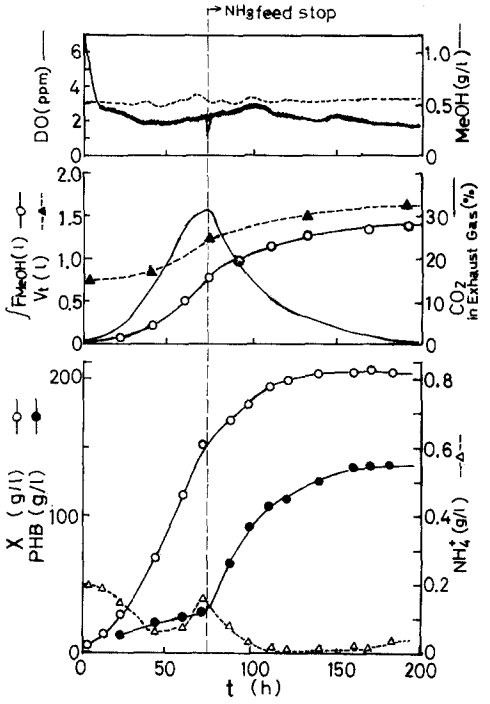


**Fig. 5.** Experimental results for mass production of PHB by fully automatic fed-batch culture. Ammonia feeding was stopped when the cell concentration reached 160 g/l at 75 h

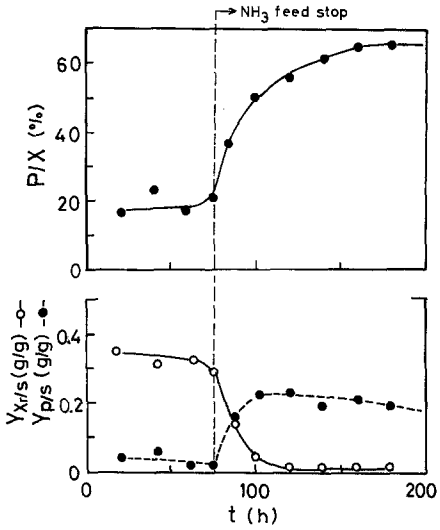


**Fig. 6.** Time courses of PHB content and correlation between the yield of PHB ($Y_{P/S}$) and the yield of residual biomass ($Y_{Xr/S}$) during the fully automatic fed-batch culture

### *Effect of DO on PHB formation*

It has been reported that PHB production is stimulated by DO limitation (Ward et al. 1977). This possibility was examined for the fed-batch culture

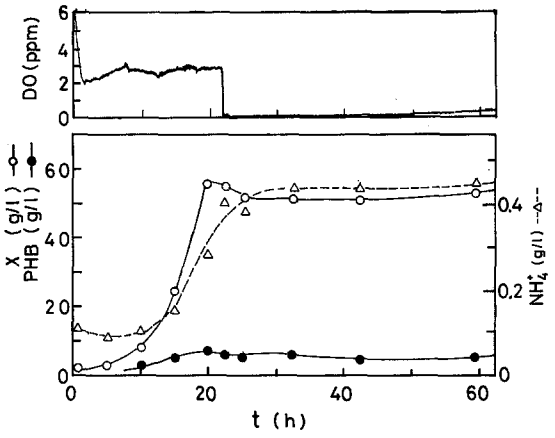


**Fig. 7.** Effect of DO on PHB formation. When the cell mass concentration reached 50 g/l, aeration condition was changed to let cells DO-deficient

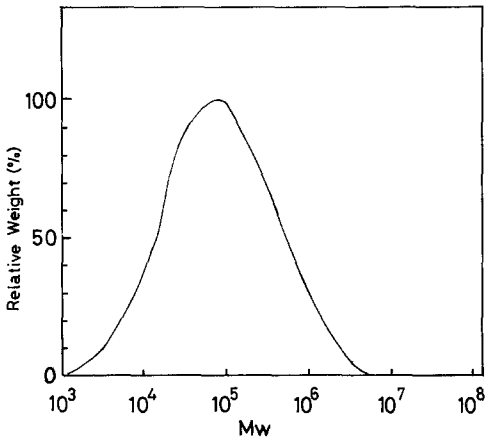


**Fig. 8.** Molecular weight distribution curve of the PHB produced by *Pseudomonas* sp. K

of *Pseudomonas* sp. K. When the cell mass concentration reached 50 g/l, the oxygen absorption condition was changed from 1000 rpm of agitation speed and 0.2 l/min of oxygen flow rate and 0.8 l/min of air flow rate to 1100 rpm and zero l/min of oxygen flow rate and 1.0 l/min of air flow rate. The experimental result is shown in Fig. 7. DO dropped at once and the cell growth decreased sharply, but PHB did not accumulate in the cells at all. DO-deficiency did not clearly stimulate PHB production in *Pseudomonas* sp. K. The cells seemd to be injured by dissolved oxygen concentration deficiency. DO should be maintained at a sufficient level not only for cell growth in the early phase but also for the PHB production at the later stage (Sonnleitner et al. 1979).

