Function of Bacteria as Vitamin B₁₂ Producers during Mass Culture of the Rotifer *Brachionus plicatilis*

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The balance of vitamin B₁₂ in massculture of the rotifer *Brachionus plicatilis* was calculated for both batch and semicontinuous cultures after the evaluation of B₁₂ concentrations in *Nannochloropsis*, yeast, rotifer and culture water. The B₁₂ output, which is taken as the total of B₁₂ harvested and left remaining in a tank, was 1.6-2.2 times larger than the B₁₂ introduced with food, water and inoculum. On the other hand, among 100 bacterial strains isolated from rotifer tanks, 35 had B₁₂ productivity and 19 of them promoted the growth of the rotifer effectively when supplied with yeast. Calculated from the feeding rate, the B₁₂ content of bacteria and the B₁₂ demand of the rotifer, it is proved that the greater part of B₁₂, the essential vitamin to the rotifer is produced by bacteria existing in water, and excess amount was transferred to the rotifer automatically by grazing.

The rotifer *Brachionus plicatilis* is indispensable as a primary food for seedling production of marine fishes and crustaceans. In the early years, only phytoplankton such as *Chlorella* or *Nannochloropsis* were fed for the rotifer, which restricted the mass production of rotifers small in scale. Since baker's yeast, however, was introduced as a complement or a substitute, the production made great strides, as was the case with seedlings.

It was found that baker's yeast had no food value for the rotifer under germ-free conditions, and the value was improved remarkably when complemented with bacterial strains which had been isolated from outdoor ponds.¹⁾ Those strains which showed the complementary effect coincided with vitamin B₁₂ producers, and a positive interrelation was observed between the reproductivity of the rotifer and the dose of bacteria added. It was suggested for the mass production of *B. plicatilis* that vitamin B₁₂ which is essential for the rotifer^{2,3)} be produced by bacteria to complement the nutritional deficiency of baker's yeast.

In this study, the changes of vitamin B₁₂ in the rotifer, particulate matter and culturing water were observed in the course of rotifer culture at a seedling production center, and the

vitamin B₁₂ balance in a pond was estimated after evaluating the vitamin B₁₂ contents of rotifer, *Nannochloropsis*, baker's yeast, waters and bacteria. Moreover, as to bacteria, 100 strains were isolated and generic composition, vitamin B₁₂ productivity and complementary effect on rotifer growth were investigated.

Materials and Methods

Rotifer Mass Culture at Seedling Production Centers

There are basically two types in the technique of rotifer culture performed at seedling production centers. One is batch culture and another is semicontinuous culture. In the former, the rotifer is raised rapidly under the sufficient food supply and heating, and wholly harvested at the top of its growth curve. In the latter, harvesting is periodically performed in order to maintain the culture density nearly constant, which, consequently, is also called eliminating culture. In both techniques, rotifer culture is initiated by the use of phytoplankton which is mostly *Nannochloropsis* in Japan. The culture medium of *Nannochloropsis* is seawater enriched by ammonium sulfate, urea, calcium superphosphate and chelator.

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Generally in seedling production centers, the chlorinity of seawater is not particularly adjusted, and it was almost 13–15‰ in the case of mass culture reported here.

1. Batch culture We performed batch culture at the Tamano Branch of Japan Sea Farming Association in October, 1986 for the evaluation of B_{12} changes among rotifers, particulate matters and waters. The culture of *Nannochloropsis* was diluted with seawater into approximately 2×10^6 cells/ml and 10 m^3 was put into concrete pond of 11 m^3 capacity. A small type rotifer of *B. plicatilis* which is called a Tamano stock was inoculated at 203 individuals/ml. On and after 24 h, baker's yeast was continually supplied at the rate of $0.7\text{--}1.3 \mu\text{g}/\text{individual}/\text{day}$. The culture was carried out at an averaged temperature of 26.0°C for 54 h.

2. Semicontinuous culture Semicontinuous culture was carried out at the Kanagawa Prefectural Sea Farming Center from June 14–23, 1987. L type rotifer called Kanagawa L stock was inoculated after having been mixed with Kanagawa S type stock and cultured by semicontinuous method in a 150 ton outdoor concrete tank. Initially, the culture of *Nannochloropsis* was introduced similarly as the batch culture mentioned above. On and after the second day, baker's yeast was supplied at the rate of $1 \mu\text{g}/\text{individual}/\text{day}$. From the 3rd day, 40 m^3 of the culture was taken out every-day as a harvest, and refilled with the same volume of *Nannochloropsis* culture containing $1.5\text{--}3.0 \times 10^7$ cells/ml, which was conducted prior to the yeast supply.

