Biological Quantification of Vitamin B\(_{12}\) Production in Anaerobic Fermentation of Livestock Wastes

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Abstract Quantification of vitamin B\(_{12}\) production in anaerobic fermentation of feces from swine was carried out by a bioassay technique using mutant E. coli 215 strain (requirement strain of Methionine and Vitamin B\(_{12}\)). The concentration of vitamin B\(_{12}\) in the anaerobic fermentation sludge increased with an increase in the sludge volume, and a close correlation between them was found (r = 0.97, p < 0.001). The BOD removal and the vitamin B\(_{12}\) production were also very closely correlated in each experimental section, as the vitamin B\(_{12}\) production increased with a decrease in the BOD removal. Bioassay using mutant E. coli 215 gave satisfactory results in quantitative estimation of vitamin B\(_{12}\) production.


Key words: biological quantification vitamin B\(_{12}\), anaerobic fermentation, livestock excrement

Vitamin B\(_{12}\) production in anaerobic fermentation\(^{7,11,24,25}\), aerobic fermentation\(^{17,18}\) and activated sludge\(^{5,8}\) has been reported by a number of investigators. The bioassay techniques for vitamin B\(_{12}\) using microorganisms have been reported in many papers\(^{5,6,14,15,26}\) since early 1950s, in which Escherichia coli has been one of the most frequently used microorganisms.

The bioassay of vitamin B\(_{12}\) using mutant strains of E. coli\(^{2,12,14}\), however, seem to have not been applied to the anaerobic fermentation of livestock wastes.

In this study, biological quantification of vitamin B\(_{12}\) production by anaerobic fermentation of swine feces was carried using E. coli 215\(^5,12,14\). The content of organic matter and the volume of fermentation sludge were varied to investigate their relationship to the production of vitamin B\(_{12}\).

Materials and Methods

1. Waste sample for anaerobic fermentation

The sample of waste used was feces from swine maintained on formula feed (a mixture of rice bran, wheat bran, formula feed for swine and formula feed for piglet
at the ratio of 3:3:3:1). The feces were diluted three times with water. The
diluted feces were stirred in a mixer and filtered through 24, 28 and 32 mesh filters.
Table 1 shows the analysis of the waste samples.
2. Seed sludge and sludge volume

Seed sludge cultured at our laboratory was used. The sludge volume was
approximately 20% (v/v) of the effective capacity. The separation of sludge
was carried out by centrifugation at 1,500 rpm for 5 minutes.

The fermentation installation used was the same as previously reported\(^{13}\). As a
fermenter, a 2-liter Erlenmyer flask with effective capacity of 1.4 l was used. The
temperature during fermentation was controlled at 34–36°C.
4. Items of analysis
4-1. Pre-treatment for quantification of vitamin B\(_{12}\)

For bioassay of vitamin B\(_{12}\), the following pre-treatment was carried out. One
gram of the sludge sample (Dry) or 1 ml of the supernatant for extraction of anaerobic
fermentation was mixed with 10 ml of 0.1 M acetic acid buffer, and 1 µg of KCN per
1 µg of estimated vitamin B\(_{12}\) was then added to this mixture for stabilization of
vitamin B\(_{12}\).

For extraction, the above-mentioned sample was heat-treated at 100°C for
20 minutes. Separation of the extract from the heat-treated sample was carried out
first by filtration and then by centrifugation at 3,500 rpm for 20 minutes. The
supernatant was used as an adjusted sample.
4-2. Bioassay technique in quantification of vitamin B\(_{12}\)

Mutant E. coli 215 (requirement strain of Methionine and vitamin B\(_{12}\)) was used
as a bacterial strain in the bioassay. In addition, methionine or vitamin B\(_{12}\) are
necessary for E. coli 215 to grow, and requirement volume of methionine is very higher
than vitamin B\(_{12}\) during growth process of E. coli 215. As SATO\(^{21}\) reported the effect
of methionine for quantification of vitamin B\(_{12}\) is not very serious except much
involved sample of methionine, the effect of methionine was not very serious in this
paper. The adjusted sample was diluted to 10\(^{-2}\), 10\(^{-3}\), 10\(^{-4}\), 10\(^{-5}\) with sterile distilled
water. A culture medium was added to 2.5 ml of each dilution to the total volume of

\begin{table}
\centering
\caption{Table 1. Analysis of waste sample}
\begin{tabular}{ll}
\hline
\textbf{Items} & \textbf{Measured value} \\
\hline
1. pH & 6.5 \\
2. SV* (%) & 27.5 \\
3. COD** (ppm) & 13,080.0 \\
4. BOD*** (ppm) & 25,010.0 \\
5. Ash (%) & 27.7 \\
6. VB\(_{12}\) & 0.678 \\
\hline
\end{tabular}
\end{table}

