FORTIMICIN A PRODUCTION BY *MICROMONOSPORA OLIVOASTEROSPORA* IN A CHEMICALLY DEFINED MEDIUM

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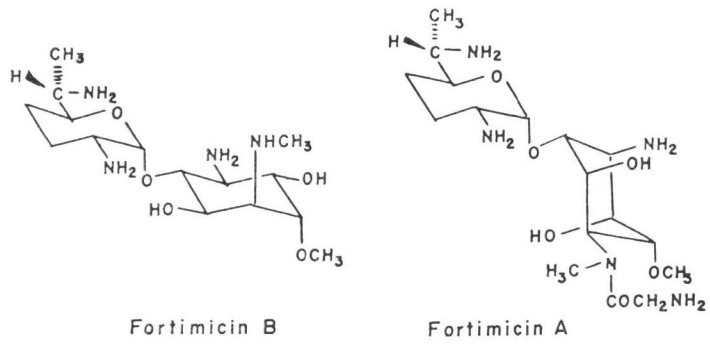
A chemically defined medium was devised in order to study the requirements for fortimicin A production by *Micromonospora olivoasterospora* KY 11515. Soluble starch was the best carbon source; NH_4Cl and NH_4NO_3 were suitable nitrogen sources both for the growth and fortimicin production. Amino acids such as L-arginine, L-asparagine, L-aspartic acid and L-glutamic acid showed some stimulatory effects on both growth and antibiotic production of *M. olivoasterospora* while L-serine stimulated only antibiotic production and L-citrulline only the growth. K_2HPO_4, $MgSO_4 \cdot 7H_2O$ and $CaCO_3$ were essential especially for the antibiotic production. The most important finding was that vitamin B_{12}, cobalt and nickel showed marked stimulatory effects on fortimicin A production.

Micromonospora olivoasterospora produced fortimicin A, fortimicin B and some other minor components in the fermentation broth[1~4]. Fortimicins A and B were isolated as purified materials[2] and the structural studies revealed that fortimicin A was a unique aminocyclitol-aminoglycoside antibiotic[5] with broad antibacterial activities[1,6]. Fortimicins A and B are pseudodisaccharides and incorporate a novel aminocyclitol fortamine, which is a 1,4-diamine, contains both N- and O-methyl groups and has an unusual stereochemistry as shown in Fig. 1.

Fig. 1. Structures of fortimicins A and B



Micromonospora have been reported to produce several antibiotics, but no report has appeared on antibiotic production in a chemically defined medium except for gentamicin production in a synthetic medium reported by TESTA *et al.*[7] Development of a chemically defined medium for *M. olivoasterospora* was investigated to determine the factors affecting the growth and/or production of fortimicins.

Materials and Methods

Producing Organism

The organism used in this investigation was a high productivity mutant, *Micromonospora olivoasterospora* KY 11515, induced from the parent *M. olivoasterospora* MK-70, ATCC 21819. The mutant had physiologically and morphologically similar characteristics to the parent strain. The culture was maintained in lyophile tubes and each fermentation grown at 30°C 2~3 weeks on an agar slant consisting of 0.4% yeast extract, 1.0% malt extract, 0.4% glucose and 2.0% agar, pH 7.2.

Fermentation

All fermentations were carried out in 300-ml Erlenmeyer flasks containing 30-ml of fermentation medium. Flasks were shaken at 30°C on a rotary shaker rotating within a 5-cm circle at 190 rpm, usually for 4~6 days. Flasks were sampled daily for assay. If fermentation time is not indicated in a table the data show maximum antibiotic potencies during that experiment. For the experiments on carbon sources and inorganic nitrogen sources, three times washed inoculum from a complex organic medium was transferred to the fermentation medium. The complex organic medium consisted of 1% soluble starch, 1% glucose, 0.1% yeast extract, 0.5% peptone and 0.1% $CaCO_3$. For all other experiments, three times washed inoculum from slants was transferred to the 50 ml of seed medium in a 300-ml Erlenmeyer flask, which was cultivated at 30°C for 4 days on a rotary shaker. The seed medium consisted of 2% soluble starch, 0.5% glucose, 0.5% NH_4Cl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% $CaCO_3$. Contents from several seed flasks were then mixed. Ten percent inoculum was transferred to the fermentation medium. All media used in this investigation were adjusted to pH 7.2 with HCl or NaOH and then sterilized by autoclaving at 120°C for 15 minutes.

