Cobalt Salt-Induced Increase in Vitamin B\textsubscript{12} Production by \textit{Escherichia coli} in Anaerobic Fermentation

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Abstract Vitamin B\textsubscript{12} producing \textit{E. coli} was isolated by anaerobic fermentation. By means of biological quantification with \textit{E. coli} mutant strain 215, the bacterial production of vitamin B\textsubscript{12} in the presence of cobalt chloride (CoCl\textsubscript{2} \cdot 2H\textsubscript{2}O) or cobalt sulfate (CoSO\textsubscript{4} \cdot 7H\textsubscript{2}O) was studied in the \textit{E. coli} cells harvested from anaerobic fermentation, using cultured cells (dry and wet) and concentrated culture.

A significant increase was found in vitamin B\textsubscript{12} content between the dry cells treated with cobalt chloride (8 mg/l) or cobalt sulfate (10 mg/l) and the control cells not treated with cobalt salts (p<0.01). The production of vitamin B\textsubscript{12} was increased by 4.5-6.2 times via the addition of cobalt salts, compared to that without the addition of these agents.


Key words : \textit{E. coli}, cobalt salts, vitamin B\textsubscript{12}, Anaerobic fermentation

Many investigators have reported vitamin B\textsubscript{12} production in anaerobic fermentation\textsuperscript{9,12,29}, aerobic fermentation\textsuperscript{18,19} and activated sludge\textsuperscript{7,8,11,13}. The production and absorption of vitamin B\textsubscript{12} in microorganisms treated with cobalt salts have also been studied\textsuperscript{6,15,27}. Furthermore, an attempt has been made to improve the ability of vitamin B\textsubscript{12} production in ruminant nutrition by the addition of cobalt salts to various bacteria\textsuperscript{5,14}.

The production of vitamin B\textsubscript{12} by \textit{E. coli} has been known about for many years. Earlier studies\textsuperscript{4,19,20} have asserted that surface receptors of \textit{E. coli} transport vitamin B\textsubscript{12}. However, it has not been reported so far that \textit{E. coli} having vitamin B\textsubscript{12} producing ability may be screened from anaerobic fermentation and that the production ability may be biologically assessed by adding cobalt salts to the culture.

The authors screened \textit{E. coli} having vitamin B\textsubscript{12} producing ability via anaerobic fermentation, added cobalt salts for culture and quantified the vitamin B\textsubscript{12} contents in the microbial cells and concentrated culture medium by a biological method using vitamin B\textsubscript{12} demanding \textit{E. coli}. The findings obtained are reported as follows.

Materials and Methods

1. Screening of and cultivation of \textit{E. coli} under anaerobic fermentation

From swine feces in an anaerobic fermentation installation as previously reported\textsuperscript{12} ten strains of \textit{E. coli} were screened. A strain
having the highest vitamin B₁₂ production capability was used. CoCl₂ · 2 H₂O and CoSO₄ · 7 H₂O were added to the basal medium at concentrations of 2, 4 and 8 mg/l and 5, 7.5 and 10 mg/l, respectively, as described by Kamikubo et al.¹⁴ and Fujii et al.⁵.

Trypticase soy broth (BBL) was used as a basal medium composed of trypticase peptone 17 g/l, phytone peptone 3 g/l, sodium chloride 5 g/l, dipotassium phosphate 2.5 g/l and dextrose 2.4 g/l. After culture for 22-24 hours at 37°C, E. coli cells were harvested using a cooling centrifuge (Kokusan Co., Ltd., Type H 103 N) at 8,000 rpm for 10 minutes at 5°C and were sonicated using a sonicator (Ohtake Co., Ltd., Type 5202) at 60-70 w for 7-8 minutes. The suspernatant of the disrupted cell suspension was used as a sample solution. Dry cell samples were prepared from wet cells by 48 hours of desiccation. The culture supernatants were concentrated to approximately 500 times using an evaporator (Shibata Co., Ltd., Type SPC No. 187) at 60-70°C.

2. Bioassay of vitamin B₁₂
Vitamin B₁₂ in the samples was assayed by the method of Sato²⁶ using mutant E. coli 215 (strain requiring methionine and vitamin B₁₂).

The medium contained 0.6 g of KH₂PO₄, 1.4 g of K₂HPO₄, 0.1 g of Na₃C₆H₅OH · 2 H₂O, 0.02 g of MgSO₄ · 7 H₂O, 0.2 g of (NH₄)₂SO₄, 0.1 g of NaCl and 2.0 g of glucose in 100 ml of distilled water.

