한국생물공학회지 제 10권 제 2호
Korean J. Biotechnol. Bioeng.
Vol. 10. No. 2. 170-175(1995)

*Methylobacterium organophilum*에 의한 메탄올로부터 메틸란의 생산에 대한 암모니아 이온의 영향

*오 덕 근 · *임 현 수 · †김 정 회
한국과학기술원 생물공학과, *우석대학교 식품공학과

Effect of Ammonium Ion on the Production of a Polysaccharide, Methylan from Methanol by *Methylobacterium organophilum*

Deok-Kun Oh*, Hyun-Soo Lim* and Jung-Hoe Kim†
Department of Biotechnology, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea
*Department of Food Science and Technology, Woosuk University, Chunju 565-800, Korea

ABSTRACT

The effect of nitrogen source on production of a high viscosity exopolysaccharide, methylan, from methanol by *Methylobacterium organophilum* was investigated in fed-batch culture. During the fermentation, cells continued to grow even after the nitrogen source added to the medium was depleted and methylan production was stimulated under the condition which ammonium ion was depleted. Cell growth increased proportionally to the initial concentration of ammonium ion in the medium, but methylan production was significantly inhibited at the high concentration of ammonium ion. As the initial concentration of ammonium ion increased, the specific growth rate, the specific product formation rate and the specific substrate consumption rate decreased due to the inhibitory effect of excess ammonium ions. In order to reduce the inhibitory effect by high concentration of ammonium ion. The control of ammonium ion concentration within the desired level(usually 0.45g/ℓ) was necessary. When ammonium ion concentration was maintained below 0.15g/ℓ by exponential feeding, methylan production could be increased up to 12.5g/ℓ.

INTRODUCTION

Biopolymers have recently attracted much attention as a research subject because of their interesting properties resulted from a wide variety of chemical structures. Microbial biopolymers including polysaccharides have been traditionally used in a wide range of applications such as a stabilizer, emulsifier, or thickener in foods, as an additive for smooth drilling of petroleum wells and recovery of petroleum by water flooding, as a selective absorbent, and as a rheology control agent.

Nitrogen is one of the major components of living organisms, and has also a key role in regulatory

† Corresponding Author

metabolisms. The regulatory effect of nitrogen on polysaccharide production is involved in nitrogen assimilation and catabolism of nitrogenous compounds. The earlier work suggested that the presence of ammonium ion inhibited specifically protein synthesis(1). In other systems such as antibiotic synthesis(2, 3) and lipid accumulation(4), both type and concentration of nitrogen source could determine the composition of intracellular pools(5) by affecting the activities of key enzyme systems involved in nitrogen assimilation. The presence of ammonium ion, as allosteric effector, could directly control carbon flow within the cells to either biomass or polysaccharide formation.

It was reported that nitrogen limitation was essential for biopolymer production, e.g. xanthan, pullulan and PHB(poly-β-hydroxybutyric acid), and high concentration of nitrogen source strongly inhibited biopolymer production while stimulating cell growth(6-8). In this study, the effect of ammonium ion on the production of a novel high viscosity exopolysaccharide, methylan which was recently found in our laboratory(9), was quantitavely investigated. Optimum range of ammonium ion concentration was determined and the substantial increase of methylan productivity was obtained by maintaining the ammonium ion concentration within the desired level.

METERIALS AND METHODS

Microorganism, Culture Medium and Culture Conditions

Methylobacterium organophilum NCIB K-1 was used in this study. Fermentation medium consisted of methanol, NH_4OH, 2.52g/ℓ KH_2PO_4, 4.24g/ℓ $Na_2HPO_4 \cdot 12H_2O$, 0.9g/ℓ $MgSO_4 \cdot 7H_2O$ and metal solution(which were fed with a ratio of 0.1 %(v.v) metal solution to 0.075g/ℓ NH_4^+). Metal solution contained 3.3mg/ℓ $CaCl_2 \cdot 2H_2O$, 1.3mg/ℓ $FeSO_4 \cdot 7H_2O$, 0.13mg/ℓ $MnSO_4 \cdot 4H_2O$, 0.13mg/ℓ $ZnSO_4 \cdot 4H_2O$, 0.04mg/ℓ $CuSO_4 \cdot 5H_2O$, 0.04mg/ℓ $Na_2MoO_4 \cdot 2H_2O$, 0.03mg/ℓ H_3BO_4.

