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Optimization of Growth Medium and Poly- β-hydroxybutyric Acid Production from Methanol in *Methylobacterium organophilum*

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메탄올로부터 *Methylobacterium organophilum*에 의한 Poly-β-hydroxybutyric Acid의 생산과 배지성분의 최적화

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***Methylobacterium organophilum*, a facultative methylotroph was cultivated on a methanol as a sole carbon and energy source. The cell growth was affected by the various components of minimal synthetic medium and the medium composition was optimized with 0.5% (v/v) methanol at pH 6.8 and at 30°C. The maximum specific growth rate of *M. organophilum* was achieved to 0.26 hr^{-1} in the optimized medium which has following composition: Methanol, 0.5% (v/v): $(NH_4)_2SO_4$, 1.0 g/*l*: KH_2PO_4, 2.13 g/*l* : KH_2PO_4, 1.305 g/*l*: $MgSO_4 \cdot 7H_2O$, 0.45 g/*l* and trace elements ($CaCl_2 \cdot 2H_2O$, 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: $Na_2MoO_4 \cdot 2H_2O$, 40 μg: $CoCl_2 \cdot 6H_2O$, 40 μg: H_3BO_3, 30 μg per liter). By the limitation of nitrogen and deficiency of Mn^{+2} or Fe^{+2}, the cell growth was significantly repressed. Methanol greatly repressed the cell growth and the complete inhibition was observed at concentration above 4% (v/v). In order to overcome the methanol inhibition and to prevent the methanol limitation, intermittent feeding of methanol was conducted by a D.O.-stat technique. PHB production by *M. organophilum* was stimulated by deficiency of nutrients such as NH_4^+, SO_4^{-2}, Mg^{+2}, K^+, or PO_4^{-3} in the medium. The maximum PHB content was obtained as 58% of dry cell weight under deficiency of potassium ion in the optimized synthetic medium.**

Poly- β-hydroxybutyric acid (PHB) is a linear polymer of β-hydroxybutyrate and occurs in refractile granules of various sizes within the cells of various microorganisms (1). PHB was known as carbon and energy-storage materials in the cells which could be accumulated when nutrients other than carbon source became limiting, and it is degraded when the nutrients are sufficient in the medium (2). PHB can be produced from relatively cheaper substrate such as methanol (3), carbon dioxide (4) or ethanol (5). Some biotechnological, biochemical and physiological studies on PHB biosynthesis have been carried out. Recently, solid PHB is attracting a great attention as a biodegradable thermoplastic polyester with several properties similar to many conventional synthetic plastics (6, 7). Although a few paper and patent have been published (4, 8) 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 examined the medium optimiza-

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Table 1. Composition of medium.

Medium Number	1 (g/l)	2 (g/l)	3 (g/l)	4 (g/l)	5 (g/l)
$(NH_4)_2SO_4$	1.0	3.6	3.6	0.3	1.0
H_3PO_4	75×10^{-3}	650×10^{-3}		650×10^{-3}	
KH_2PO_4			1.305		1.305
Na_2HPO_4			2.13		2.13
$MgSO_4 \cdot 7H_2O$	30×10^{-3}	450×10^{-3}	450×10^{-3}	450×10^{-3}	450×10^{-3}
$CaCl_2 \cdot 2H_2O$	3.3×10^{-3}	4.95×10^{-3}	4.95×10^{-3}	4.95×10^{-3}	3.3×10^{-3}
$FeSO_4 \cdot 7H_2O$	1.3×10^{-3}	1.95×10^{-3}	1.95×10^{-3}	1.95×10^{-3}	1.3×10^{-3}
$MnSO_4 \cdot 4H_2O$	0.13×10^{-3}	0.2×10^{-3}	0.2×10^{-3}	0.2×10^{-3}	0.13×10^{-3}
$ZnSO_4 \cdot 7H_2O$	0.13×10^{-3}	0.2×10^{-3}	0.2×10^{-3}	0.2×10^{-3}	0.13×10^{-3}
$CuSO_4 \cdot 5H_2O$	40×10^{-6}	60×10^{-6}	60×10^{-6}	60×10^{-6}	40×10^{-6}
$Na_2MoO_4 \cdot 2H_2O$	40×10^{-6}	60×10^{-6}	60×10^{-6}	60×10^{-6}	40×10^{-6}
$CoCl_2 \cdot 6H_2O$	40×10^{-6}	60×10^{-6}	60×10^{-6}	60×10^{-6}	40×10^{-6}
H_3BO_33	30×10^{-6}	45×10^{-6}	45×10^{-6}	45×10^{-6}	30×10^{-6}