On the occasions of both rotifer culture, the water temperature, pH, specific gravity and rotifer density were checked every day. The rotifer density was estimated from the number of individuals in five replicates of 1 ml samples which were taken randomly and fixed with 1 drop of 10% formalin solution. A water sample was filtered through $0.45 \mu\text{m}$ glass fiber filter, and the filtrate was stored at -10°C . Nitrite, nitrate, and phosphate were determined by the usual colorimetric method described by Meteorological Agency of Japan.⁴⁾ Ammonia was measured by the method of Nimura⁵⁾ and un-ionized ammonia ($\text{NH}_3\text{-N}$) was calculated according to Hampson⁶⁾ and Bower and Bidwell.⁷⁾ For the analysis of vitamin B_{12} , three samples such as the culture water filtrated with $0.45 \mu\text{m}$ Millipore filter, $100 \mu\text{m}$ plankton mesh

net and without any filtration were prepared and stored at -10°C .

Isolation and Identification of Bacteria

Bacteria in culture water was calculated and isolated with Marine Agar 2216 (Difco) plates. After incubation at 25°C for one week, the bacterial colony forming unit (CFU) in 0.1 ml of diluted sample was determined by counting the colonies on the plate. The colonies were also categorized according to characteristics such as colony shape, size, elevation, structure, surface, edge, color and opacity. Every strain was purified and stored in 2216 semisolid medium. Subcultures were made every month.

Biochemical characteristics were tested as follows: Oxidase and catalase production, Cowan's⁸⁾ method; the mode of glucose utilization (*i.e.*, oxidatively or fermentatively), marine oxidation-fermentation medium (MOF)⁹⁾; Na^+ requirement, the method of Lee *et al.*¹⁰⁾; gelatin liquefaction, the method of Cowan⁹⁾. Deoxyribonuclease (DNAase) was detected by the method of Jeffries *et al.*¹¹⁾ Gram-negative bacteria were identified by the generic level according to Sugita *et al.*¹²⁾ Gram-positive bacteria were identified according to Cowan.⁹⁾ All cultures and growth tests were set at 25°C and test media were prepared with seawater except for the Na^+ requirement test.

Measurement of Vitamin B_{12}

The amount of vitamin B_{12} contained in bacteria culture, seawater, tapwater, *Nannochloropsis*, yeast or rotifers was determined by the same *Euglena* bioassay method as mentioned by Hayashi and Kamikubo.¹³⁾ As to the dissolved form, *Euglena* was inoculated in the filtration with $0.45 \mu\text{m}$ Millipore filter after proper dilution. Extraction by KCN method¹⁴⁾ was made on $100 \mu\text{m}$ filtrated and non-filtrated samples. $100 \mu\text{m}$ filtration was performed for eliminating the rotifer. The amount of particulate B_{12} was calculated by subtracting the amount of dissolved form from the value in $100 \mu\text{m}$ filtrate.

Vitamin B_{12} productivity of bacteria was determined after the one-week culture in Marine Broth 2216 (Difco) medium and extraction by KCN method was employed.

Batch Culture Test for Determining the Nutritive Complementary Effect of Bacteria

For evaluating the nutritive complementarity

of vitamin B₁₂ producing bacteria on rotifer growth, a batch culture test was carried out as stated in the previous report.¹³⁾

The rotifer strain used here was the same large type stock that employed previously.¹⁸⁾ Prior to a batch culture test, many parthenogenetic eggs were collected from a culture which had been under a rapid growing stage. Those eggs were washed with sterilized seawater 4–5 times and treated with both a methylene blue solution of 16 ppm and an antibiotic mixture of AM9¹⁹⁾ for 2–3 h. Then, the remainder of antibiotics were washed off with sterilized seawater 4–5 times. Those treated eggs were finally kept at 25°C in the dark, and neonates for experiments were picked up after 24 h. The sterility of the treated eggs was proved by STP medium.¹⁷⁾

Seawater had its salinity adjusted to 21.8‰ with distilled water, and vitamin B₁₂ was removed by 2 hour's mixing well with active carbon (190 g/l). Then, the seawater was filtered through a glass fiber (GF/C) and Millipore (0.45 µm) filters and autoclaved at 115°C for 10 min.