The measuring samples for vitamin B₁₂ were diluted 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ with sterile distilled water. The medium (2.5 ml) was mixed with the diluted samples (2.5 ml) to the total volume of 5.0 ml. The mutant E. coli 215 was cultivated at 37°C for 22-24 hours in each of the mixtures.

The optical density in each mixture was measured at 570 nm using a spectrophotometer (Hitachi Model 100-10). The concentration of vitamin B₁₂ in the samples was determined from the predetermined threshold curve of vitamin B₁₂.

**Result**

1. Vitamin B₁₂ content in dry and wet E. coli cells
After adding of CoSO₄ · 7 H₂O and CoCl₂ · 2 H₂O to each culture medium, the vitamin B₁₂ contents in the dried and wet cells were measured. Table 1 shows the mean±SD values of vitamin B₁₂, the contents of which in the samples prepared under the presence of cobalt salts were 1.9-5.2 times (p<0.01) higher than those in the control without cobalt salts. Also, in the wet cells, the vitamin B₁₂ producing ability of E. coli was increased by adding cobalt salts. In addition, CoCl₂ is known to enhance vitamin B₁₂ production at a concentration lower than that of CoSO₄.

Fig. 1 shows the vitamin B₁₂ producing ability of the E. coli cells in the presence of cobalt salts. Vitamin B₁₂ content in the CoCl₂ (4 and 8 mg/l) added dry cells were significantly higher than those in the CoSO₄ added cells at a level of p<0.01. Higher values in mean±SD were revealed in the presence of CoCl₂ · 2 H₂O than those treated with CoSO₄ · 7 H₂O.

2. Vitamin B₁₂ content in concentrated culture supernatant
Vitamin B₁₂ content in each concentrated culture supernatant was determined and shown in Table 2.

As clearly seen in this table too, the vitamin B₁₂ producing ability of E. coli was enhanced more by CoCl₂ at a concentration lower than that of CoSO₄.

**Discussion**

The production of vitamin B₁₂ by Bacillus²⁵, Prominobacter²⁵, Butylibacterium rettgeri²¹, Pseudomonas¹,²³, Propionibacterium¹, Corynebacterium⁵, Methanosalina²² and Rhodopseudomonas¹⁴ is well known. It has been reported⁸,¹²,²⁸ that the production of a large amount of vitamin B₁₂ occurs in anaerobic fermentation. Brot et al.¹⁴ and Rosales
Vitamin B12 Production of E. coli by cobalt

**Table 1.** Changes of vitamin B12 content in dried bacterial cell by addition of cobalt salts

<table>
<thead>
<tr>
<th>No.</th>
<th>Cobalt salts</th>
<th>Addition volume (mg)</th>
<th>Vitamin B12 Contents (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dry Sample Mean±SD</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>86.6±2.5</td>
</tr>
<tr>
<td>2</td>
<td>CoSO4 7H2O</td>
<td>5</td>
<td>107.0±4.3</td>
</tr>
<tr>
<td>3</td>
<td>CoSO4 7H2O</td>
<td>7.5</td>
<td>201.4±8.1</td>
</tr>
<tr>
<td>4</td>
<td>CoSO4 7H2O</td>
<td>10.0</td>
<td>263.0±15.5</td>
</tr>
<tr>
<td>5</td>
<td>CoCl2 2H2O</td>
<td>2.0</td>
<td>176.5±27.7</td>
</tr>
<tr>
<td>6</td>
<td>CoCl2 2H2O</td>
<td>4.0</td>
<td>205.4±32.2</td>
</tr>
<tr>
<td>7</td>
<td>CoCl2 2H2O</td>
<td>8.0</td>
<td>275.0±21.8</td>
</tr>
</tbody>
</table>

