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Studies on the Production of Amino Acids by Methanol-Utilizing Bacteria

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Methanol 資化細菌에 依한 아미노酸의 生成에 關한 硏究

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ABSTRACT

About 600 strains of bacteria which can utilize methanol as the sole source of carbon were isolated from natural sources, and their ability to produce amino acids on the medium containing methanol as the sole source of carbon was tested by paper chromatography, by which 3 strains were screened by virtue of their relatively superior amino acid production. These were tentatively identified as follows; one strain belonged to the genus *Methylomonas*, while the remaining two were members of the genus *Hyphomicrobium*. Further studies on their growth characteristics were carried out with relation to amino acid production. According to these experiments, the following results were obtained.

1) No vitamin added was effective for the growth enhancement.

2) Optimum initial concentration of methanol was proved to be one per cent (v/v), and no conspicuous effect of feeding methanol on the growth of these bacteria could be observed, provided that the initial methanol concentration was controlled to 1 per cent.

3) Among the nitrogen sources tested, 0.1 per cent of $(NH_4)_2SO_4$ enabled the best growth.

4) Addition of 0.001 per cent $MgSO_4 \cdot 7H_2O$ largely increased the growth of all three strains. Other metal ions tested were either not effective or strongly inhibitory for the growth.

5) Addition of 0.1 per cent each of yeast extract, corn steep liquor, beef extract, and peptone also increased the growth markedly.

6) About 75μg/ml of alanine could be obtained finally after 64 hr culture of *Hypm*. A on the medium containing 0.1 per cent yeast extract and 1 percent methanol. Approximately 40μg/ml of glutamic acid by *Hypm*. GA and 43μg/ml of alanine by *Mtlm*. A were produced at the same culture conditions.

INTRODUCTION

The history of human interest on one-carbon compounds such as methane or methanol as a sole carbon source for microbial growth is not new at all,

although no much works had been done until quite recent days. After Söhngen's first isolation of *Bacillus methanicus* in 1906, only three other new species had been isolated until 1966, after which Whittenbury and others isolated over 100 strains of methanol-utilizers (Quayle, 1972). But even after that, the main interest was paid only on the physiological and taxonomical properties of these one carbon utilizers, although some attempts were also made to produce single cell protein by them. In 1973, Oki *et al.* stated that methanol has a potential application in fermentation production of amino acids, reporting 0.68g/dl of L-glutamic acid production by *Methanomonas methylovora* with methanol as the sole carbon source, during their attemps to search for low-cost carbon material for the fermentation production of amino acids. Since then, however, there has appeared no other report on the amino acid production by methanol-utilizers.

In the present work, screening of some methanol-utilizing amino acid producers was carried out, and their cultural characteristics for the growth and production of amino acids were investigated.

MATERIALS AND METHODS

Enrichment culture and isolation of methanol-utilizers.

Samples were collected from wide range of natural sources such as soil, sewage, animal feces, decayed woods and others. Test tubes (16.5mm) containing 5 ml basal medium were inoculated with the samples and incubated aerobically at 28°C for 2 to 3 days until the bacterial growth could be recognized. These turbid suspensions were streaked directly on agar plates of the same medium composition except for the addition of 1.5 per cent agar. Repeated streaking was carried out until pure isolation could be assured.

Stock cultures were maintained on the slants of the same medium composition. Throughout this experiment, all the cultures were done aerobically at 28°C, with 5ml medium in 16.5mm test tubes. Composition of the basal medium is as follows: $(NH_4)_2SO_4$, 3g; K_2HPO_4, 2g; NaCl, 1g; $MgSO_4 \cdot 7H_2O$, 0.2g; methanol, 10ml; thiamine hydrochloride, 0.1mg; riboflavin, 0.2mg; pyridoxine hydrochloride, 0.1mg; nicotinic acid, 0.2mg; Ca-pantothenate, 0.2mg; biotin, 0.01mg; para-aminobenzoic acid, 0.1mg; tap water, 1 liter; *p*H 7.0.

Paper chromatography of amino acids.

Isolated strains were again incubated for 3 days on the basal medium. Amino acids in the culture liquids were detected by the paper chromatographic methods. The solvent system was n-butanol:acetic acid:water (4 : 1 : 1, v/v). Amino acids were identified by cochromatographic technique. For the estimation of the amount of amino acids produced, 0.5 per cent (w/v) ninhydrin in acetone:water(7 : 3, v/v)was flooded, followed by the color development at 50°C for 30 min. The spots were eluted with 5 ml of 0.005 per cent $CuSO_4 \cdot 5H_2O$ in 75 per cent ethanol for 15—30 min, after which the color intensity was measured at 500 nm(Hitachi 101 spectrophotometer).