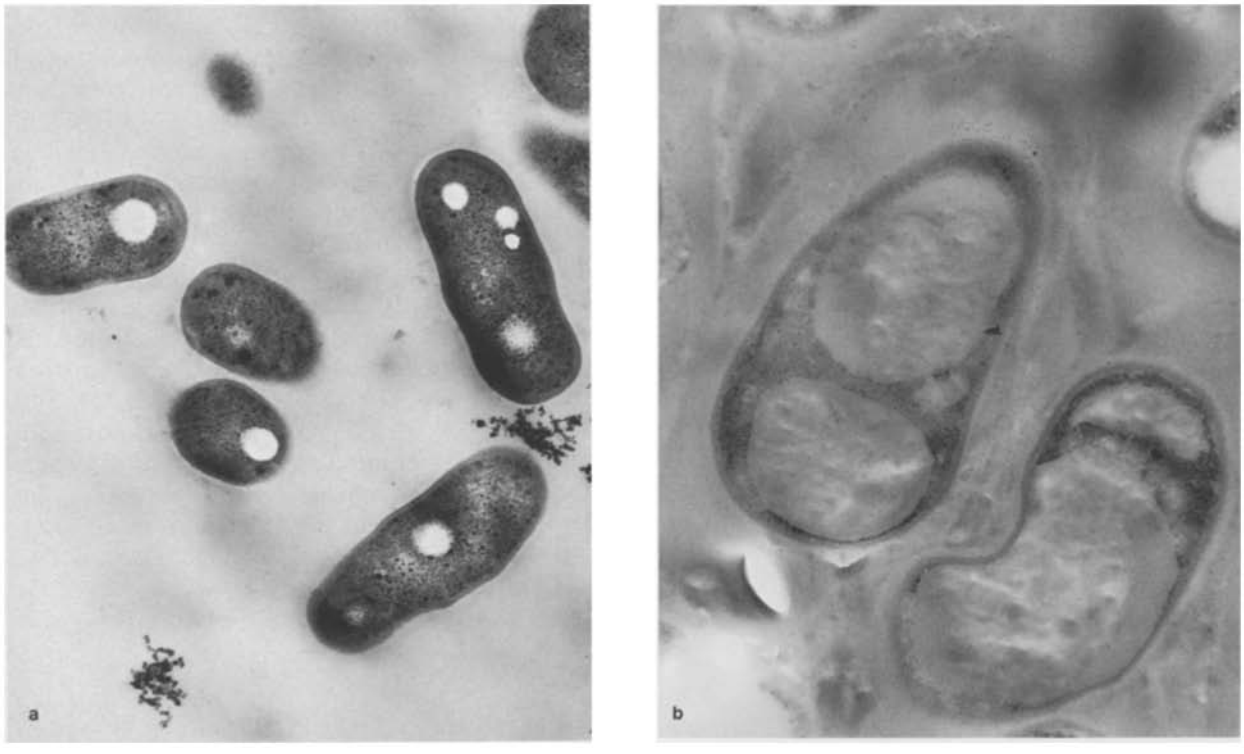


**Fig. 9.** Transmission electron micrograph of the cells of *Pseudomonas* sp. K **(a)** at the initial stage of the fed-batch culture and **(b)** at the final stage of nitrogen-deficiency

*Properties of PHB produced*

Melting point of solid PHB produced from methanol by fully automatic fed-batch culture of *Pseudomonas* sp. K was 176° C. Its infrared spectrum was identical with that of the authentic sample. Figure 8 shows the molecular weight distribution curve of the PHB dissolved in chloroform. The average molecular weight was about $3.0 \times 10^5$ and the range was rather broad.

*Electron microphotograph of cell*

Figure 9 shows the transmission electron micrographs of the cells of *Pseudomonas* sp. K at the initial stage of the fed-batch culture (a) and at the final stage of nitrogen-deficiency (b). It is uncertain whether the white globules seen in the initial stage are PHB globules or not, but their size alone suggests that the one or more large globules seen at the final stage must be PHB globules.

## General discussion

In this paper we have reported mass production of PHB up to 136 g/l by a methylotroph, *Pseudomonas* sp. K. The PHB production process used here has several advantages. Methanol is a abundant carbon source. A lot of methylotrophs can accumulate high intracellular content of PHB. Its concentration in the culture liquid is easily detectable and can be controlled by automatic feedback using the tubing method combined with both a detector and a microcomputer. Shift of the culture condition in the fed-batch culture from the cell growth phase to the PHB biosynthesis phase can be accomplished simply by nitrogen deficiency, i.e. by stopping the feed of ammonia water. The technique developed in this study is applicable to various microbial processes where concentration of any nutrient affects the yield of metabolites.

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