Basic food for the rotifer culture was baker's yeast *Saccharomyces cerevisiae* propagated on Potato Dextrose Agar (Difco) plate at 25°C for 3–4 days. It was washed 3–4 times and diluted to 200 µg/ml with sterilized seawater.

Bacterial strains were cultured on Marine Agar 2216 plate at 25°C for one week. Then, the bacterial colony was taken, washed 2–3 times and put into test tubes to a density of 10⁷–10¹⁰ CFU/ml.

On every test, 10 individuals of newly hatched neonates were selected and put into test tubes containing 5 ml of the abovementioned yeast and bacteria suspension. The culture was conducted at 25°C for 7 days under dark conditions. During the culture, test tubes were shaken once a day to keep the yeast and bacteria suspended. Initial density of bacteria was checked with Marine Agar 2216 plates.

Results

Water Quality Parameters

Mean values of culture condition and rotifer density in the batch and semicontinuous cultures in Tamano and Kanagawa are shown in Table 1. Water temperature varied 25.5–26.6 (mean 26.2) and 20.0–25.0 (mean 22.6)°C respectively. Because of the introduction of *Nannochloropsis* into a rotifer tank, the pH was usually high at the initiation such as 7.3–8.8 (mean 7.8) and 7.5–9.1 (mean 8.1). Salinity was usually kept the levels around 27.6–28.5 (mean 28.1) and 34.0–34.8 (mean 34.6)‰. NO₂-N ranged 1.61–5.61 (mean 3.26) and 0.73–1.31 (mean 1.01) µg-at/l, which increased gradually during the culture. Total NH₄-N increased rapidly from the start, and ranged 1.43–548.9 (mean 206.0) and 244.0–774.3 (mean 532.8) µg-at/ml. Meanwhile, un-ionized form (NH₃-N) were 0.01–0.08 (mean 0.05) and 0.10–1.00 (mean 0.35) ppm.

V. B₁₂ Contents of Mass Produced *Nannochloropsis*, Rotifer and Water

The concentrations of vitamin B₁₂ in tap-water and seawater were averaged 0.88 and 11.0 pg/ml respectively. *Nannochloropsis* culture contained 16.1 pg/ml, and baker's yeast and *ω*-yeast contained 0.12 and 0.48 µg/kg respectively (Fig. 1). As to bacteria culture (10⁸ CFU/ml), it was 7.4 µg/l. The B₁₂ content of the rotifer differed with stocks: 1.0 pg/individual for Tamano S type; 2.7 pg/individual for Nagasaki L type. As for the stock cultured in Kanagawa, it was composed of both L and S type stocks, and the mean value of B₁₂ was 1.6 pg/individual.

Changes of B₁₂ and Its Budget during Rotifer Culture

1. Batch culture The rotifer density increased gradually from 203 to 292 individuals/ml in 36 h, and decreased afterward. Changes of form and concentration of vitamin B₁₂ were

Table 1. Mean values of environmental parameters and rotifer density in mass cultures in Tamano and Kanagawa

Station	Data number	Rotifer density (No./ml)	Temperature (°C)	Salinity (‰)	pH	NO ₂ -N (µg-at/l)	NO ₃ -N (µg-at/l)	NH ₄ -N (ppm)	NH ₃ -N (ppm)
Tamano	12	245.7	26.2	28.1	7.8	3.26	8.79	206.0	0.05
Kanagawa	10	89.8	22.6	34.6	8.1	1.01	—	532.8	0.35

Fig. 1. Vitamin B₁₂ contents in culture fluids, foods and rotifers.

Fig. 2. Changes of rotifer density and vitamin B₁₂ concentrations during batch culture in Tamano.

given in Fig. 2. Dissolved and particulate B₁₂ increased from 19.7 and 32.6 pg/ml to 195.6 and 114.1 pg/ml respectively in 54 h. The B₁₂ contained in the rotifer did not show a remarkable change, however, and the level ranged 0.6–1.2 (mean 1.0) pg/individual.