*0.5% (v/v)-methanol was supplemented in each medium.

tion for a PHB-producing methylotroph, *Methylobacterium organophilum* growing on a methanol as a sole carbon and energy source and fed-batch culture of *M. organophilum* was conducted in order to improve the PHB production by overcoming the methanol inhibition.

Materials and Methods

Microorganism

The strain used in this study was *Methylobacterium organophilum* K-1, shared by Dr. J.M. Lebeault, Universite de Technologie de Compiegne, France (9).

Culture media and conditions

The basal culture media are minimal synthetic media supplemented with 0.5% (v/v) methanol described by Maclennan, *et al.* (10) and Tsuchiya, *et al* (11). The modification of culture medium was followed as described by Maclennan, *et al.* (10), and by the procedure described in this paper. The compositions of various media were given in Table 1.

The batch cultures were performed in 2*l* jar fermentor (New Brunswick Scientific Co.) with a working volume of 1.3 liter or in 250 m*l* flask. Aeration and agitation were conducted to maintain the dissolved oxygen above 40% of air saturation using an oxygen probe (Ingold). The culture pH was maintained 6.8 with 4 N NaOH/KOH (1:1 mixture) at the temperature of 30°C.

Analytical methods

Optical density was measured at 570 nm for the estimation of cell growth. Dry cell weight was determined gravimetrically and ammonium nitrogen was analyzed by Indo-Phenol method (12). The concentration of residual methanol in culture broth was measured by gas chromatography in Intersmat IGC 121 DFL with a column packed with chromosorb 101, 80/100 mesh at 150°C and a flame ionization detector was used. The PHB granule was prepared by the method of Williamson and Wilkinson (13) and the quantitative estimation was performed by gravimetry or gas chromatography by the method of Braungg G., *et al.* (3). Total contents of nitrogen in organic sources (yeast extract, peptone, corn steep liquor, and casamino acid) were measured by micro-Kjeldahl method (14).

Results and Discussion

Optimization of medium composition for cell growth

In order to develop and optimize the synthetic medium in flask culture, *M. organophilum* was cultivated on five kinds of minimal basal medium supplemented with 0.5% (v/v) of methanol. First of all, phosphorus source was tested for cell growth, since phosphorus was known as a crucial growth-

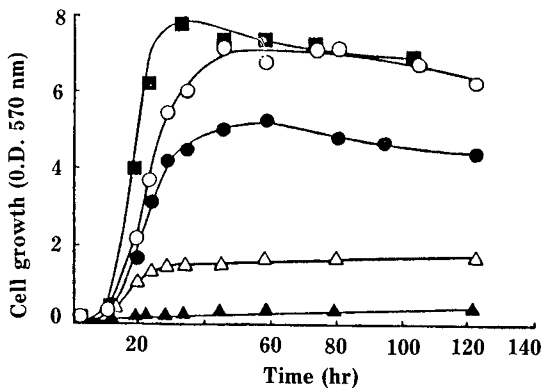

Fig. 1. Time courses of the cell growth on medium 1 (▲), medium 2 (△), medium 3 (○), medium 4 (●) and medium 5 (■).

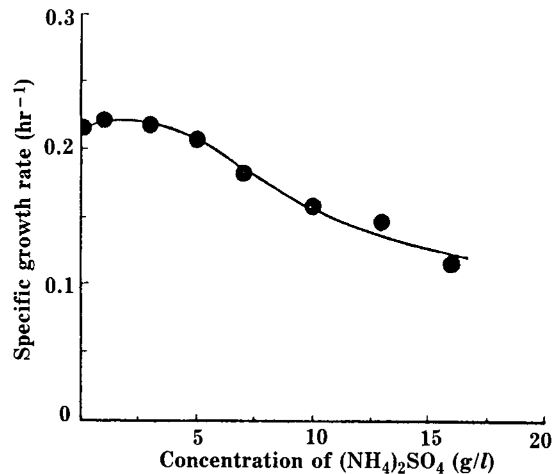

Fig. 2. Effect of initial concentration of $(NH_4)_2SO_4$ on the specific growth rate.

