Vitamin B₁₂ Production by a Methanol-Utilizing Bacterium

TETSUO TORAYA, BUSABA YONGSMITH,¹ ATSUO TANAKA, AND SABURO FUKUI*  
Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Yoshida,  
Sakyo-ku, Kyoto, Japan

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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 utilization, 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·nH₂O, 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 0.08 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).

¹ Present address: Department of Microbiology, Faculty of Sciences and Art, Kasetsart University, Bangkok, Thailand.
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 | Nutrienta | Growth (g of dry cells/liter) | Vitamin $B_{12}$ produced (µg/liter) | Vitamin $B_{12}$ produced (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
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 B₁₂ is promising and attractive from a practical point of view.

Paper electrophoretic behaviors of the B₁₂ fraction from strain FM-02T demonstrated that the vitamin exists mainly in the forms of adenosylcobalamin (coenzyme B₁₂) and methylcobalamin. The coenzyme activity of the fraction in the coenzyme B₁₂-dependent diol dehydrase (β-D-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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**Table 2. Optimization of vitamin B₁₂ 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 B₁₂ produced μg/liter | μg/g of dry cells |
|--------------------|---------------------------|---------------------|-----------------------|----------------------|-----------------------------|-------------------------------|------------------|
| Batch*             | 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    | 0.0–0.05                  | –                   | –                     | 26.0                 | 19.8                        | 400                           | 20               |
| batch*             | 0.0–0.95                  | –                   | –                     | 31.3                 | 17.2                        | 863                           | 50               |
|                    | 0.40–1.38                 | + c                 | –                     | 48.2                 | 25.0                        | 2,560                         | 102              |

* 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).

* 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.

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