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Vitamin B_{12} Production by a Methanol-Utilizing Bacterium

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Vitamin B_{12} production by a newly isolated strain of a methanol-utilizing bacterium was studied. The maximal yield of the vitamin, 2.6 mg/liter of medium, was attained by optimization.

A pink-pigmented bacterial strain capable of utilizing methanol as a sole source of carbon and energy was isolated from soil of the oil field in Niigata, Japan. This new isolate, strain FM-02T, was found to produce vitamin B_{12} significantly. It would be of much practical significance to produce this expensive and complicated vitamin from cheap and simple noncarbohydrate substrates, such as methanol, since vitamin B_{12} is at present produced exclusively by fermentation of carbohydrates using certain bacteria. Recently, formation of vitamin B_{12} by methanol-utilizing bacteria has been reported by us (6) and by Nishio et al. (3, 4). However, the vitamin B_{12} productivities reported by them were too poor for industrial application (less than 0.3 mg/liter). Therefore, we attempted to optimize vitamin B_{12} production using our newly isolated strain of a methanol-utilizing bacterium and obtained a maximal yield of 2.6 mg/liter of medium.

The composition of the basal medium used for the methanol utilizer, strain FM-02T, in batch culture experiments is identical to that described before (6): $NH_4H_2PO_4$, 2.0 g; KH_2PO_4, 2.0 g; $Na_2HPO_4 \cdot 12H_2O$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $MnSO_4 \cdot nH_2O$, 0.005 g; $CoSO_4 \cdot 7H_2O$, 0.001 g; and carbon source, 10 ml (liquid substrate) or the amount corresponding to 0.3 g-atom of carbon in 1 liter of tap water (pH 7.0 to 7.2). Cultivation was carried out aerobically at 30 C. Vitamin B_{12} compounds (consisting of adenosylcobalamin and methylcobalamin) in cultured broth were extracted as cyanocobalamin by boiling in 0.08 M acetate buffer (pH 5.5) containing 0.01% KCN, and the amount was determined microbiologically using *Escherichia coli* 215, a vitamin B_{12}-L-methionine auxotroph, as a test organism (2).

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Strain FM-02T is gram negative, pink pigmented, and rod shaped. The absorption spectrum of the main pigment fraction from the bacterium, which was extracted with absolute ethanol, was quite similar to that from *Pseudomonas* AM1 (5). Strain FM-02T was able to utilize 1,2-propanediol and lactate, as well as methanol, as sole carbon sources, indicating that this organism is a facultative methylotroph. Poor growth was observed on succinate, fumarate, malate, and tartrate. Glycerol, methylamine, and dimethylamine also permitted some growth after a long lag period. Among the carbon sources tested, the following compounds did not appreciably serve as growth substrates: formate, trimethylamine, ethanol, ethylene glycol, acetate, glycine, *n*-propanol, propionate, serine, glutamate, citrate, and glucose.

Effects of growth conditions on vitamin B_{12} productivity were then studied. When cultured on a rotary shaker (200 rpm) at 30 C, the growth reached maximum in 3 days in the basal medium (1% methanol [vol/vol]), whereas the amount of vitamin B_{12} produced reached maximum in 2 days (late exponential phase). Almost all of the vitamin was found to be included in the cells. Vitamin B_{12} productivity was dependent on Co^{2+} concentration, and 1 mg of $CoSO_4 \cdot 7H_2O$ per liter was used as the optimal concentration for usual experiments. Strain FM-02T grew most rapidly in the medium containing methanol at an initial concentration of 0.8 to 1.2% (Fig. 1). The growth was markedly inhibited by a higher concentration of methanol, and essentially no growth was observed on 4.8% methanol. It seems interesting that maximal production of the vitamin was obtained at 2.4%, where the bacterial growth was inhibited to some extent. Effects of addition of various nutrients on vitamin B_{12} productivity were examined (Table 1). Among the natural nutrients tested, Casamino Acids (Difco) enhanced vitamin B_{12} production significantly. L-Methio-

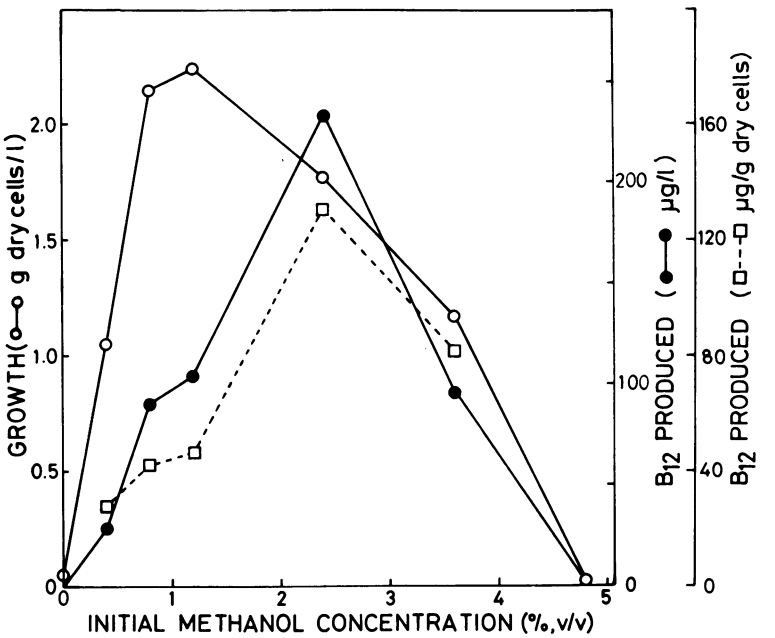

FIG. 1. *Effects of initial concentration of methanol on growth and vitamin B_{12} production. The bacterium was cultivated for 3 days with shaking.*