* SV: Sludge Volume
** COD: Chemical Oxygen Demand
*** BOD: Biochemical Oxygen Demand
Bioassay of Vitamin B₁₂ in Anaerobic Fermentation

Table 2. The medium for Vitamin B₁₂ bioassay

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>0.6 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>1.4 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Na₃C₆H₅O₇·2H₂O</td>
<td>0.1 g</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Table 3. Analysis of samples (Experimental sections 1, 2 and 3)

<table>
<thead>
<tr>
<th>Sample</th>
<th>pH</th>
<th>COD (ppm)</th>
<th>BOD (ppm)</th>
<th>Ash (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Section-1</td>
<td>6.4</td>
<td>5,110</td>
<td>9,895</td>
<td>10.8</td>
</tr>
<tr>
<td>Section-2</td>
<td>6.4</td>
<td>6,385</td>
<td>12,090</td>
<td>14.1</td>
</tr>
<tr>
<td>Section-3</td>
<td>6.5</td>
<td>8,850</td>
<td>16,216</td>
<td>17.4</td>
</tr>
</tbody>
</table>

5 ml each. The medium was inoculated with mutant *E. coli* 215 and incubated at 37°C for 20–24 hours. After incubation, the optical density was measured at 570 nm by a spectrophotometer. The concentration of vitamin B₁₂ in the sample was determined from the standard curve of vitamin B₁₂ that had been prepared beforehand. Table 2 shows the composition of the medium used for quantification of vitamin B₁₂.

4-3. Items of other analysis

To follow the anaerobic fermentation process, pH, COD (Chemical Oxygen Demand), BOD (Biochemical Oxygen Demand), ORP (Oxidation Reduction Potential), gas production and methane gas concentration were measured once a week. Analysis of the waste samples was based on the JIS method¹⁰).

5. Experimental design

To investigate the relationships of the vitamin B₁₂ production to the BOD load and to the sludge volume, the experiment was divided into 3 sections. The sample shown in Table 1 was diluted to 3 concentrations. These dilutions were based on the BOD values in this experiment. Table 3 shows the 3 samples, No. 1, No. 2, and No. 3, used in experimental sections 1, 2 and 3 respectively.

Results

1. Relation between the total production of vitamin B₁₂ and the period of fermentation

Figure 1 shows the weekly total production of vitamin B₁₂ in the sections 1, 2 and 3. Especially in the sections 2 and 3, the total production of vitamin B₁₂ increased with increases in the volume of anaerobic fermentation sludge and BOD concentration of the sample.

2. Correlation between the amount of vitamin B₁₂ and the volume of fermentation
Figure 2 shows the relation between the amount of vitamin B₁₂ production and the sludge volume. A close correlation was obtained between them with a correlation coefficient of 0.97 (p<0.001). The correlation coefficients in sections 1, 2 and 3 were 0.96, 0.97 and 0.97 respectively.

3. Correlation between the vitamin B₁₂ production and the BOD removal
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A close correlation was noted between the production of vitamin B₁₂ and the BOD removal in all the experimental sections. The correlation coefficients were -0.74, -0.75 and -0.98 for the experimental sections 1, 2 and 3 respectively.

Figure 3 shows the correlation in section 3. The correlation coefficient was especially high (r = -0.98, p < 0.005).

Discussion

Microorganisms that produce vitamin B₁₂ include *Streptomyces olivaceus*, *Streptomyces griseus*, *Streptomyces antibioticus*, *Bacillus megatherium*, *Flavobacterium devorans*, *Mycobacterium smegmatis* and *Lactobacillus arabinosus*, Methane-producing bacteria in anaerobic fermentation also produce vitamin B₁₂.