Measurement of the bacterial growth.

Growth of the bacteria was measured spectrophotometrically at 610 nm.

Vitamin requirements.

Requirement for each of the vitamins

contained in the basal medium was tested to check its effect on the growth of the bacteria. The same concentrations as indicated in the basal medium were used.

Optimum concentration and feeding effect of methanol.

The cultivation with different initial methanol concentrations such as 0.5, 1.0 and 2.0 per cent was tried, and various combinations of feeding concentrations were examined (Table 2).

Effect of nitrogen sources.

In place of the initial 0.02 per cent of $MgSO_4 \cdot 7H_2O$, 0, 0.001, 0.01, 0.02, 0.05, and 0.1 per cent each of $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4$—$6H_2O$, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $ZnSO_4 \cdot 7H_2O$ were added to the basal medium, and the growth was followed.

Effect of growth factors.

Yeast extract, corn steep liquor, beef extract, and peptone, 0.1 per cent each, were added to the basal medium and the growth was measured.

RESULTS AND DISCUSSION

Isolation of methanol-utilizing bacteria.

Some colonies appeared on the first agar plates failed to develop in the second or third streaking, indicating that the impurities from the enrichment culture step could be removed effectively in the repeated streaking, although suspensions from the enrichment culture were streaked directly to the agar plates. By this way, total of 623 strains were isolated finally which could utilize methanol as the sole carbon source. About half of them produced some amino acids more or less. After paper chromatographic examinations for the extracellular production of amino acids, 3 strains, all soil-born, were screened for further studies, by virtue of their relatively superior amino acid productivity.

Description of the isolates.

One strain was a gram-negative, pink colored straight rod measuring 0.8—1.0 $\times$2—3μ, and was actively motile in the liquid culture. Cells occurred singly and were grown aerobically. No endospore was found. Catalase reaction was positive. According to the Bergey's Manual of Determinative Bacteriology (8th ed., 1974), this organism was tentatively classified as a member of the genus *Methylomonas*, and was designated *Methylomonas* A.

The other two strains produced the typical prosthecae from one or both ends of the poles, upon the end of which daughter cell was produced whose size was not equal to that of the mother cell. Both were aerobic, and motile in the liquid culture. Cells were egg-shaped to long elipsoidal, measuring 0.5—1.0$\times$1.5—2.5μ. With these distinctive characters and their ability to grow on methanol as the sole source of carbon, they were thought to belong to the genus *Hyphomicrobium*. One was designated *Hyphomicrobium* A and the other, *Hyphomicrobium* GA.

All three strains grew best at 28°C within neutral *p*H range.

Vitamin requirements.

As shown in Table 1, there was almost no effect of any vitamin or vitamin mixture (7 kinds) on the growth of all three strains. Although addition of some vitamins such as 0.2mg/l of Ca-pantothenate increased the growth a little, the effect was thought to be negligible. No difference between the

Table 1. Effect of Vitamin on the Growth. (O.D. at 610nm)

Strain / Time(day) / Vitamin added (mg/l)	*Methylomonas* A				*Hyphomicrobium* A				*Hyphomicrobium* GA			
	0	1	2	3	0	1	2	3	0	1	2	3
none	0.125	0.315	0.750	1.23	0.152	0.640	0.95	2.35	0.135	0.620	2.35	2.80
thiamine-HCl (0.1)	0.115	0.265	0.540	1.05	0.198	0.670	1.00	2.28	0.142	0.600	2.30	2.55
riboflavin (0.2)	0.125	0.205	0.355	0.90	0.142	0.640	0.97	2.18	0.135	0.590	2.30	2.80
nicotinic acid (0.2)	0.140	0.285	0.475	1.23	0.180	0.630	0.90	2.23	0.115	0.515	2.05	2.55
Ca-pantothenate (0.2)	0.132	0.280	0.480	1.38	0.165	0.630	0.93	2.53	0.144	0.580	2.50	2.93
pyridoxine-HCl (0.2)	0.135	0.310	0.550	1.23	0.210	0.710	1.05	2.03	0.135	0.690	2.40	2.60
biotin (0.01)	0.173	0.320	0.660	1.25	0.175	0.710	1.10	2.20	0.115	0.640	2.55	2.95
paraaminobenzoic acid (0.1)	0.170	0.300	0.540	1.30	0.210	0.650	0.95	2.15	0.140	0.630	2.45	2.85
mixture*	0.162	0.255	0.570	1.18	0.195	0.610	0.95	2.35	0.125	0.595	2.40	2.80