regulating nutrient in microorganism (15). The better cell growth was obtained by substitution of phosphate salts (combination of KH_2PO_4 and Na_2HPO_4) for phosphoric acid in medium 1,2, and 3 proposed by Maclennan (10) as shown in Fig. 1. This result was probably due to the buffering action of phosphate. In fact, pH was only changed from 6.8 to 6.5 during the culture. From the growth profiles on medium 2 and 4, it was found that high concentration of ammonium sulfate inhibited the cell growth. In case of medium 3 and 5, the higher growth rate was obtained on medium 5 containing 1.0g-$(NH_4)_2SO_4/l$ instead of 3.6g-$(NH_4)_2SO_4/l$ in medium 3, even though the final cell mass was similar between two different media. To determine the appropriate concentration of ammonium sulfate, the cultures were carried out in the range of concentration from 0.5g/l to 16g/l. The maximum specific growth rate decreased significantly as the concentration of $(NH_4)_2SO_4$ increased over 2.5g/l of $(NH_4)_2SO_4$ (Fig. 2). It is a very rare case which a ammonium sulfate inhibits cell growth in range of 2.5-10g/l. However, nitrogen regulations on metabolites accumulation were reported in several cases of amino acid and secondary metabolite production (16, 17). To find out the better nitrogen sources, the growth of *M. organophilum* was examined on the medium containing various nitrogen sources and the results were represented in Table 2. As the inorganic nitrogen sources such as ammonium and nitrate salts were rapidly assimilated by the cell, the high specific growth rates ranged from 0.22 to 0.26 hr^{-1} were obtained. The specific growth rates

Table 2. Effect of nitrogen sources on specific growth rate.

Nitrogen sources*	μ (hr^{-1})	Dry cell weight (g/l)
$(NH_4)_2SO_4$	0.254	1.954
$NaNO_3$	0.224	1.796
Urea	0.120	1.572
Casamino acid	0.076	1.414
CSL	0.109	1.486
Yeast extract	0.072	1.442
Peptone	0.041	0.664

*Nitrogen content: Amount equivalent to 0.064g-total nitrogen/l in each case.

Table 3. Effect of trace elements on specific growth rate.

Deficient component	Specific growth rate (hr^{-1})
Ca^{+2}	0.228
Fe^{+2}	0.206
Mn^{+2}	0.205
Zn^{+2}	0.246
Co^{+2}	0.234
Mo^{+2}	0.276
Cu^{+2}	0.221
H_3BO_3	0.242
Control	0.261

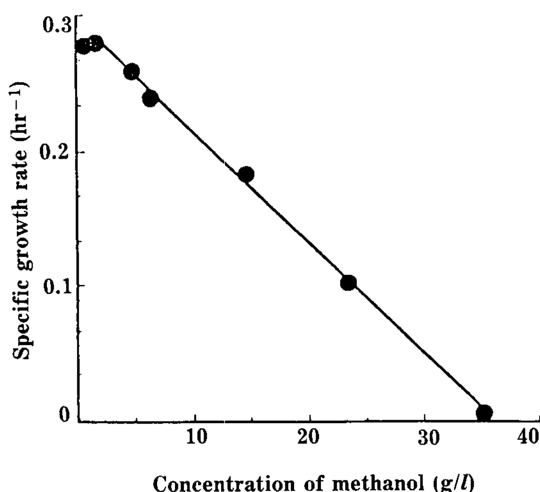

Fig. 3. Effect of methanol concentration on the specific growth rate.

