Further Studies of Vitamin B₁₂ Production by Methanol Utilizing Bacterium, *Klebsiella* sp. No. 101[†]

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The accumulation of vitamin B₁₂ by *Klebsiella* sp. No. 101 grown on methanol was investigated furthermore. Addition of urea together with ammonium sulfate or ammonium nitrate was more effective than other nitrogen sources tested. Simultaneous addition of metal ions such as ferrous, calcium and manganese, and addition of amino acids such as alanine, valine, leucine, cysteine, phenylalanine, tyrosine and proline promoted the bacterial growth and vitamin B₁₂ production. Optimal pH for vitamin B₁₂ production was near 7.0. pH adjustment and methanol feeding were effective for the growth and the vitamin formation. Vitamin B₁₂ production was decreased by addition of CN-B₁₂ to the medium at the concentrations more than 100 µg per liter.

In our previous paper,¹⁾ the isolation of an excellent methanol-assimilating bacterium capable of producing vitamin B₁₂, the microbiological identification of the organism as *Klebsiella* sp. No. 101, and the effects of organic nutrients as well as B-vitamins on the bacterial growth and vitamin B₁₂ production were reported.

This paper deals with the further investigation on the effects of various culture conditions such as nitrogen sources, methanol feeding, metal ions, and pH adjustment on the growth of and vitamin B₁₂ production by *Klebsiella* sp. No. 101.

MATERIALS AND METHODS

Microorganism. The organism used in this study was *Klebsiella* sp. No. 101 isolated from soil by the authors. The organism was maintained on the methanol agar slant of the medium shown in Table I.

Cultivation method. As previously mentioned,¹⁾ a loop of the microorganism was inoculated into 100 ml of the basal medium in a 500 ml-shaking flask (see

Table I), and then cultured on a reciprocal shaker (115 rev./min) at 28°C for 6 days. Two ml of the broth was inoculated into 100 ml of the medium in a 500 ml-shaking flask and cultured on the shaker at 28°C.

TABLE I. COMPOSITION OF BASAL MEDIUM

(NH ₂) ₂ CO	1.5	g
(NH ₄) ₂ SO ₄	1.5	g
NH ₄ NO ₃	1.5	g
KH ₂ PO ₄	1.5	g
K ₂ HPO ₄	1.5	g
MgSO ₄ ·7H ₂ O	0.3	g
CaCl ₂ ·2H ₂ O	0.01	g
ZnSO ₄ ·7H ₂ O	0.01	g
FeSO ₄ ·7H ₂ O	0.01	g
MnSO ₄ ·7H ₂ O	0.001	g
Yeast extract	0.3	g
CoCl ₂ ·6H ₂ O	0.2	mg
Methanol	20	ml
Deionized water	1	liter
pH	7.0	

Analysis. The growth of the microorganism was determined by measuring optical density at 570 nm of the cultured broth diluted to one tenth or one twentieth. As shown in Fig. 1, a linear relation was obtained between the cell yield and the optical density.

Determination of vitamin B₁₂ activity in the whole culture broth or supernatant and cells was done using *Escherichia coli* 215, a vitamin B₁₂ auxotroph, as a test

[†] Outline of this paper was orally reported at the 26th Annual Meeting of the Society of Vitamin of Japan, in 1974. Studies on Methanol Metabolism. Part II. See Reference 1).

FIG. 1. Relationship between Dry Cell Weight and Cell Concentration.

●—●, O.D. at 570 nm of the cultured broth diluted to one tenth; ○—○, O.D. at 570 nm of the cultured broth diluted to one twentieth.

organism. Prior to the determination, the samples were extracted by adding KCN at the final concentration of 0.01%, acidifying to pH 6.0, and autoclaving at 15 lb for 15 min.

RESULTS AND DISCUSSION

The effects of various factors on the bacterial growth and vitamin B₁₂ production were examined.