* Mixture of all the vitamins above at the designated concentrations.

growth with and without vitamin mixture could be observed. By Kouno *et al.* (1973), *Methylomonas methylovora* M8-5 requires relatively high concentration of thiamine hydrochloride. But such a relation could not be found in the present experiment. On the other hand, Anthony and Zatman (1965) reported that none of the various vitamins and cofactors, including all 7 vitamins examined here, has any effect on the alcohol dehydrogenase activity of a methanol-utilizer, *Pseudomonas* sp. M27. Stock and McCleskey (1964) also indicated that no vitamin added was effective for increasing the colony size of *Methanomonas methanooxidans* growing on the agar plate.

Optimum concentration and feeding effect of methanol.

Growth of 3 strains by various combinations of methanol feeding is seen in Table 2. For *Methylomonas* A, 1 per cent of initial methanol concentration without any further feeding seemed to be the best for the maximum growth. When the initial concentration was lowered to 0.5 per cent, much poor growth was observed in 2 and 3 days after inoculation even if the total amount of methanol added was equivalent to 1 per cent or more. Feeding 0.5 per cent methanol on the second day in addition to the initial 1 per cent didn't make much difference compared to the one without any feeding. But if 0.5 per cent each of methanol was added on 1 and 2 days after inoculation, the growth became much lower. In such a way the growth decreased as the amount of feeded methanol increased. The most distinct result could be seen when the initial concentration was adjusted to 2 per cent and feeding 1 per cent methanol was continued from the next day after inoculation, indicating the inhibitory effect of high methanol concentration on the growth.

The tendency was almost the same in case of *Hyphomicrobium* GA and *Hypm.* A. The only difference was that the growth was not altered significantly even when up to 1.5 per cent more of methanol was added to the initial 1 per cent concentration. This is believed to be

Table 2. Effect of Feeding on the Growth. (O.D. at 610nm)

strain / time (day) / methanol feeding (%)			*Methylomonas* A			*Hyphomicrobium* A			*Hyphomicrobium* GA		
initial	1 day	2 day	0	2	3	0	2	3	0	2	3
0.5	0	0	0.130	0.130	0.142	0.162	0.140	0.115	0.165	0.130	0.025
0.5	0	0.5	0.110	0.135	0.225	0.210	0.145	0.515	0.086	0.170	0.385
0.5	0.5	0.5	0.125	0.255	0.490	0.170	0.470	0.680	0.098	0.375	0.870
1.0	0	0	0.122	0.700	1.200	0.142	0.940	2.150	0.120	2.200	2.630
1.0	0	0.5	0.105	0.495	0.660	0.178	0.930	2.130	0.108	2.150	2.500
1.0	0.5	0.5	0.122	0.560	0.670	0.140	0.940	2.050	0.120	2.050	2.400
1.0	0	1.0	0.135	0.530	0.650	0.170	0.920	1.900	0.118	1.950	2.100
1.0	0.5	1.0	0.105	0.495	0.620	0.140	0.920	2.130	0.155	2.000	2.200
1.0	1,0	1.0	0 130	0.460	0.510	0.165	0.810	1.900	0.125	1.900	2.170
2.0	1.0	1.0	0.127	0.225	0.270	0.155	0.860	1.700	0.108	1.850	2.190

the result of much heavier growth of these two strains compared to *Mtlm.* A. But even in these two strains, too high concentration of methanol was obviously harmful as in case of *Mtlm.* A. Oki *et al.* (1973) reported that they used total of 10 per cent methanol by repeated feeding for 8 times with the initial concentration of 2 per cent in their 48 hr culture of *Methanomonas methylovora* M12-4. Compared to the results of the present experiment, the concentration is surprisingly high. But regarding the fact that their bacterium shows much faster growth (more than 10 times as much) than the present ones, such a difference in methanol requirement is supposed to be easily explained. Peel and Quayle (1961) are reporting that the growth rate of *Pseudomonas* AM1 is reduced at methanol concentration greater than 1 per cent, and totally inhibited at 5 per cent. Chalfan and Mateles (1972), after their experiment on the combined effect of temperature and methanol concentration, reported that the doubling of pseudomonad C is much more lengthened at 40°C as the methanol concentration is increased from 0.5 per cent through 1 per cent to 2 per cent. But at 30 and 37°C, no considerable difference at these three concentrations was observed. Although the relation between the temperature and methanol concentration was not followed in the present experiment, it seems to be obvious that high initial concentration such as 2 per cent is quite harmful for the growth of all three strains.