Determination of Growth

The method reported by WAGMAN *et al.*[8] was used.

Preparation of Samples for Antibiotic Assay

Four tenths ml of 6 N HCl was added to 10 ml of fermented broth, which was then stirred thoroughly to release fortimicins bound to the mycelia and finally centrifuged. The supernatant was adjusted to pH 8 with 6 N NH_4OH or diluted by 20 times with 0.1 M Tris-HCl buffer, pH 8, for bioassay. For the chemical assay, the intact acidified supernatant was used.

Chemical Assay of Fortimicins

For chemical assay of each component of fortimicins, a modified method of BENJAMIN *et al.*[9] was used. Samples were applied to silica-gel plates which were then developed with lower phase of chloroform - methanol - ammonia (1 : 1 : 1). The plate was then developed in a solution of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD chloride, E. Merck & Co.) in acetone (2 mg/ml). The plate was heated at 120°C for 10 minutes, and then rechromatographed in acetone, repeatedly. Fluorescent spots were measured by a fluorometer (Shimazu CS-900) and the fortimicins were calculated by a standard curve. Details of the chemical assay of fortimicins will be reported elsewhere.

Bioassay for Total Antibiotic Potency

An agar diffusion assay using *Bacillus subtilis* KY 4273 was used. Potency was expressed in μg/ml of a standard fortimicin A sulfate of 537 μg/mg.

Results

Effects of Inorganic Nitrogen Sources

Effects of several kinds of inorganic nitrogen compounds on the growth and antibiotic production were studied in a basal fermentation medium containing 2% soluble starch, 0.5% glucose, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% $CaCO_3$ (Table 1). NH_4Cl, NH_4NO_3 and $(NH_4)_2SO_4$ increased growth slightly more than KNO_3 and $NaNO_3$. This organism could not utilize $NaNO_2$. On the other hand, ammonium ion increased antibiotic production more significantly than nitrate or nitrite. NH_4Cl

Table 1. Effects of inorganic nitrogen sources

Inorganic nitrogen source (M)		4 days			7 days		
		pH	Growth (mg/ml)	Antibiotics (μg/ml)	pH	Growth (mg/ml)	Antibiotics (μg/ml)
NH_4Cl	0.1	5.5	4.25	4	4.4	3.40	5
	0.05	5.4	3.39	4	3.3	2.57	4
$(NH_4)_2SO_4$	0.1	5.8	5.09	3	4.5	4.26	<1
	0.05	5.6	4.24	4	4.6	3.38	3
NH_4NO_3	0.1	5.5	3.41	4	4.7	3.37	4
	0.05	5.4	3.38	5	4.8	3.40	6
KNO_3	0.1	7.3	3.29	<1	8.5	2.57	<1
	0.05	7.5	2.54	<1	8.4	2.53	<1
$NaNO_3$	0.1	7.3	2.49	<1	8.3	4.28	<1
	0.05	7.6	2.60	<1	8.4	2.55	<1
$NaNO_2$	0.1	6.8	0.00	<1	7.0	0.00	<1
	0.05	6.8	0.00	<1	7.0	0.00	<1
none		6.7	0.00	<1	7.0	0.00	<1

Basal fermentation medium: soluble starch 2%, glucose 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.1%.