The strain was cultivated in a 5-liter fermentor and the working volume was 3 liters. Temperature was kept at 30℃ and pH was controlled at 7.0 by adding 5N NaOH. With NBS Dissolved Oxygen(DO) probe(galvanic probe) and DO analyzer(Medel DO-50 USA), aeration rate and agitation speed were varied in the range of 0.5~1.5vvm and 400~1200rpm, respectively, to maintain the DO level above 20% of air saturation. Methanol was initially added at 1.0%(v/v) and then intermittently fed at 0.5%(v/v) by DO-stat technique in order to overcome an inhibitory effect by high concentration of methanol.

Analytical Methods

Ammonium ion concentration was determined by the indo-phenol method(7). Methylan concentration was measured by the following method. Fermentation broth was diluted to about 1g/ℓ of polysaccharide with distilled water and centrifuged at 10,000rpm for 30min. The supernatant was collected and two volumes of ethanol was added. The precipitated polysaccharide was collected by centrifugation at 10,000rpm for 15min and washed with ethanol. The washed polysaccharide was dried and weighed. Viscosity of fermentation broth was measured by a Haake Rotovisco Rheometer(Model RV 2) equipped with MV1 or NV sensor system. PHB content was determined by a gas chromatographic method(Model GC-8A, Shimadzu, Japan)(11) with benzoic acid as an internal standard. The column used was Chromosorb WHP(100-120 mesh) coated with 6 % SE-30. Cellular protein content was determined according to the modified Biuret method (12) after the solubilization with 2N NaOH at 100℃ for 10min. Nitrogenase activity was measured by the acetylene reduction assay(13). Other analytical methods were the same as described in the previous paper(9).

RESULTS AND DISCUSSON

Kinetics of Nitrogen Source Utilization During Methylan Fermentation

In order to understand the fermentation pattern of methylan with *M. organophilum*, time

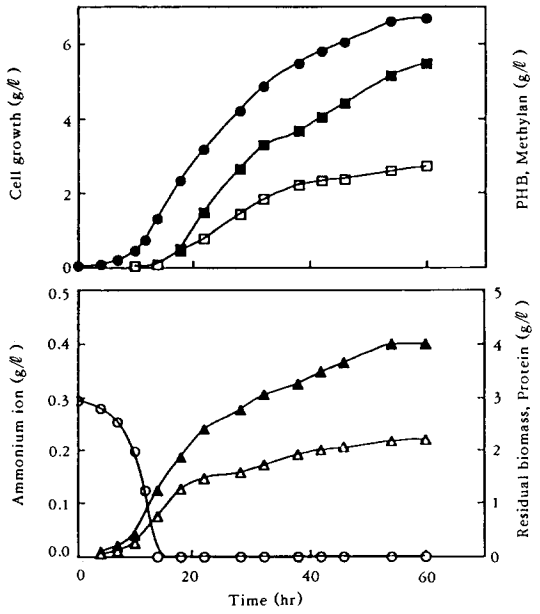

Fig. 1. Time courses of fermentation with *M. organophilum* when the initial concentration of ammonium ion was 0.3g/ℓ. Cell growth(●), PHB(□), methylan(■), residual biomass(▲), protein(△), ammonium ion(○).

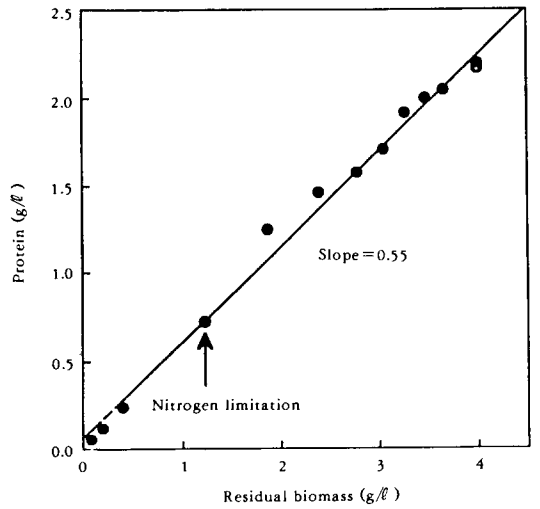

Fig. 2. Correlation between residual biomass and intracellular protein.

courses of fermentation were examined in fed-batch culture. As shown in Fig. 1, cells continued to grow even after the ammonium ion added to the medium was depleted and methylan production was stimulated when ammonium ion was depleted. Since cells contain intracellular PHB (about 30~40%), residual biomass was assumed to be the difference between the total dry cell weight and the content of PHB, and it could be considered as the physiologically active biomass including proteins and nucleic acids. The content of intracellular protein and residual biomass continued to increase even after the depletion of the initially supplied ammonium ion(0.3g/ℓ).