Effect of nitrogen sources.

Among $(NH_4)_2SO_4$, $NaNO_3$, NH_4Cl, and NH_4NO_3, $(NH_4)_2SO_4$ enabled the maximum growth of all three strains. Moreover, among the three concentrations tested (0.05, 0.1, and 0.3 per cent), 0.1 per cent was proved to be most suitable. The results are shown in Table 3. Comparing the effect of ammonium ion and nitrate ion, it is clearly seen that ammonium ion is much superior to nitrate

Table 3. Effect of Nitrogen Sources on the Growth. (O.D. at 610nm)

Nitrogen Sources (%) \ Strain \ Time(day)		*Methylomonas* A				*Hyphomicribium* A				*Hyphomicrobium* GA			
		0	1	2	3	0	1	2	3	0	1	2	3
$(NH_4)_2SO_4$	0.3	0.225	0.330	0.590	1.200	0.230	0.570	1.05	2.13	0.225	0.680	1.45	2.60
	0.1	0.240	0.295	0.615	1.720	0.240	0.555	1.00	2.95	0.245	0.660	1.41	3.07
	0.05	0.225	0.227	0.395	0.930	0.225	0.500	0.95	2.35	0.230	0.620	1.35	3.02
$NaNO_3$	0.1	0.235	0.250	0.330	0.450	0.251	0.460	0.82	1.56	0.243	0.450	0.75	1.50
NH_4Cl	0.1	0.235	0.290	0.490	0.760	0.205	0.525	0.95	2.41	0.235	0.615	1.48	2.42
NH_4NO_3	0.1	0.230	0.283	0.510	1.330	0.257	0.525	0.92	2.78	0.240	0.595	1.36	2.57

ion for the growth.

Patel and Hoare (1971) and Feel and Quayle (1972) indicated that ammonium ion is essential for the activation of primary alcohol dehydrogenase of *Methylomonas capsulatus* (Texas strain) and *Pseudomonas* sp. M27. Anthony and Zatman (1964), and Johnson and Quayle (1964) also found that ammonium ion is necessary for the activation of methanol dehydrogenase in the extracts of *Pseudomonas* sp. M27 and *Pseudomonas* AM1, respectively, although Anthony and Zatman at later works (1967) hesitated to interpret the role of ammonia as an activator of alcohol dehydrogenase.

Anyway, the present result that ammonium ion is much superior to nitrate ion for the growth of these three methanol-utilizers matches quite well with all these observations. Stocks and McCleskey (1964) in their works with *Methanomonas methanooxidans*, a methane (and methanol)-utilizer, described that $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3, KNO_3, and some other amino acids were all satisfactory as the nitrogen source for this bacterium. But no quantitative data was available from their report.

Effect of metal ions.

Among the 5 metal ions tested, only $MgSO_4 \cdot 7H_2O$ showed the growth accelerating effect, and all others failed to show any positive effect(Table 4, 5, and 6). In case of $MgSO_4 \cdot 7H_2O$, the concentration of 0.001 per cent was proved to be the best. Almost all the others manifested some harmful effect, and naturally, the extent of growth inhibition was increased as the concentration became higher.

Table 4. Effect of Metal Ions on the Growth of *Methylomonas* A.

(O.D. at 610nm)

Metal ions(%)		Time (day)			
		0	1	2	3
none		0.145	0.147	0.187	0.210
$MgSO_4 \cdot 7H_2O$	0.1	—	0.090	0.115	0.252
	0.05	—	0.115	0.117	0.422
	0.02	0.110	0.185	0.272	0.520
	0.01	0.120	0.195	0.301	0.900
	0.001	0.150	0.260	0.375	1.230
$MnSO_4 \cdot 4\text{-}6H_2O$	0.1	—	—	—	—
	0.001	0.145	0.175	0.190	0.232
$FeSO_4 \cdot 7H_2O$	0.1	0.123	0.075	0.075	0.093
	0.001	0.133	0.195	0.212	0.255
$CuSO_4 \cdot 5H_2O$	0.1	0.100	0.100	0.100	0.055
	0.001	0.102	0.170	0.176	0.218
$ZnSO_4 \cdot 7H_2O$	0.1	—	—	—	—
	0.001	0.108	0.185	0.174	0.247

Table 5. Effect of Metal Ions on the Growth of *Hyphomicrobium* A.