Table 2. Effects of carbon sources

Carbon source	pH	Growth (mg/ml)	Antibiotics (μg/ml)	Carbon source	pH	Growth (mg/ml)	Antibiotics (μg/ml)
Soluble starch	4.9	3.38	3	D-Mannose	6.0	tr	<1
D-Raffinose	6.8	tr*	<1	D-Xylose	6.0	tr	<1
Maltose	5.7	3.25	2	D-Arabinose	6.1	tr	<1
Sucrose	6.4	0.85	<1	Glycerine	6.8	tr	<1
Lactose	6.5	tr	<1	Sorbitol	6.9	tr	<1
D-Galactose	6.3	tr	<1	Mannitol	6.9	tr	<1
Glucose	5.3	1.75	<1	Inositol	6.9	tr	<1
L-Rhamnose	6.4	tr	<1	None	6.8	tr	<1
D-Fructose	6.2	tr	<1				

Basal fermentation medium: NH_4Cl 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.1%. *tr: trace
Each carbon source was added 4% in a basal medium.

and NH_4NO_3 were slightly better for increasing fortimicin production than $(NH_4)_2SO_4$.

Effects of Carbon Sources

The effects of carbohydrates on the growth and antibiotic production shown in Table 2 were studied in a fermentation medium containing 4% carbohydrate, 0.5% NH_4Cl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.1% $CaCO_3$. Soluble starch, maltose, sucrose and glucose showed stimulatory effects on the growth. Other carbohydrates were not utilized by the organism. Organic acids such as sodium citrate, sodium fumarate and sodium acetate also failed to support growth. Among the four carbohydrates which stimulated the growth, only soluble starch and maltose produced antibiotic activity in the broth. When 0.5% of L-arginine was added to the fermentation medium, the effect of soluble starch was distinguished more clearly from the other carbohydrates by both growth and antibiotic production.

Effects of Amino Acids

The effects of more than 20 amino acids were tested in a basal fermentation medium containing

4% soluble starch, 0.5% NH_4Cl, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% NaCl and 0.1% $CaCO_3$. Among them, L-arginine, L-asparagin, L-aspartic acid and L-glutamic acid showed stimulatory effects both on the growth and antibiotic production. L-Citrulline stimulated only growth while L-serine increased only fortimicin production. The data on L-arginine, L-serine, and L-asparagine are shown in Table 3. Many combinations of these amino acids were tested but no further improvement was found.

Table 3. Effects of amino acids

Amino acid	pH	Growth (mg/ml)	Antibiotics (μg/ml)
0.5% L-Arginine	5.1	9.40	34
0.2% L-Serine	5.3	4.23	30
1.0% L-Asparagine	6.9	9.33	32
None	5.1	4.22	24

Basal fermentation medium: soluble starch 4%, NH_4Cl 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.1%, $CaCO_3$ 0.1%

Fig. 2. Combination effects of mannitol with soluble starch
Basal fermentation medium: soluble starch 4%, NH_4Cl 0.5%, L-arginine 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.1%
Fortimicins were assayed by bioassay.

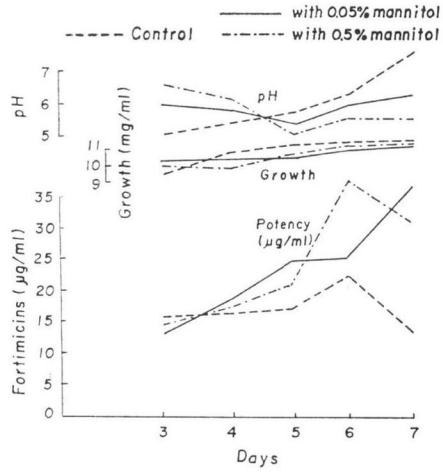
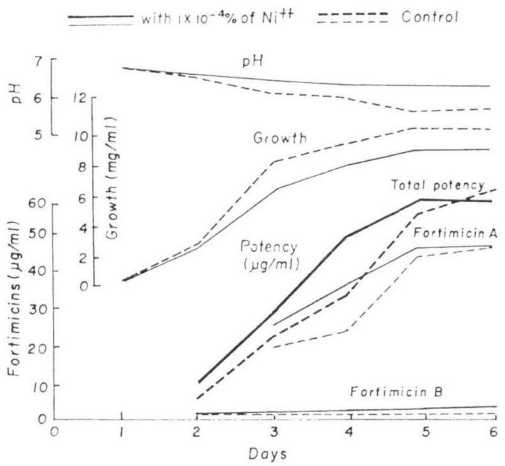