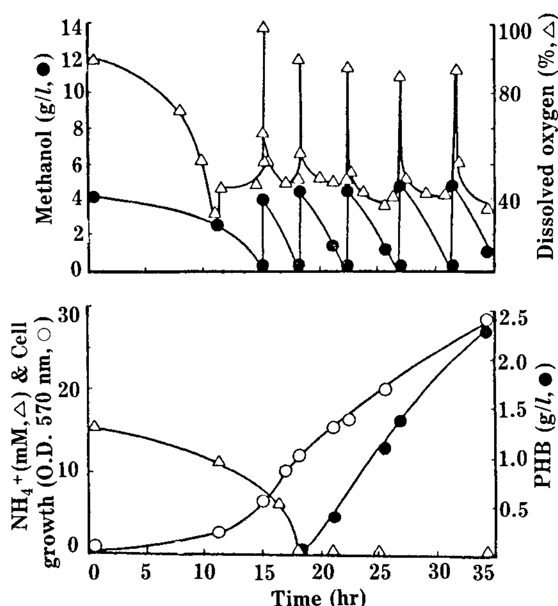

Fig. 4. Time course of cell growth and PHB production in intermittent feeding fed batch culture.

were significantly decreased when only organic sources were used. Thus, the ammonium sulfate was selected as a nitrogen source for the further experiments.

The major functions of metal ions are maintaining an osmotic balance and providing a cationic environment. For the maintenance of the active physiological state of cells, it was known that twentyseven elements are required at least (18). In order to determine the requirements of metal ions, the culture medium 5 was used with deficiency of a specific metal ion. The deficiency of metal ions affected the specific growth rate and the data were summarized in Table 3. The deficiency of Fe^{+2} and Mn^{+2} significantly repressed the cell growth, whereas the deficiency of Co^{+2}, Cu^{+2}, and Ca^{+2} tended to repress slightly. From these results, the Fe^{+2} and Mn^{+2} were found to be essential for the cell growth. These results might agree with the fact that Fe^{+2} plays an important role in an electron transport system and acts as a cofactor of heme (18).

As a result, the composition of optimal growth medium for *M. organophilum* was determined as follows; Methanol, 0.5% (v/v): $(NH_4)_2SO_4$, 1.0g/*l*: Na_2HPO_4, 2.13g/*l*: KH_2PO_4, 1.305g/*l*: $MgSO_4 \cdot 7H_2O$, and trace elements ($CaCl_2 \cdot 2H_2O$, 3.3 mg: Fe $SO_4 \cdot 7H_2O$, 1.3 mg: $MnSO_4 \cdot 4H_2O$, 130 μg: $ZnSO_4 \cdot 4H_2O$, 130 μg: $CuSO_4 \cdot 5H_2O$, 40 μg: Na_2 $Mo_4O_4 \cdot 2H_2O$, 40 μg: $CoCl_2 \cdot 6H_2O$, 40 μg: H_3BO_3, 30 μg per liter).

Effect of methanol concentration

In general, methanol is a toxic substrate for bacteria and for methanol utilizing organisms even 1.0% of methanol could inhibit the growth of many strains (15, 19). The effect of methanol concentration on the cell growth was shown in Fig. 3. The maximum specific growth rate was tended to drop as methanol concentration increased beyond 0.3% (v/v) and the cell growth was completely inhibited at the concentration above 4% (v/v) of methanol. For the optimal cell growth, it is necessary to keep the methanol concentration under 0.3% (v/v) during the culture.

Accumulation of PHB

To overcome the methanol toxicity and inhibition, intermittent feeding of methanol was conducted by D.O.-stat technique. When methanol in culture medium was completely used up and became a growth limiting factor, the dissolved oxygen tension in culture broth increased abruptly. This change of D.O. level was used as a control indicator of feeding. A typical data of the culture was shown in Fig. 4. In this experiment, pH and temperature were controlled to 5.7 and 37°C, respectively. Since these conditions were more favorable for the accumulation of PHB (data not shown). The PHB accumulation was

Table 4. Production of PHB under deficiency of each nutrient in the medium.

Deficient Nutrient	Cell Mass* (g/l)	Accumulated PHB (g/l)	PHB/Cell Mass (%)
NH_4^+	28.9	5.97	20.7
Mg^{+2}	27.4	10.95	40.0
PO_4^{-3}	10.3	3.50	34.0
K^+	12.2	7.10	58.2
SO_4^{-2}	15.3	4.28	28.0

*: Initial concentration of resuspended cells was adjust to 2g/l and the cultures were carried out for 20 hr.

significantly stimulated by limitation of nitrogen source and 2.3g/l of PHB was obtained after 35 hr and the PHB content reached about 20% of dry cell weight.