The increase of intracellular protein and residual biomass after the depletion of ammonium ion was shown more distinctly(Fig. 2). The straight line had a slope of intracellular protein content in residual biomass which remained almost constant(about 55%) even after the depletion of ammonium ion. It sugests that *M. organophilum* might utilize the gaseous nitrogen. Nitrogenase activity was not detected before the depletion of ammonium ion, but during the period of the depletion of ammonium ion, nitrogenase activity of the cells was found even though the activity was very low(about 14mmols C_2H_4/g-cell /hr) compared to other nitrogen fixing cells (about 500mmols C_2H_4/g-cell/hr)(14). Thus, it seemed that the cells utilized atmospheric nitrogen as a nitrogen source for the cell growth and cellular function once after the ammonium ion supplied was totally consumed.

Effect of Ammonium ion Concentration on Cell Growth and Methylan Production

In general, ammonium ion is a commonly used nitrogen source in fermentation processes because it can be easily assimilated by cells. However, there are some reports that cell growth is adversely affected by ammonium ion concentration(15, 16). To determine the effect of ammonium ion concentration on cell growth and methylan production, fed-batch cultures were carried out from 0.075 to 7.5g/ℓ (Fig. 3).

Final cell concentration was increased by increasing ammonium ion concentration. However, the maximal production of methylan was

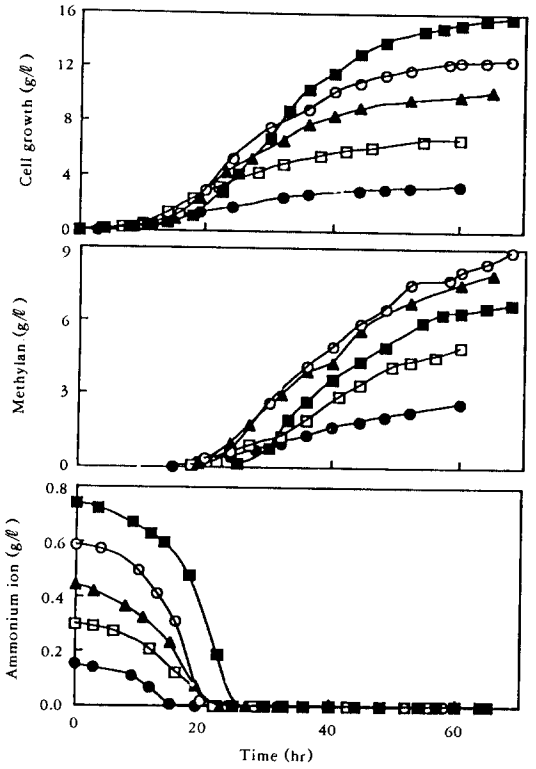

Fig. 3. Effect of the initial concentration of ammonium ion in fed-batch culture on cell growth and methylan production. Initial concentrations of ammonium ion were 0.15g/ℓ (●), 0.3g/ℓ (□), 0.45g/ℓ (▲), 0.6g/ℓ (○), 0.75g/ℓ (■).

achieved as 9.0g/ℓ in the culture with 0.6g/ℓ of ammonium ion concentration. The specific growth rate(μ), specific product formation rate(q_p) and specific substrate consumption rate(q_s) were calculated by polynomial regression from the fermentation data of Fig. 3 and summarized in Table 1. The maximal specific growth rate decreased slightly as ammonium ion concentration increased in the range of ammoniumion concentration tested. The effect on the maximal specific substrate consumption rate showed the similar trend to that on the maximal specific growth rate due to the almost constant yield factor for cell growth on methanol($Y_{x/s}=0.23$, $q_s^{max}=\mu^{max}/Y_{x/s}$). Inhibitory effects on the maximal specific production rate of methylan and methylan yield (methylan concentration/cell concentration, P/X) were not critical up to 0.45g/ℓ of ammonium ion concentration, but the adverse effect was observed to be significant above 0.45g/ℓ. Up to 0.45g/ℓ of ammonium ion concentration, the maximal specific production rate and methylan yield were determined to be 0.065g/g-hr and 0.77g/g, respectively. From the experimental results, it was concluded that the concentration of ammonium ion during fed-batch culture should be controlled at least below 0.45g/ℓ in order to prevent the cells from the inhibiton.