Fig. 3. Effects of Ni^{++} in the presence of Co^{++} on fortimicin production
Basal fermentation medium: soluble starch 4%, mannitol 0.05%, NH_4Cl 0.5%, L-arginine 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.1%, $CaCO_3$ 0.1%, Co^{++} 1×10^{-4}%
Fortimicins A and B were assayed by chemical assay. Co^{++} and Ni^{++} were added as $CoCl_2 \cdot 6H_2O$ and $NiCl_2 \cdot 6H_2O$, respectively.



Combination Effects of Sugars with Soluble Starch

Maltose, sucrose, mannose, xylose, sorbitol, mannitol and glucose were added to a fermentation medium containing 4% soluble starch, 0.5% NH_4Cl, 0.5% L-arginine, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.1% $CaCO_3$ and their combination effects with soluble starch were examined.

None showed any effects on the growth, while mannitol and mannose showed almost the same effect on antibiotic production.

The combination effects of mannitol with soluble starch are shown in Fig. 2. The growth levels in the three conditions were the same. Whereas the broth pH of the control condition rose above pH 7, the pH of the 0.05% or 0.5% mannitol-containing media remained below pH 6 at the 7th day. In the control medium, the antibiotic yield increased more slowly and decreased suddenly at the 7th day. In the mannitol-containing media, fortimicin increased more rapidly to a 75% higher maximum and

Table 4. Effects of inorganic salts

K_2HPO_4 (%)	pH	Growth (mg/ml)	Antibiotics (μg/ml)
0.0	7.8	5.96	<10
0.05	5.4	11.0	22
0.1	5.3	10.2	22
0.2	5.1	11.1	23
0.4	5.1	11.9	21
1.0	7.0	8.6	<10

$MgSO_4 \cdot 7H_2O$ (%)	pH	Growth (mg/ml)	Antibiotics (μg/ml)
0.0	7.6	7.64	<10
0.05	6.0	8.51	24
0.1	5.9	8.52	32
0.2	6.0	11.3	20
0.4	7.1	12.8	<10
1.0	7.1	11.2	<10

$CaCO_3$ (%)	pH	Growth (mg/ml)	Antibiotics (μg/ml)
0.0	5.2	5.94	<10
0.05	5.6	5.95	20
0.1	5.1	13.6	25
0.2	6.9	15.2	19
0.4	7.2	12.7	15
1.0	7.4	12.7	<10

NaCl (%)	pH	Growth (mg/ml)	Antibiotics (μg/ml)
0.0	5.2	11.0	24
0.05	5.1	11.1	27
0.1	5.1	11.9	28
0.2	5.4	11.0	27
0.4	5.4	11.9	24
1.0	7.3	11.0	11

Basal fermentation medium: soluble starch 4%, mannitol 0.05%, NH_4Cl 0.5%, L-arginine 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.1%, $CaCO_3$ 0.1%.
Antibiotics were assayed by the agar diffusion assay using *B. subtilis* KY 4273.

Table 5. Effects of trace metal mixtures

Trace metal mixtures[1]	Concentration[2] (%)	pH	Growth (mg/ml)	Fortimicins (μg/ml)
none		4.9	17	<20
Fe^{++}, Fe^{+++}, Mo^{++}, Co^{++}, Ni^{++}	1×10^{-7}	5.0	8	38
	1×10^{-6}	5.0	16	58
	1×10^{-5}	5.0	15	62
	1×10^{-4}	5.1	12	90
	1×10^{-3}	6.5	3	<20
Mn^{++}, Cr^{+++}, Cu^{++}, Zn^{++}	1×10^{-7}	4.9	15	<20
	1×10^{-6}	5.3	15	29
	1×10^{-5}	6.6	20	<20
	1×10^{-4}	5.3	9	<20
	1×10^{-3}	5.1	3	<20