TABLE 1. *Effects of various nutrients on vitamin B_{12} production*

Expt	Nutrient[a]	Growth (g of dry cells/liter)	Vitamin B_{12} produced	
			μg/liter	μg/g of dry cells
1	None	2.4	110	46
	Casamino Acids	2.4	153	64
	Corn steep liquor	2.4	120	50
	Malt extract	2.4	116	48
	Meat extract	2.4	96	40
	Peptone	1.9	85	45
	Yeast extract	2.2	110	50
2	None	2.2	109	50
	Glycine	2.4	72	30
	L-Serine	2.3	109	47
	L-Aspartate	1.9	126	66
	L-Glutamate	1.9	80	42
	L-Methionine	1.9	140	74
	L-Threonine	0.5	14	28

[a] Natural nutrient, 0.1%; amino acid, 1 mM. The bacterium was cultivated for 3 days with shaking.

nine also stimulated the vitamin production. The following compounds did not affect the vitamin productivity: glycine and/or succinate (precursors of corrin ring); adenosine, adenine, and related compounds (precursors of adenosyl group of coenzyme B_{12}); betaine or choline (methyl donor); and various vitamins. Penicillin G and various surfactants also did not affect vitamin B_{12} production.

The yield of the vitamin was not as high even after optimization of the growth conditions in the shaking culture. Therefore, the cultivation method was improved to obtain the cells in much larger quantities. "Exponential-fed batch cultivation" was found to be significantly favorable for bacterial growth as well as vitamin B_{12} formation. In this cultivation method, the feed rate of methanol was increased exponentially in accord with the exponential growth of the microorganism by use of a rotating drum-type programmer, thus keeping the methanol concentration in the culture medium at a constant low level. (Details of this method will be published elsewhere by F. Yoshida and his co-workers). By using this method, about 20 g of dry cells and 0.4 mg of vitamin B_{12} were obtained per liter of the medium (Table 2). As far as bacterial growth is concerned, it would be preferable to keep the methanol concentration as low as possible. However, the higher methanol concentration was more suitable for vitamin B_{12} production under our experimental conditions. The yield of vitamin B_{12} further increased to about 2.6 mg/liter of medium by

TABLE 2. *Optimization of vitamin B_{12} production*

Cultivation method	Methanol concn (%, vol/vol)	L-Methionine (1 mM)	Casamino Acids (0.1%)	Cultivation time (h)	Growth (g of dry cells/liter)	Vitamin B_{12} produced	
						μg/liter	μg/g of dry cells
Batch[a]	1 (initial)	−	−	72	2.2	108	49
	1 (initial)	+	+	72	1.7	130	76
	2.4 (initial)	−	−	72	2.3	116	50
	2.4 (initial)	+	+	72	2.7	172	64
Exponential-fed batch[b]	0−0.05	−	−	26.0	19.8	400	20
	0−0.95	−	−	31.3	17.2	863	50
	0.40−1.38	+[c]	−	48.2	25.0	2,560	102

[a] The bacterium was cultivated in a 500-ml shaking flask containing 100 ml of the medium (initial pH, 7.0 to 7.2) at 30 C on a rotary shaker (200 rpm).

[b] The bacterium was cultivated in a 10-liter jar fermenter (working volume, 5 liters) at 30 C with agitation speed of 300 to 1,500 rpm and aeration rate of 2 or 10 liters/min.

[c] Methionine (3.3 mM) was added at a cell concentration of 10 g of dry cells per liter (at 25.8 h).

increasing the methanol concentration and adding L-methionine. Although the value is still lower than the highest values obtained by industrial-type microorganisms cultivated on carbohydrate media (23 mg/liter by *Propionibacterium shermanii;* 5.7 mg/liter by *Streptomyces* sp.) (7), the use of methanol for microbial production of vitamin B_{12} is promising and attractive from a practical point of view.

Paper electrophoretic behaviors of the B_{12} fraction from strain FM-02T demonstrated that the vitamin exists mainly in the forms of adenosylcobalamin (coenzyme B_{12}) and methylcobalamin. The coenzyme activity of the fraction in the coenzyme B_{12}-dependent diol dehydrase (D,L-1,2-propanediol hydro-lyase, EC 4.2.1.28) system (1) from *Aerobacter aerogenes* ATCC 8724 gave additional clear evidence for the presence of adenosylcobalamin in the cells of strain FM-02T (data not shown).

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