In order to improve the product yield of PHB, the experiments using the medium with deficiency of specific nutrients were carried out. For these experiments, two-stage culture technique was applied by the resuspension of the cells harvested from the first stage with growth medium. As shown in Table 4, the product yield of PHB on cell mass was significantly improved when the nutrients such as NH_4^+, Mg^{+2}, K^+, or PO_4^{-3} were deficient in the culture medium. The maximum PHB yield was achieved to 58% of dry cell weight by the deficiency of potassium ion.

요 약

Facultative methylotroph 인 *Methylobacterium organophilum* 을 유일한 탄소원으로 메탄올을 0.5% (v/v) 함유한 최소 합성배지를 이용하여 pH 6.8 과 30℃에서 배양하였다. 세포의 증식은 배지 내의 여러 성분들에 의해 영향을 받았으며 이로부터 최대 증식속도를 얻을 수 있는 최적 합성배지 조성을 확립하였다. 배지조성 중 질소원이 결핍되면 세포의 증식이 감소하며 Fe^{+2} 또는 Mn^{+2}이온의 결핍은 세포의 비증식속도를 감소시켰다. 탄소원인 메탄올은 농도를 증가시킬수록 세포의 비증식속도가 감소하는 메탄올의 기질 저해성을 확인하였으며, 4%(v/v) 이상의 메탄올 농도에서는 세포의 증식이 완전히 저해되었다. 이러한 메탄올의 기질 저해성은 간헐식 유가배양법을 이용하여 극복하고 PHB의 축적을 촉진하는 영양분 제한(nutrient-deficiency) 실험을 수행하였다. 합성배지의 영양분 중 NH_4^+, SO_4^{-2}, K^+ 또는 PO_4^{-3}이온을 결핍시켰을 때 *M. organophilum* 의 PHB 생산이 증가하였으며 칼륨이온을 결핍시켰을 때 세포의 건조 중량에 대한 PHB 함량은 최대 58%를 얻었다.

References

1. Dawes, E.A. and P. J. Senior: *Adv. Microbiol. Physiol.*, **10**, 203 (1973).
2. Rose, A.H. and D.W. Tempest: *Adv. Microbiol. Physiol.*, **10**, 136 (1973).
3. Braungg, G., B. Sonnleitner and R.M. Lafferty: *Eur. J. Appl. Microbiol. Biotechnol.*, **6**, 29 (1978).
4. Schegel, H.G., G.Gottschalk and R. Bartha: *Nature*, London, **191**, 463 (1961).
5. Taylor, I.J. and C. Anthony: *J. Gen. Microbiol.*, **95**, 134 (1976).
6. Lafferty, R.M. and E. Heinzle: *Chem. Rundsch.*, **30**, 14 (1977).
7. Holmes, P.A.: *Phys. Technol.*, **16**, 32 (1985).
8. Pawell, K.A., B.A. Collinson and K.R. Richardson: *Jap. Patent* 117 793 (1981).
9. Daniel. M., Ph. D Dissertation, Universite de Technologie de Compiegne, France (1987).
10. Maclennan, D.G., R.B. Vasey and N.T. Cotton: *J. Gen. Microbiol.*, **69**, 395 (1971).
11. Tsuchiya, Y., N. Nishio and S. Nagai: *Eur. J. Appl. Microbiol. Biotechnol.*, **9**, 121 (1980).
12. Weatherburn, M.W.: *Anal. Chem.*, **39**, 971 (1967).
13. Williamson, D.H. and J.F. Wilkinson: *J. Gen. Microbiol.*, **19**, 198 (1958).
14. Srikar, L. and R. Chandru: *J. Fd. Technol.*, **18**, 129 (1983).
15. Tou, C.T., A.I. Laskin and R.N. Patel: *Appl. Environ. Microbiol.*, **37**, 800 (1979).
16. Kole, M.M., B.G. Thompson and D.E. Gerson: *J. Ferment. Technol.*, **63**, 211 (1985).
17. Masuma, R., Y. Tanaka and S. Omura: *J. Ferment. Technol.*, **61**, 607 (1983).
18. Harrison, P.M. and R.J. Hoane: In "Metals in Biochemistry", Chepman & Hall, London & New York, 14-35 (1980).
19. Van Dijken, J.P. and W. Harder: *J. Gen. Microbiol.*, **84**, 409 (1974).

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