1) Each ions was added as the following salts
Fe^{++}: $FeSO_4 \cdot 7H_2O$ Co^{++}: $CoCl_2 \cdot 6H_2O$ Cr^{+++}: $Cr_2(SO_4)_3 \cdot nH_2O$
Fe^{+++}: $Fe_2(SO_4)_3 \cdot nH_2O$ Ni^{++}: $NiCl_2 \cdot 6H_2O$ Cu^{++}: $CuSO_4 \cdot 5H_2O$
Mo^{++}: $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ Mn^{++}: $MnSO_4 \cdot 4H_2O$ Zn^{++}: $ZnSO_4 \cdot 7H_2O$
2) Final concentration of each ions in a medium

did not decrease as suddenly as in the control medium.

Effects of Inorganic Salts

The effects of such components as K_2HPO_4, $MgSO_4 \cdot 7H_2O$, NaCl and $CaCO_3$ were also examined (Table 4). All of them showed stimulatory effects both on the growth and antibiotic production at an optimum concentration of around 0.1%. Though the effect of NaCl on the growth and maximum anti-

Table 6. Single addition of trace metals

Trace metals	Concentration (%)	pH	Growth (mg/ml)	Fortimicins (μg/ml)
none		4.5	16	<20
Fe^{++}	1×10^{-5}	6.9	11	<20
	1×10^{-4}	4.7	13	<20
	1×10^{-3}	5.9	15	<20
Fe^{+++}	1×10^{-5}	5.3	11	24
	1×10^{-4}	5.4	10	23
	1×10^{-3}	5.4	11	24
Co^{++}	1×10^{-5}	5.1	10	62
	1×10^{-4}	5.5	10	86
	1×10^{-3}	6.0	6	26
Ni^{++}	1×10^{-5}	6.2	12	44
	1×10^{-4}	6.3	10	75
	1×10^{-3}	6.6	10	40
Mo^{++}	1×10^{-5}	5.3	11	30
	1×10^{-4}	5.3	9	30
	1×10^{-3}	5.4	13	24
Fe^{++} Fe^{+++} Mo^{++} Co^{++} Ni^{++}	1×10^{-4}	5.1	13	95

Table 7. Effects of vitamin mixtures

Vitamin mixtures[1)]	Concentration[2)] (%)	pH	Growth (mg/ml)	Fortimicins (μg/ml)
none		5.3	14	30
[A]	1×10^{-7}	7.6	4	<16
	1×10^{-6}	5.3	11	42
	1×10^{-5}	5.4	12	72
	1×10^{-4}	5.4	13	80
	1×10^{-3}	7.6	5	<16
[B]	1×10^{-7}	5.6	18	22
	1×10^{-6}	6.1	13	17
	1×10^{-5}	5.5	10	28
	1×10^{-4}	5.8	15	26
	1×10^{-3}	6.7	12	<16

1) [A] pyridoxine, pyridoxal, niacin, biotin, vitamin B_{12}
[B] Ca-pantothenate, riboflavin, thiamine, *p*-aminobenzoic acid, folic acid
2) Final concentrations of each vitamin in a medium

biotic production was not clear, it was effective in stabilizing the fermentation. With 0.1% of NaCl, the maximum antibiotic yield continued from the 4th to 6th day, while under other conditions it did not.