1) Nitrogen compounds

The basal medium contains urea, ammonium sulfate and ammonium nitrate as nitrogen sources at the concentration of 1.5 g/liter, respectively. The effect of single or combined use of the 3 kinds of nitrogen compounds was examined at different levels. As shown in Table II, single dose of each compound was not very effective, but addition of urea together with ammonium sulfate or ammonium nitrate at the concentration of 1.5 g/liter resulted in the best growth and vitamin B₁₂ production. Under these conditions, the pH values of the culture broth ranged from 8.1 to 8.2. This finding suggested that pH of the broth as well as nitrogen compound utility was an important factor for the growth and vitamin B₁₂ formation.

2) Metal ions

Since the basal medium contains divalent metal ions such as calcium, zinc, ferrous and manganese, the effect of the 4 kinds of metal

TABLE II. EFFECT OF NITROGEN COMPOUNDS ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

N-Compound	Amt. added (g/liter)	pH of broth	Maxi- mal ^{a)} growth	Vita- min B ₁₂ μg/liter
None	—	7.0	0.15	16
(NH ₂) ₂ CO	1.5	8.3	0.20	28
(NH ₂) ₂ CO	4.5	8.5	0.03	—
(NH ₄) ₂ SO ₄	1.5	5.2	0.34	60
(NH ₄) ₂ SO ₄	4.5	5.3	0.35	62
NH ₄ NO ₃	1.5	5.2	0.34	53
NH ₄ NO ₃	4.5	5.5	0.37	51
(NH ₂) ₂ CO +	1.5	8.2	0.77	138
(NH ₄) ₂ SO ₄	1.5			
(NH ₄) ₂ SO ₄ +	1.5	5.4	0.34	55
NH ₄ NO ₃	1.5			
(NH ₂) ₂ CO +	1.5	8.2	0.75	131
NH ₄ NO ₃	1.5			
(NH ₂) ₂ CO +	1.5	8.1	0.74	128
(NH ₄) ₂ SO ₄ +	1.5			
NH ₄ NO ₃	1.5			
(NH ₂) ₂ CO +	2.5	8.7	0.13	17
(NH ₄) ₂ SO ₄	2.5			
(NH ₄) ₂ SO ₄ +	2.5	5.5	0.36	55
NH ₄ NO ₃	2.5			
(NH ₂) ₂ CO +	2.5	8.5	0.16	32
NH ₄ NO ₃	2.5			

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth. Cultivation was carried out on a reciprocal shaker for 6 days at 28°C.

ions was examined in further detail. The result obtained after 6 day culture is shown in Table III. Zinc ion inhibited the growth and vitamin B₁₂ production. Single addition of ferrous or calcium ion as well as simultaneous addition of the both ions was stimulatory for the growth and the vitamin production. Simultaneous addition of calcium, ferrous and manganese ions resulted in the best effects.

Cobalt ion is essential for the formation of vitamin B₁₂ considering from the structure of the vitamin. Therefore, the basal medium for vitamin B₁₂ production contains cobalt chloride at the concentration of 0.2 mg/liter. The effect of the concentration of cobalt ion for vitamin B₁₂ formation by *Klebsiella* sp. No. 101 was examined at the levels of 0 to 2.0 mg/liter. As shown in Table IV, the optimal concentration for vitamin B₁₂ formation was from 0.2 to 2.0 mg/liter. Without