(O.D. at 610nm)

Metal ions(%)		Time (day)			
		0	1	2	3
none		0.107	0.305	0.305	0.332
$MgSO_4 \cdot 7H_2O$	0.1	—	0.375	0.530	1.730
	0.05	—	0.375	0.610	2.250
	0.02	—	0.530	0.860	2.360
	0.01	0.086	0.550	0.870	2.650
	0.001	0.088	0.590	0.950	3.220
$MnSO_4 \cdot 4\text{-}6H_2O$	0.1	—	—	—	—
	0.001	0.090	0.342	0.335	0.372
$FeSO_4 \cdot 7H_2O$	0.1	0.120	0.150	0.168	0.230
	0.001	0.116	0.370	0.360	0.357
$CuSO_5 \cdot 5H_2O$	0.1	0.076	0.070	0.068	0.062
	0.001	0.095	0.360	0.362	0.390
$ZnSO_4 \cdot 7H_2O$	0.1	—	—	—	—
	0.001	0.085	0.130	0.270	0.298

Table 6. Fffect of Metal Ions on the Growth of *Hyphomicrobium* GA.

(O.D. at 610nm)

Metal ions(%)		Time (day)			
		0	1	2	3
none		0.111	0.580	0.810	0.800
$MgSO_4 \cdot 7H_2O$	0.1	—	0.595	0.970	3.100
	0.05	—	0.600	1.140	3.000
	0.02	0.103	0.760	1.500	3.300
	0.01	0.120	0.720	1.700	3.350
	0.001	0.135	0.820	1.800	4.370
$MnSO_4 \cdot 4\text{-}6H_2O$	0.1	—	—	—	—
	0.001	0.113	0.630	0.850	0.790
$FeSO_4 \cdot 7H_2O$	0.1	0.125	0.245	0.325	0.360
	0.001	0.137	0.490	0.560	0.485
$CuSO_4 \cdot 7H_2O$	0.1	0.095	0.100	0.092	0.085
	0.001	0.123	0.480	0.640	0.650
$ZnSO_4 \cdot 7H_2O$	0.1	—	—	—	—
	0.001	0.120	0.360	0.420	0.415

By Oki *et al.* (1973), *Methanomonas methylovora* M12-4 requires 5 ppm of ferrous ion as an optimal concentration for the growth and glutamic acid production. At the same time, the bacterium requires 0.05 per cent of $MgSO_4 \cdot 7H_2O$ for the maximal glutamic acid production. The growth also increased as the concentration of $MgSO_4 \cdot 7H_2O$ was raised from 0.01 to 0.2 per cent. Compared to the present result, such a value is quite a high one which can easily result in the growth inhibition of the present three strains.

Effect of organic growth factors.

All the organic growth factors added markedly inhanced the growth of three strains at 0.1 per cent concentration (Table 7). Because of the relatively high concentration of these substances, and because of the lack of further studies, it is difficult to say whether such a high result is due to their availability as a carbon or nitrogen source or not, regarding the peculiar pattern of nutrient requirement of the genera *Methylomonas* and *Hyphomicrobium*.

It is known that members of the genus *Methylomonas* do not require any organic growth factor. Some of them, furthermore, may be inhibited in growth by the presence of complex organic compounds. The genus *Hyphomicrobium* is famous for its oligocarbophical growth pattern (Buchanan and Gibbons, 1974). Regarding these facts, it is quite interesting that the present microorganisms all grew much better in the presence of high concentrations of complex organic compounds. Further studies will be necessary to solve this problem.

Production of amino acids.

Based on the results above, the production of amino acids by these three strains was measured. Vitamin mixtures

Table 7. Effect of Organic Growth Factors on the Growth. (O.D. at 610nm)

Strain	Growth factor (0.1%)	Time (Day)			
		0	1	2	3
Methylomonas A	none	0.24	0.33	0.59	1.20
	yeast extract	0.25	0.45	0.82	2.20
	corn steep liquor	0.27	0.52	1.05	3.10
	beef extract	0.27	0.50	1.13	2.90
	peptone	0.25	0.38	0.80	2.30
Hyphomicrobium A	none	0.24	0.61	1.07	2.15
	yeast extract	0.23	1.16	1.49	3.10
	corn steep liquor	0.24	1.25	1.45	2.80
	beef extract	0.24	1.23	1.50	3.35
	peptone	0.23	1.15	1.49	2.75
Hyphomicrobium GA	none	0.255	0.68	1.50	2.76
	yeast extract	0.22	0.70	1.70	4.10
	corn steep liquor	0.23	0.75	1.45	3.52
	beef extract	0.24	0.80	1.70	4.10
	peptone	0.23	0.78	1.70	3.80

were subtracted from the basal medium and the concentration of $MgSO_4 \cdot 7H_2O$ was adjusted to 0.001 per cent. Instead, organic growth factors were added and the results were compared to the ones obtained by the cultures without organic growth factors.

