Vitamin B₁₂ Production by a Methanol-Utilizing Bacterium

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Vitamin B₁₂ 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 B₁₂ 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 B₁₂ is at present produced exclusively by fermentation of carbohydrates using certain bacteria. Recently, formation of vitamin B₁₂ by methanol-utilizing bacteria has been reported by us (6) and by Nishio et al. (3, 4). However, the vitamin B₁₂ productivities reported by them were too poor for industrial application (less than 0.3 mg/liter). Therefore, we attempted to optimize vitamin B₁₂ 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): NH₄H₂PO₄, 2.0 g; KH₂PO₄, 2.0 g; Na₂HPO₄·12H₂O, 3.0 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.01 g; FeSO₄·7H₂O, 0.005 g; MnSO₄·H₂O, 0.005 g; CoSO₄·7H₂O, 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 B₁₂ compounds (consisting of adenosylcobalamin and methylcobalamin) in cultured broth were extracted as cyanocobalamin by boiling in 0.06 M acetate buffer (pH 5.5) containing 0.01% KCN, and the amount was determined microbiologically using Escherichia coli 215, a vitamin B₁₂-L-methionine auxotroph, as a test organism (2).

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, methyamine, 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 B₁₂ 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 B₁₂ produced reached maximum in 2 days (late exponential phase). Almost all of the vitamin was found to be included in the cells. Vitamin B₁₂ productivity was dependent on Co²⁺ concentration, and 1 mg of CoSO₄·7H₂O 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 B₁₂ productivity were examined (Table 1). Among the natural nutrients tested, Casamino Acids (Difco) enhanced vitamin B₁₂ production significantly. L-Methio-

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TABLE 1. Effects of various nutrients on vitamin B₁₂ production

| Expt | Nutrienta | Growth (g of dry cells/liter) | Vitamin B₁₂ 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 B₁₂); betaine or choline (methyl donor); and various vitamins. Penicillin G and various surfactants also did not affect vitamin B₁₂ 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 B₁₂ 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 B₁₂ 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 B₁₂ production under our experimental conditions. The yield of vitamin B₁₂ further increased to about 2.6 mg/liter of medium by
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\textsubscript{12} is promising and attractive from a practical point of view.

Paper electrophoretic behaviors of the B\textsubscript{12} fraction from strain FM-02T demonstrated that the vitamin exists mainly in the forms of adenosylcobalamin (coenzyme B\textsubscript{12}) and methylcobalamin. The coenzyme activity of the fraction in the coenzyme B\textsubscript{12}-dependent diol dehydrase (3,4,5,6-tetrahydrofolate reductase, EC 1.5.1.4) 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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