TABLE III. EFFECT OF DIVALENT METAL IONS ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Metal ion	pH of broth	Growth ^{a)}	Vitamin B ₁₂ (μg/liter)
None	8.3	0.13	25
Mn ²⁺	8.4	0.17	27
Fe ²⁺	8.6	0.14	34
Zn ²⁺	7.3	0.06	10
Ca ²⁺	8.5	0.16	31
Fe ²⁺ + Mn ²⁺	8.3	0.23	43
Zn ²⁺ + Mn ²⁺	7.5	0.08	10
Zn ²⁺ + Fe ²⁺	8.3	0.14	26
Ca ²⁺ + Mn ²⁺	8.1	0.33	46
Ca ²⁺ + Fe ²⁺	8.0	0.44	114
Ca ²⁺ + Zn ²⁺	8.5	0.09	33
Zn ²⁺ + Fe ²⁺ + Mn ²⁺	8.2	0.28	40
Ca ²⁺ + Fe ²⁺ + Mn ²⁺	7.7	0.80	142
Ca ²⁺ + Zn ²⁺ + Mn ²⁺	8.6	0.14	20
Ca ²⁺ + Zn ²⁺ + Fe ²⁺	8.5	0.18	41
Ca ²⁺ + Zn ²⁺ + Fe ²⁺ + Mn ²⁺	7.8	0.75	122

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth. Cultivation was carried out on a reciprocal shaker for 6 days. Zn²⁺, Fe²⁺ and Mn²⁺ were added as the forms of sulfate hydride and Ca²⁺ was added as the form of chloride hydride. Fe²⁺, Zn²⁺ and Ca²⁺ were added at the concentration of 0.01 g/liter and Mn²⁺ was added at the concentration of 0.001 g/liter as the forms of sulfate hydride or chloride hydride.

TABLE IV. EFFECT OF CONCENTRATIONS OF COBALT ION ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Amount added (mg/liter)	pH of broth	Maximal growth ^{a)}	Vitamin B ₁₂ (μg/liter)
None	8.0	0.67	— ^{b)}
0.02	8.0	0.68	23
0.2	8.0	0.68	69
2.0	7.9	0.67	75

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth.

^{b)} Not detected.

Cobalt ion was added as the form of chloride hydride. Cultivation was carried out for 8 days at 28°C.

addition of cobalt ion, vitamin B₁₂ was not produced at all, but the bacterial growth was normal. The results seem to suggest that vitamin B₁₂ might not be required for methanol metabolism. However, cobalt deficient condition seems to be incomplete in the medium,

TABLE V. EFFECT OF CONCENTRATIONS OF MAGNESIUM ION ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Amount added(g/liter)	pH of broth	Maximal growth ^{a)}	Vitamin B ₁₂ (μg/liter)
None	7.9	0.35	45
0.03	8.2	0.59	70
0.1	8.2	0.68	64
0.3	8.0	0.67	74
1.0	7.7	0.72	86
3.0	6.8	0.80	78

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth.

Magnesium ion was added as the form of MgSO₄·7H₂O. Cultivation was carried out for 8 days at 28°C.

since trace amount of cobalt ion might possibly be present as a contaminant accompanied with the other metals used or from the sub-culture broth.

The effect of magnesium concentration was examined. As shown in Table V, the bacterial growth increased with magnesium concentrations, but vitamin B₁₂ production was not affected so much at the concentrations from 0.03 to 3.0 g/liter.

3) Aeration

Aeration is in general an important factor for microbial growth. In methanol fermentation, dispersion of methanol is also an important factor. The loss of methanol during fermentation must be considered, since Ogata *et al.*²⁾ reported 30% and 70% losses of methanol after 5 day incubation on a rotary shaker and on a reciprocal shaker, respectively. The effect of aeration on the growth and vitamin B₁₂ production was examined. As shown in Table VI, the growth and vitamin B₁₂ production in the cultivation by rotary shaking were much inferior to those by reciprocal shaking. In the latter, satisfactory results were obtained except for 50 ml and 300 ml-culture.

4) pH

Klebsiella sp. No. 101 grew well on the media of weakly acidic pH values and produced vitamin B₁₂, as seen in Table VII. When the

TABLE VI. EFFECT OF AERATION ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Cultivation was carried out until the bacterial growth reached maximum.

The revolutions per minute were 220 on the rotary shaker and 115 on the reciprocal shaker, respectively.