Methylan Production by Feeding of Ammonium Ion

Since the high concentration of ammonium ion in the medium inhibited both cell growth and methylan production, ammonium ion during the growth phase was maintained at low level in order to reduce the inhibitory effect. Two different cultures were compared; a culture starting with initial 0.75g/ℓ of ammonium ion concentration without any further supplement as control

Table 1. Effect of the initial concentration of ammonium ion on fermentation parameters. Fermentation time is 60hr.

NH_4^+ (g/ℓ)	μ^{max} (hr^{-1})	q_p^{max} (g/g-hr)	q_s^{max} (g/g-hr)	X (g/ℓ)	P (g/ℓ)	P/X (g/g)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)	K (mPa · s^n)
0.15	0.240	0.068	1.014	3.245	2.535	0.781	0.236	0.164	968
0.30	0.229	0.064	1.008	6.623	5.084	0.768	0.227	0.177	2896
0.45	0.219	0.065	0.973	9.846	7.526	0.764	0.217	0.193	7058
0.60	0.200	0.057	0.829	12.37	8.020	0.673	0.242	0.212	7309
0.75	0.1803	0.0310	0.6147	15.91	6.348	0.418	0.293	0.150	4909

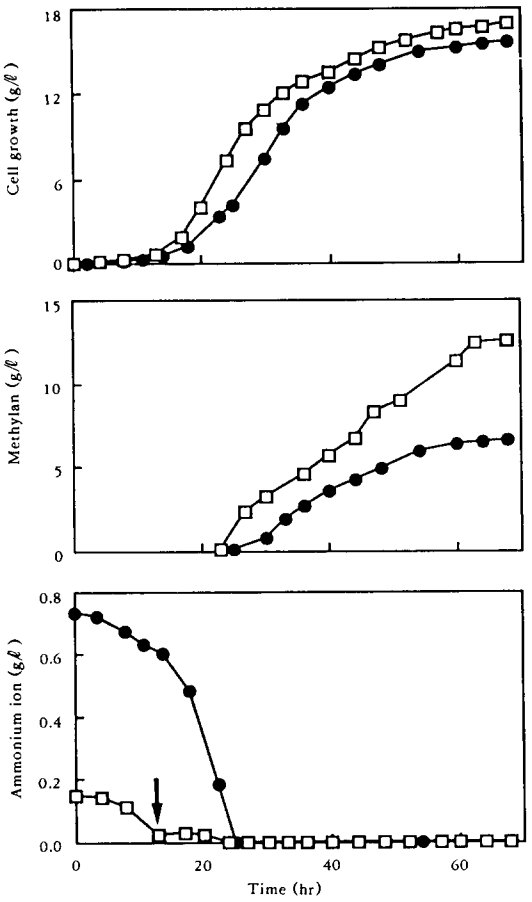

Fig. 4. Effect of the control of ammonium ion in fed-batch culture on cell growth and methylan production. Initial 0.75g/ℓ of ammonium concentration(●), and exponential feeding maintained within 0.15g/ℓ with total amount of 0.75g/ℓ (□). Arrow indicates the starting point of the exponential feeding.

and a culture starting with initial 0.15g/ℓ of ammonium ion and then residual 0.6g/ℓ of ammonium ion was fed by exponential feeding when ammonium ion(0.15g/ℓ) become limited(Fig. 4). Thus the total concentration of ammonium ion supplied was 0.75g/ℓ throughout the cultures. The exponential feeding of ammonium ion was performed by the feeding program of ammonium ion based on the kinetic data on ammonium ion consumption. When, ammonium ion concentration was maintained below 0.15g/ℓ during the culture, a slight increase in cell growth was observed and methylan production increased almost two fold compared with that obtained at the initial 0.75g/ℓ of ammonium ion concentration (from 6.6g/ℓ to 12.5g/ℓ of methylan). In the cultures maintaining ammonium ion concentration within 0.15g/ℓ, the maximal specific production rate and methylan yield were increased from 0.03g/g-hr to 0.065g/g-hr and from 0.43g/g to 0.73g/g, respectively.