By *Hypm.* A, about 75μg/ml and 68μg/ml of alanine could be obtained after 3-day culture on the basal medium with and without 0.1 per cent of yeast extract, respectively. In case of *Mtlm.* A and *Hypm.* GA, the resultant values were much lower. 43μg/ml of alanine on the basal medium with 0.1 per cent of yeast extract was recorded by *Mtlm.* A. By *Hypm.* GA, 40μg/ml of glutamic acid was obtained at the same culture conditions as in case of *Mtlm.* A.

Although only one report on the production of amino acids by methanol-utilizers is known, that paper has already reported the production of 0.68g/dl of L-glutamic acid by *Methanomonas methylovora* M12-4. The present results are far beyond comparison to the result of that paper (Oki *et al.*, 1973). But some more improvements are still expected by the alteration of culture conditions and by some other methods like mutation, which remains to be done from now on.

摘 要

Methanol을 資化하여 아미노酸을 生成하는 菌株를 얻으려는 試圖의 結果 約 600餘種의 methanol資化菌을 選別할 수 있었으며, 이들중 methanol 資化에 依한 아미노酸 生成能이 比較的 優秀한 3菌株를 選擇하여 이들의 增殖 및 아미노酸 生成에 미치는 數種 營養源의 影響을 調査하였다. 이들은 暫定的으로 *Methylomonas A*, *Hyphomicrobium* A 및 *Hyphomicrobium* GA로 各其 命名되었다. 調査의 結果 이들은 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4 \cdot 7H_2O$와 各各 0.1%의 yeast extract, corn steep liquor, beef

extract, 및 peptone의 添加에 依하여 增殖이 크게 늘어났으며. 最適 methanol 濃度는 培養始作時 1%의 濃度였다. 生長을 위한 vitamin 要求性은 認定되지 않았고 methanol의 feeding에 의한 增殖效果도 나타나지 않았다. 뿐만 아니라 methanol, 金屬 ion 等은 比較的 낮은 濃度에서도 심한 阻害現狀을 나타냄을 觀察할 수 있었다. 培養最適 無機培地에 1%의 methanol과 0.1%의 yeast extract를 加한 條件下에서 64時間 培養한 結果, *Hyphomicrobium* A에 依하여 75μg/ml의 alanine, *Hyphomicrobium* GA에 依하여 40μg/ml의 glutamic acid, 그리고 *Methylomonas* A에 依하여 43μg/ml의 alanine을 各各 얻을 수 있었다.

REFERENCES

1. Anthony, C. and L.J. Zatman. 1964. *Biochem. J.* 92 : 614.
2. _____. 1965. *Biochem. J.* **96** : 808.
3. _____. 1967. *Biochem. J.* **104** : 953.
4. Buchanan, R.E. and N.E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. The williams and Wilkins Co., Baltimore.
5. Chalfan, Y. and R.I. Mateles. 1972. *Appl. Microbiol.* **23** : 135.
6. Johnson, P.A. and J.R. Quayle. 1964. *Biochem. J.* **93** : 281.
7. Kouno, K., T. Oki, H. Nomura, and A. Ozaki. 1973. *J. Gen. Appl. Microbiol.* **19** : 79.
8. Oki, T., A. Kitai, K. Kouno, and A. Ozaki. 1973. *J. Gen. Appl. Microbiol.* **19** : 79.
9. Patel, R.N. and D.S. Hoare. 1971. *J. Bacteriol.* **107** : 187.
10. Patel, R.N., H.R. Bose, W.J. Mandy, and D.S. Hoare. 1972. *J. Bacteriol.* **110** : 570.
11. Peel, D. and J.R. Quayle. 1961. *Biochem. J.* **81** : 465.
12. Quayle, J.R. 1972. *Advon. Microbiol. Physiol.* **7** : 119.
13. Stocks, P.K. and C.S. McCleskey. 1964. *J. Bacteriol.* **88** : 1071.