Shaking	Volume of medium (ml)	pH of broth	Growth ^{a)}	Vitamin B ₁₂ (μg/liter)
Rotary shaking	200	7.1	0.06	4.6
	100	7.1	0.05	4.3
Reciprocal shaking	300	7.1	0.08	6.8
	250	8.4	0.71	97
	200	7.9	0.58	85
	150	8.3	0.73	101
	100	8.3	0.73	94
	50	8.5	0.65	67

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth.

final pH of the culture broth reached higher than 8.7, both the bacterial growth and vitamin B₁₂ production were low. The finding led to attempt to improve the both by adjusting pH values during the fermentation. As shown in Table VIII, when the pH values were adjusted to the initial ones, satisfactory growth was observed also at the alkaline pH. Remarkable increase in the growth and vitamin B₁₂ production was observed at the neutral and slightly alkaline pH values after pH adjustment. The findings suggest that the optimal pH for the present purpose may be near 7.0.

5) Methanol feeding

It is supposed that methanol could diffuse by shaking during fermentation and a high concentration of methanol might inhibit the microbial growth. Investigation was made on the effect of additional methanol feeding at a rate of 2% (v/v) once at the appropriate time. As shown in Table IX, methanol feeding, especially that on the 4th day of cultivation, gave the best vitamin B₁₂ production. In addition, the effect of the amounts of methanol supplemented was also examined. Additional methanol feedings were carried out once on the 4th day, twice on the 4th and 5th day, and three times on the 4th,

TABLE VII. EFFECT OF INITIAL pH ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Initial pH	Final pH	Maximal growth ^{a)}	Vitamin B ₁₂ (μg/liter)
7.6	9.1	0.09	17
7.3	8.7	0.17	24
6.9	7.9	0.76	124
6.5	7.4	0.75	136
6.2	7.2	0.74	129
6.1	7.0	0.72	123

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth.

Cultivation was carried out reciprocal shaker for 8 days at 28°C.

TABLE VIII. EFFECT OF pH CONTROL ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Initial pH	Final ^{a)} pH	Maximal growth ^{b)}	Vitamin B ₁₂ (μg/liter)
6.0	5.7	0.91	128
6.5	6.6	0.93	125
7.0	7.1	0.93	182
7.5	7.8	0.83	168
6.7	7.5 ^{c)}	0.87	155

^{a)} pH was adjusted to initial pH at an interval of one day for 7 days with 10% HCl or 30% NaOH.

^{b)} O.D. at 570 nm of the cultured broth diluted to one tenth.

^{c)} pH was not adjusted to the initial pH.

Cultivation was carried out on a reciprocal shaker for 8 days at 28°C.

TABLE IX. EFFECT OF TIMES OF METHANOL FEEDING ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Day of methanol feeding ^{a)}	pH of broth	Maximal growth ^{b)}	Vitamin B ₁₂ (μg/liter)
No addition	7.2	0.51	143
0	5.1	0.68	157
1	5.4	0.70	168
2	5.0	0.73	177
3	5.4	0.71	193
4	5.1	0.74	228
5	5.1	0.75	205
6	4.9	0.75	207
7	4.8	0.78	190
8	4.7	0.75	166
9	5.0	0.72	166

^{a)} Methanol was fed at the concentration of 2% (v/v) to the growing culture once on the day indicated in the table.

^{b)} O.D. at 570 nm of the cultured broth diluted to one twentieth.

Cultivation was carried out for 10 days at 28°C.

5th and 6th day of cultivation, each time at 2% concentration. As shown in Table X, one time feeding resulted in the best vitamin B₁₂ production. Satisfactory result was obtained also in the other feedings. As shown in Table IX and Table X, methanol feeding lowered pH of the broth, while no feeding resulted in the rise of pH. The effects of methanol feedings and pH adjustment were examined in the same way as in Table X.