In this study, bioassay of the vitamin B₁₂ production in anaerobic fermentation of swine feces was undertaken using mutant *E. coli* 215 strain. In the experiment, the content of organic matter and the volume of fermentation sludge were varied to investigate their relationship to the production of this vitamin.

The results revealed a close correlation (r = 0.97, p < 0.001) between the production of vitamin B₁₂ and the volume of fermentation sludge in all the experimental sections. This suggests correlations between the sludge volume and the amount as well as the concentration of organic matter. Furthermore, a correlation between the sludge production and vitamin B₁₂ production was also suggested in Fig. 2. The production of vitamin B₁₂ seemed to have increased with an increase in the sludge production. This may be attributed to the existence of a large amount of methane bacteria that produce vitamin B₁₂ in case of a large amount of fermentation sludge.

As another aspect of the results, high correlation coefficients were obtained between the production of vitamin B₁₂ and the BOD removal in experimental sections 1,
2 and 3 \((r = -0.74, -0.75, -0.98\) respectively). It is assumed that dissolved organic matter had an effect of inducing multiplication of vitamin \(B_{12}\)-producing bacteria. The production of vitamin \(B_{12}\) increased, when the BOD removal was reduced, namely, when dissolved organic matter existed in a large amount. Anaerobic fermentation in this stage was very much promoted. Therefore, the volume of gas production and the concentration of methane were high. As described by Wolins et al.\(^{27}\) and Wood and Wolf\(^{28}\), the formation of methane from methylcobalamin depends upon ATP (Adenosine Triphosphate). In other words, they obtained similar results to those of Blatlock and Stadtman\(^{13}\) who demonstrated ATP-dependent formation of \(CH_4\) with coM of Methanobacillus omelianskii. Distribution of ATP seemed to activate methyl-coM-reductase. However, the mechanism still remains unknown.

The total production of vitamin \(B_{12}\) amounted to 1,720 \(\mu g/l\) (for 7 weeks), 2,167 \(\mu g/l\) (for 7 weeks) and 1,563 \(\mu g/l\) (for 3 weeks) in experimental sections 1, 2 and 3 respectively. Values earlier reported were 2,600 \(\mu g/l\) by Toraya et al.\(^{26}\), 200-2,000 \(\mu g/l\) by Hoover et al.\(^{8}\) in treatment of feces and urine, and 2.1 \(\mu g/g\) (dry matter) by Hayakawa\(^{7}\) in sludge. Szeemler and Szekeely\(^{24}\) reported the values of 6,000-7,000 \(\mu g/l\) in sludge of waste water by screening and improvement of vitamin \(B_{12}\)-producing bacteria. The values obtained in this experiment seemed in agreement with the earlier reported values except that of Szeemler and Szekeely\(^{24}\).

The results of anaerobic fermentation using swine feces as a waste sample may indicate that the bioassay using \(E.\ coli\) 215 enabled considerably accurate quantification of the vitamin \(B_{12}\) production.

Acknowledgement

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1981.


家畜排泄物を用いた嫌気性発酵でのビタミン B\textsubscript{12}

生産の生物学的定量実験

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家畜排泄物（豚ふん）の嫌気性発酵で生産されるビタミン B\textsubscript{12} の定量を E. coli 215 株を用いる Bioassay によって検討した。

発酵汚泥量と投入 BOD 値を変えた三実験区を設けて実験を開始した。

その結果、実験区 1, 2, 3 を通じて、発酵汚泥量とビタミン B\textsubscript{12} 生産との間に r = 0.97 (p < 0.001) で非常に高い相関関係を認めた。次に BOD 除去率とビタミン B\textsubscript{12} 生産との間にも高い負の相関が認められ、その中でも実験区 3 では r = -0.98 (p < 0.005) と特に高い相関係数がえられた。また、発酵時間とビタミン B\textsubscript{12} 総生産との間では、汚泥量が増加し、投入サンプル中の BOD 濃度が低い実験区 2, 3 でビタミン B\textsubscript{12} の総生産量が発酵時間の経過とともに増加した。

これらの結果、突然変異大腸菌 215 株を用いる Bioassay 法で、精度高いビタミン B\textsubscript{12} の生産量を求めることができた。

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