*1: Wet sample-control  
*2-7: Wet sample-cobalt salts added groups

**Table 2.** Changes of Vitamin B12 content in condensed culture by addition of cobalt salts

<table>
<thead>
<tr>
<th>No.</th>
<th>Cobalt salts</th>
<th>Addition volume (mg)</th>
<th>Vitamin B12 Content (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>20.7±3.50</td>
</tr>
<tr>
<td>2</td>
<td>CoSO4 7H2O</td>
<td>5</td>
<td>19.3±4.10</td>
</tr>
<tr>
<td>3</td>
<td>CoSO4 7H2O</td>
<td>7.5</td>
<td>15.7±3.40</td>
</tr>
<tr>
<td>4</td>
<td>CoSO4 7H2O</td>
<td>10.0</td>
<td>12.7±1.80</td>
</tr>
<tr>
<td>5</td>
<td>CoCl2 2H2O</td>
<td>2.0</td>
<td>24.7±8.90</td>
</tr>
<tr>
<td>6</td>
<td>CoCl2 2H2O</td>
<td>4.0</td>
<td>24.0±8.10</td>
</tr>
<tr>
<td>7</td>
<td>CoCl2 2H2O</td>
<td>8.0</td>
<td>23.0±7.70</td>
</tr>
</tbody>
</table>

**Fig. 1.** Changes of vitamin B12 content in dried cells in the presence of CoCl2 or CoSO4.

et al. have also found vitamin B12 production by E. coli. DONNA et al., JOHN et al., and PAULA et al. have reported that receptors on the surface of E. coli were closely associated with the intake and transport of vitamin B12.

The authors screened E. coli strains having high vitamin B12 producing ability via anaerobic fermentation with cobalt salts, referring to the studies of MORIKAWA et al. and FUJII et al. who attempted to augment the vitamin B12 producing ability of various bacteria by treatment with cobalt salts.

In this study, vitamin B12 producing ability...
was compared between cells cultured with and cells cultured without cobalt salts.

Vitamin B₁₂ production of as much as 2,220 mg/l was obtained by SATO et al. using Protopaminobacter ruber. TORAYA et al. obtained the production of 2.6 mg/l using methanol-utilizing bacteria. However, the bacterial production of vitamin B₁₂ was usually less than 1 mg/l.

In earlier experiments with cobalt salts as used in this study, MORIKAWA et al. found the production of 122 μg/100 ml cell by adding 0.1 mg/100 ml of CoSO₄·7H₂O to Pseudomonas aeruginosa. FUJII et al. obtained the production of 500-600 μg/l by adding 10 mg/l of cobalt sulfate of Corynebacterium simplex. MARWAHA et al. obtained the production of 5.34 mg/l by adding 5 mg/l of cobalt nitrate to Propionibacterium shermanii.

In the present experiment, E. coli strains having vitamin B₁₂ production ability were isolated from anaerobic fermentation and treated with CoSO₄·7H₂O and CoCl₂·2H₂O. As a result, vitamin B₁₂ production of 200-300 μg/l was confirmed in the dry cells. This producing ability was 1.9-5.0 times as much as that in the control cells not treated with cobalt salts (p<0.01). This finding indicates that the addition of cobalt salts greatly augmented the production of vitamin B₁₂ in these cells. By adding 5 mg/l of CoSO₄·7H₂O the production was, on the average, 1.6-1.9 times higher than production via the addition of 2-4 mg/l of CoCl₂·2H₂O. Thus, a significant difference (p<0.01) was demonstrated. In the E. coli cells used in this study, addition of the cobalt chloride seemed to result in higher vitamin B₁₂ production than production via the addition of the cobalt sulfate.

Further studies are being undertaken by the authors on maximum vitamin B₁₂ producing ability using other kinds of cobalt salts and other bacterial species having vitamin B₁₂ producing ability in ruminant nutrition.

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嫌気性発酵由来 E. coli のコバルト塩添加による
Vitamin B₁₂ 生産の増加

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嫌気性発酵状態由来で、ビタミン B₁₂ 生産能力をもつ E. coli をスクリーニングし、塩酸コバルト（CoCl₂・2H₂O）と硫酸コバルト（CoSO₄・7H₂O）を単独で増菌培地に添加し、培養した菌体（乾燥および湿潤）および濃縮培養上清中のビタミン B₁₂ 含量を大腸菌突然変異株 215 を用いた微生物学的定量法により測定した。

その結果、CoCl₂・2H₂O の 8 mg/l 添加と CoSO₄・7H₂O の 10 mg/l 添加の乾燥菌体中のビタミン B₁₂ 含量はコバルト無添加の乾燥菌体中のビタミン B₁₂ 含量に対して 1% の危険率で有有意に高く、それぞれのサンプルの平均値±標準偏差は 263±15.5 μg および 275±21.8 μg であり、無添加対照区に対して、4.6-5.2 倍生産性が向上した。また、濃縮培養上清中のビタミン B₁₂ 含量は対照区に比べて、差がなかった。