TABLE X. EFFECT OF THE AMOUNTS OF METHANOL FEEDING ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Amount of methanol		pH of broth	Maximal growth ^{a)}	Vitamin B ₁₂ (μg/liter)
Initial (ml)	Additional ^{b)} (ml)			
2	0	7.1	0.56	153
2	2	5.8	0.73	204
2	4	5.2	0.77	174
2	6	5.4	0.77	183

^{a)} O.D. at 570 nm of the cultured broth diluted to one twentieth.

^{b)} Additional methanols were fed to the growing culture once on the 4th day in 2 ml, twice on the 4th and 5th day in 4 ml and three times on the 4th, 5th and 6th day in 6 ml at a rate of 2 ml. Cultivation was carried out for 8 days at 28°C.

TABLE XI. EFFECTS OF pH CONTROLS AND METHANOL FEEDINGS ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Initial pH	Final ^{a)} pH	Amount of methanol		Maximal growth ^{b)}	Vitamin B ₁₂ (μg/liter)
		Initial (ml)	Additional ^{c)} (ml)		
6.6 ^{d)}	7.7 ^{e)}	2	0	0.56	141
7.0	7.0	2	2	0.98	223
7.0	7.0	2	4	1.00	199
7.0	7.0	2	6	0.95	184

^{a)} pH was adjusted to 7.0 during the growth with 10% HCl or 30% NaOH.

^{b)} O.D. at 570 nm of the cultured broth diluted to one twentieth.

^{c)} Methanol was fed to the growing culture by the same ways as shown in Table X.

^{d)} pH 6.6 was the best initial pH when pH was not adjusted during the growth.

^{e)} pH was not adjusted to 7.0 during the growth. Cultivation was carried out on a reciprocal shaker for 9 days at 28°C.

As shown in Table XI, the both treatments seem to improve the growth and vitamin B₁₂ production.

6) Amino acids

Since organic nutrients such as yeast extract and casamino acids exerted a stimulatory effect on the vitamin production as mentioned previously,¹⁾ the effect of some amino acids on the growth and vitamin B₁₂ production was studied in the place of yeast extract of the basal medium. In this study, each amino acid was added to the medium at the concentration of 50 mg/100 ml as a L-form. As shown in Table XII, alanine, valine, leucine, cysteine, cystine, phenylalanine, tyrosine and proline stimulated the growth and vitamin B₁₂ pro-

TABLE XII. EFFECT OF AMINO ACIDS ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Amino acid	pH of broth	Growth ^{a)}	Vitamin B ₁₂ (μg/liter)
None	8.6	0.11	6
Glycine	9.0	0.02	4
Alanine	8.1	0.65	114
Valine	7.9	0.68	90
Leucine	8.0	0.65	97
Isoleucine	7.3	0.03	4
Serine	8.2	0.15	11
Threonine	8.7	0.04	—
Cystine	7.1	0.75	97
Cysteine	7.0	0.80	126
Methionine	8.9	0.14	31
Glutamic acid	8.7	0.04	6
Aspartic acid	8.2	0.19	21
Lysine	8.6	0.11	8
Arginine	8.9	0.15	6
Histidine	8.4	0.19	10
Phenylalanine	8.0	0.49	63
Tyrosine	7.4	0.66	90
Tryptophan	8.6	0.13	14
Proline	8.3	0.61	102
Hydroxyproline	8.3	0.16	12
Ethionine	6.9	0.03	—
All added	8.5	0.08	12
Yeast extract ^{b)}	7.7	0.71	112

^{a)} O.D. at 570 nm of the cultured broth diluted to one tenth.

^{b)} Yeast extract was added at the concentration of 30 mg/100 ml.

All amino acids were added at the concentration of 50 mg/100 ml as a L-form.

duction. Cysteine was more effective than yeast extract.