Effects of Trace Metals

Trace metals were tested in two groups: (1) Fe^{++}, Fe^{+++}, Mo^{++}, Co^{++} and Ni^{++}, and (2) Mn^{++}, Cr^{+++}, Cu^{++} and Zn^{++}. The mixtures were added to a fermentation medium containing 4% soluble starch, 0.05% mannitol, 0.5% NH_4Cl, 0.5% L-arginine, 0.05% K_2HPO_4, 0.1% $MgSO_4\cdot7H_2O$, 0.1% NaCl and 0.1% $CaCO_3$, at concentrations of 1×10^{-7}% to 1×10^{-3}% as each ion. As shown in Table 5, group 1 exhibited a significant effect on the antibiotic production at a concentration of 1×10^{-4}%,

Table 8. Effect of vitamin B_{12}

Vitamin B_{12} (%)	pH	Growth (mg/ml)	Antibiotics (μg/ml)		
			Total fortimicins	Fortimicin A	Fortimicin B
none	5.5	10	20	19	tr
1×10^{-5}	5.6	9	40	32	tr
2×10^{-5}	5.3	9	40	30	tr
5×10^{-5}	6.4	12	48	32	tr
1×10^{-4}	4.8	10	54	45	tr
2×10^{-4}	5.2	12	33	32	tr
5×10^{-4}	5.0	10	36	35	tr
1×10^{-3}	5.4	11	40	37	tr

Table 9. Simultaneous addition of vitamin B_{12} and Co^{++}

Co^{++} (%)	Vitamin B_{12} (%)	pH	Growth (mg/ml)	Antibiotics (μg/ml)		
				Total fortimicins	Fortimicin A	Fortimicin B
1×10^{-5}	—	5.1	9	60	48	tr
1×10^{-4}	—	5.4	8	74	69	tr
1×10^{-3}	—	7.7	7	30	25	tr
—	1×10^{-5}	5.6	9	40	31	tr
—	1×10^{-4}	5.0	9	57	46	tr
—	1×10^{-3}	5.3	10	39	35	tr
1×10^{-4}	1×10^{-5}	5.4	10	64	52	tr
1×10^{-4}	1×10^{-4}	5.0	10	70	68	tr
1×10^{-4}	1×10^{-3}	5.3	8	58	43	tr
—	—	5.5	10	20	16	tr

although the growth was slightly inhibited. The maximum total potency was 90 μg/ml as total fortimicins while the no addition control was 20 μg/ml. Group 2 showed no effect on antibiotic production.

Co^{++} and Ni^{++} were subsequently selected as effective metals by single omission and single addition tests (Table 6). In Tables 5 and 6, the increase in total antibiotic production were mainly due to the increase of fortimicin A, with only a slight effect on the production of fortimicin B.

The slight stimulatory effect of simultaneous addition of nickel with cobalt was also evident. As shown in Fig. 3, when 1×10^{-4}% of Ni^{++} was added to the cobalt-containing fermentation medium, the total potency and fortimicin A formation were higher than cobalt-containing control condition on the 4th day, but the effect of nickel disappeared finally. On the 6th day the total potency and fortimicin A in a nickel-containing condition were the same as those of the control; pH's of flasks containing nickel were also higher from 3 to 6 days. The production of fortimicin B was almost the same for the two conditions.

Effects of Vitamins

A mixture [A] of pyridoxine, pyridoxal, niacin, biotin and vitamin B_{12}, and another mixture [B] containing calcium-pantothenate, riboflavin, thiamine, *p*-aminobenzoic acid and folic acid were added to a basal fermentation medium containing 4% soluble starch, 0.05% mannitol, 0.5% NH_4Cl, 0.5% L-arginine, 0.05% K_2HPO_4, 0.1% $MgSO_4\cdot7H_2O$, 0.1% NaCl and 0.1% $CaCO_3$, at concentrations of 1×10^{-7}% to 1×10^{-3}% of each vitamin. The first mixture [A] gave two to three times higher total antibiotic potency than the control, while the second mixture [B] showed no effect on the potency (Table 7). By single omission and single addition tests in the first mixture [A], it was shown that vitamin B_{12} could be substituted for the mixture. As shown in Table 8, vitamin B_{12} was found effective for stimulating the total potency by bioassay which was mainly due to the increase of fortimicin A without any effect on the growth. Vitamin B_{12} had much less effect on the fortimicin B production. The effect of simultaneous addition of vitamin B_{12} with cobalt was almost the same as that with cobalt alone (Table 9).