This study attempted to investigate the kinetics of nitrogen source utilization, and effect of ammonium ion concentration on cell growth and methylan production. It was found that during the fermentation, cells continued to grow even after the nitrogen source added to the medium was depleted. Cell growth increased proportionally to the initial concentration of ammonium ion in the medium, but methylan production was inhibited significantly at the high concentration of ammonium ion. In order to improve productivity of the polysaccharide, ammonium ion concentration was maintained within the desired level(usually 0.15g/ℓ). By maintaining ammonium ion concentration below 0.15g/ℓ, 12.5g/ℓ of methylan production was achieved. This concentration corresponds to about 24,000mPa s^n of consistency index resulted from the pseudoplastic behavior of the solution due to its high molecular weight(2-4 $\times 10^6$ dalton). A development of the more effective bioreactor is needed to produce methylan over this concentration.

요 약

*Methylobacterium organophilum*을 이용하여 메탄올로부터 고점성 다당류의 생산에 대한 질소원 이용의 동력학적 분석을 유가식 배양에서 수행하였다. 발효중 세포는 배지 중에 첨가된 질소원이 고갈된 후에도 계속 성장하였고, 메틸란은 질소원 고갈조건 하에서만 생성되었다. 세포 성장은 배양초기에 첨가한 질소원인 암모니아의 농도에 비례적으로 이루어졌으나, 메틸란 생산은 첨가한 암모니아 농도가

0.75g/ℓ 이상에서는 오히려 현저하게 감소하였다. 초기 암모니아 농도가 높아질수록, 비세포증식 속도, 비산물생성 속도, 비기질소비 속도가 감소하였다. 높은 암모니아 농도의 발효 속도들에 대한 저해작용을 감소시키기 위해, 질소원을 일정농도(암모니아 농도 =0.15g/ℓ) 이하로 유지시키면서 공급하는 유가식 배양법으로 메틸란의 농도를 12.5g/ℓ 까지 증가시킬 수 있었다.

NOMENCLATURES

μ^{max} =maximal specific growth rate(hr^{-1})
q_p^{max} =maximal specific product formation rate (g/g-hr)
q_s^{max} =maximal specific substrate consumption rate(g/g-hr)
X =cell concentration(g/ℓ)
P =polysaccharide concentration(g/ℓ)
P/X =polysaccharide concentration/cell concentration(g/g)
$Y_{x/s}$ =cell growth/methanol consumed(g/g)
$Y_{p/s}$ =polysaccharide produced/methanol consumed(g/g)
K =consistency index($mPa \cdot s^n$)

ACKNOWLEDGEMENT

This work was supported by grant from the Ministry of Science and Technology.

REFERENCES

1. M. A. Bulmer, B. J. Catley and P. J. Kelley (1987), *Appl. Microbiol. Biotechnol.*, **25**, 362.
2. S. Inoue, Y. Nishizawa and S. Nagai(1983), *J. Ferment. Technol.*, **61**, 7.
3. R. Masuma, Y. Tanaka and S. Omura(1983), *J. Ferment. Technol.*, **61**, 607.
4. C. T. Evans and C. Ratledge(1984), *J. Gen. Microbiol.*, **130**, 1693.
5. J. G. O'Neill and J. F. Wilkinson(1977), *J. Gen. Microbiol.*, **100**, 407.
6. D. P. F. Auer and R. J. Seviour(1990), *Appl. Microbiol. Biotechnol.*, **32**, 637.
7. P. Souw and A. L. Demain(1979), *Appl. Envron. Microbiol.*, **37**, 1186.
8. T. Suzuki, T. Yamane and S. Shimizu(1986), *Appl. Microbiol. Biotechnol.*, **23**, 322.
9. J. H. Choi, D. K. Oh, J. H. Kim and J. M. Lebeault(1991), *Biotechnol. Lett.*, **13**, 417.
10. W. T. Bolleter, C. J. Bushman and P. W. Tidwell(1961), *Anal. Chem. 1961*, **33**, 592.
11. G. Braunegg, B. Sonnleitner and R. M. Lafferty(1978), *European J. Appl. Microbiol. Biotechnol.*, **6**, 29.
12. J. J. Sedmak and S. E. Grossberg(1977), *Anal. Biochem. 1977*, **79**, 544.
13. G. L. Turner and A. H. Gibson(1980), Measurement of nitrogen fixation by indirect means.(F. I. Bergerson ed.), 111, John Wiley & Sons, New York.
14. G. Daesch and L. E. Mortenson(1972), *J. Bacteriol.*, **110**, 103.
15. M. M. Kole, B. G. Thompson and D. F. Gerson(1985), *J. Ferment. Technol.*, **63**, 121.
16. R. Whittenbury, K. C. Phillips and J. F. Wilkinson(1970), *J. Gen. Microbiol.*, **61**, 205.