7) DBI*

It is reported that addition of DBI, the base moiety of vitamin B₁₂, enhanced the vitamin production in carbohydrate fermentation by *Streptomyces*³⁾ and *Propionibacterium shermanii*⁴⁾ and in hydrocarbon fermentation by *Pseudomonas aeruginosa*.⁵⁾ The present authors examined the effect of DBI on the growth and vitamin B₁₂ production by *Klebsiella* sp. No. 101. DBI was added at the concentrations from 0 to 10 mg/100 ml. As shown in Table XIII, addition of DBI at the

TABLE XIII. EFFECT OF DBI^{a)} ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Amount added (mg/liter)	pH of broth	Maximal growth ^{b)}	Vitamin B ₁₂ (μg/liter)
0	7.4	0.78	135
1	7.5	0.76	143
10	7.8	0.70	139
50	8.0	0.65	141
100	6.5	0.15	11

a) DBI; 5,6-Dimethylbenzimidazole.

b) O.D. at 570 nm of the cultured broth diluted to one tenth.

Cultivation was carried out on a reciprocal shaker for 8 days at 28°C.

levels of 0.1 to 5 mg/100 ml did not promote the growth and the vitamin production. However, the addition at the level of 10 mg/100 ml rather inhibited strongly the growth and the vitamin production.

8) CN-B₁₂

Several microorganisms produced vitamin B₁₂ in the amounts of 200 to 300 μg/liter in methanol fermentation.^{1,5,6)} But the amount of vitamin B₁₂ produced was much lower than in the carbohydrate fermentations by *Prop. shermanii*,⁷⁾ *Str. Olivaceus*⁸⁾ and *Pseudomonas denitrificans*,⁹⁾ which produced the vitamin in the order of mg or more per liter.

* DBI; 5,6-Dimethylbenzimidazole.

Kamikubo *et al.*¹⁰⁾ reported that vitamin B₁₂ production by *Prop. shermanii* was depressed by the addition of CN-B₁₂ to the medium at the concentrations of 10 to 60 mg/liter. These results suggest that vitamin B₁₂ formation might be controlled by the extracellular vitamin B₁₂ supplement in methanol fermentation.

The present authors examined the effect of CN-B₁₂ on the growth and vitamin B₁₂ production. As shown in Table XIV, the

TABLE XIV. EFFECT OF CN-B₁₂ ON THE GROWTH AND VITAMIN B₁₂ PRODUCTION

Amt. of B ₁₂ added (μg/liter)	Maximal growth ^{a)}	Vitamin B ₁₂ in			
		cells (μg/liter)	supernatant (μg/liter)	total ^{b)} (μg/liter)	de novo ^{c)} (μg/liter)
0	0.80	139	—	139	139
1	0.79	146	—	146	145
5	0.77	128	—	128	125
12	0.85	139	1	140	128
114	0.79	126	60	186	72
519	0.87	141	456	597	78
993	1.00	146	928	1074	81

a) O.D. at 570 nm of the cultured broth diluted to one tenth.

b) Calculated as B₁₂ in cells plus supernatant.

c) Calculated as total B₁₂ minus amount of B₁₂ added.

Cultivation was carried out on a reciprocal shaker for 7 days at 28°C.

amount of vitamin B₁₂ in the cells was about 140 μg/liter and the values were almost constant regardless of addition of vitamin B₁₂. The amount of CN-B₁₂ incorporated into the cells was approximately 60 μg/liter. Thus, when CN-B₁₂ was added at the concentrations of 114, 519 and 993 μg/liter, the amounts of vitamin B₁₂ in the supernatant were 60, 456 and 928, respectively. Assuming that the amount of vitamin B₁₂ *de novo* synthesized corresponds to the difference of total vitamin B₁₂ minus added vitamin B₁₂, the *de novo* synthesis of the vitamin was depressed by the addition of CN-B₁₂ at the levels of 100 to 1000 μg/liter. The amount of vitamin B₁₂ repressing the *de novo* synthesis seems to be much smaller than that in *Prop. shermanii*.

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