Discussion

Among various kinds of inorganic nitrogen compounds, ammonium ion improved growth more

than nitrate. This organism was not able to utilize nitrite. The results shown in Table 1 agree with those reported for *Micromonospora purpurea* by WAGMAN *et al.*[8] with the exception of $(NH_4)_2SO_4$. They reported that *M. purpurea* could not utilize $(NH_4)_2SO_4$. *M. olivoasterospora* however could utilize the compound as well as NH_4NO_3 or NH_4Cl. Many other compounds which improve the growth of *M. olivoasterospora* agree with those of *M. purpurea*. K_2HPO_4, $MgSO_4$ and $CaCO_3$ showed some effect on the growth of *M. olivoasterospora* (Table 4). $NaHCO_3$ and Na_2CO_3 could be substituted for $CaCO_3$, although the growth in both instances was not so good as with $CaCO_3$. $CaCl_2$ resulted in no growth. The effective carbon sources of soluble starch, glucose, maltose and sucrose, and the effective amino acids of L-asparagine, L-citrulline, L-arginine and L-glutamic acid were the same as in the case of *M. purpurea*. Both organisms thus show almost the same nutritional requirements for growth, although some differences were noted.

The addition of Ni^{++} at the concentration of $1\times10^{-4}\%$ to the fermentation medium without cobalt showed better antibiotic production than the control (Table 6). Initially it was felt that the effect of nickel, might be due to cobalt contaminating the nickel, as in the case of coumermycin A.[10] The nickel chloride used in the experiments contained, however, only 0.049% of Co^{++}. The effect of nickel could be hardly explained by contamination by cobalt in the nickel chloride. $1\times10^{-4}\%$ of Ni^{++} added with $1\times10^{-4}\%$ of Co^{++} showed better effect on both total antibiotic production and fortimicin A production in the earlier fermentation phase (around 4 days, Fig. 3), although the effect on fortimicin B was far less than on fortimicin A.

Fortimicin A in phosphate buffer of alkaline pH is unstable, and degrades easily to fortimicin B at the temperature around 30°C, although degradation of fortimicin B is very small. Degradation increases as the pH rises, and is more rapid as the temperature increases. Therefore, it is very likely that accumulation of fortimicin B in the later phase of fermentation carried out at 30°C, is due to the chemical degradation of fortimicin A produced in the earlier phase of fermentation. Mannitol was found effective to keep pH of the broth lower than the control. The total potency of the broth with mannitol might be higher than the control then, by preventing the degradation of fortimicin A. Although glucose was more effective in keeping the pH of broth lower than mannitol and, of course than the control, it actually decreased the total antibiotic potency slightly.

Vitamin B_{12} also showed a stimulatory effect on antibiotic production (Table 8). The effect was however hidden thoroughly by cobalt added simultaneously with it (Table 9). Cobalt must contribute to formation of vitamin B_{12} by *M. olivoasterospora* as reported in other species of the genus *Micromonospora*[11]. A part of the effects of cobalt can be thus ascribed to that of vitamin B_{12}. As the effect of cobalt on antibiotic production is however greater than that of vitamin B_{12}, as shown in Table 9, the former may be involved in another mechanism for the antibiotic production besides formation of the latter.

TILLEY *et al.* reported the involvement of cobalt in methylation for gentamicin fermentation with a mutant of *M. purpurea*.[12]

Effect of L-methionine as a possibility was then examined. However, L-methionine could not be substituted for cobalt or vitamin B_{12}.

The bioassay value for total antibiotics was usually higher than the chemical assay value for fortimicin A, as the case of Fig. 3. As fortimicin B has almost no antibiotic activity against *B. subtilis*, the test organism, the difference between the two assay methods may suggest the existence of some other active components against *B. subtilis* in the broth besides fortimicins A and B.

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