2. Semicontinuous culture On and after the 3rd day, 40 ton of culture water was taken out for the harvest and the same volume of *Nannochloropsis* culture ($1.5\text{--}3.0 \times 10^7$ cells/ml) was added everyday. Therefore, the rotifer density kept at 80–120 individuals/ml (Fig. 3). The dissolved and particulate B₁₂ did not show a remarkable change and the level ranged from 12.0–34.4 (mean, 21.6) and 10.8–53.5 (mean,

Fig. 3. Changes of rotifer and bacterial density and vitamin B₁₂ concentrations during semicontinuous culture in Kanagawa.

30.8) pg/ml respectively.

B₁₂ Balance in Rotifer Mass Culture

From the numerical values stated above, the balance of vitamin B₁₂ was calculated (Table 2, 3). The calculation on both types of rotifer culture shows that the total gain of B₁₂ was 1.6–2.2 times of the total input.

Changes of Bacterial Density During Rotifer Culture

The total bacteria increased from $10^{6.2}$ to $10^{8.2}$ CFU/ml during semicontinuous culture in Kanagawa and the B₁₂ producing bacteria increased from $10^{5.9}$ to $10^{7.3}$ CFU/ml (Fig. 3). The initial flora composed of *Pseudomonas* (30%), *Flavobacterium* (20%) and un-identified genus (50%) turned into the dominance of *Pseudomonas* (100%) after 10 days.

Generic Composition and B₁₂ Productivity of Bacteria

One hundred strains of bacteria were isolated from *Nannochloropsis* or rotifer culture tanks in Kanagawa and Tamano (Table 4). Those were classified into 7 groups, which were composed of *Pseudomonas* (49%), *Vibrio* (22%), *Flavobacterium* (10%), *Moraxella* (6%), *Acinetobacter* (5%), *Bacillus* (5%), and *Staphylococcus* (3%). Among them, total 35 strains had B₁₂ productivity, and composed of *Pseudomonas* (23), *Mo-*

Table 2. Balance of vitamin B₁₂ in batch culture in Tamano

Input	(μ g)	Output	(μ g)
Rotifer inoculated	2486	Rotifer harvested	1639
Particle suspended*	359	Particle suspended	1255
Water	217	Water	2152
ω -yeast	42		
Total	3104		5046

* including *Nannochloropsis*.**Table 3.** Balance of vitamin B₁₂ in semicontinuous culture in Kanagawa

Input	(μ g)	Output	(μ g)
Rotifer inoculated	13960	Rotifer harvested	36676
Particle suspended	5300	Particle suspended	12836
Water	1200	Water	7300
<i>Nannochloropsis</i> *	4833		
ω -yeast	3		
Total	25296		56812

* fluid and cells.

Table 4. Generic composition of bacteria isolated from culture waters of *Nannochloropsis* and rotifer

Bacteria	Origin of bacteria		
	<i>Nannochloropsis</i>	Rotifer	
	Tamano	Tamano	Kanagawa
<i>Pseudomonas</i>	5 (4)	8 (3)	36 (16)
<i>Vibrio</i>	0	2 (1)	20 (2)
<i>Moraxella</i>	3 (2)	2 (2)	1
<i>Flavobacterium</i>	3	2	5
<i>Acinetobacter</i>	4 (1)	0	1
<i>Bacillus</i>	1 (1)	0	4 (2)
<i>Staphylococcus</i>	0	1	2 (1)
Total	16 (8)	15 (6)	69 (21)

Numbers in parentheses represent vitamin B₁₂ producing bacteria.**Table 5.** Vitamin B₁₂ productivity of 31 bacterial strains isolated from batch culture in Tamano and their promotion in rotifer growth*^{1,2}

Strain	No. of rotifers	B ₁₂ productivity	Strain	No. of rotifers	B ₁₂ productivity
1	11	—	17	10	—
2	10	—	18	56	+
3	39	—	19	58	+
4	89	+	20	15	+
5	27	—	21	27	—
6	139	+	22	43	+
7	111	+	23	10	—
8	38	—	24	28	—
9	28	—	25	42	—
10	10	—	26	10	—
11	35	—	27	52	+
12	18	—	28	62	+
13	12	—	29	25	+
14	10	—	30	49	+
15	39	+	31	91	+
16	10	+	Control	10	

*¹ Yeast, 200 μ g/ml; Temperature, 25°C.*² 10 individuals of rotifer were received in 5 ml of yeast suspension with one bacterial strain for 7 days. Bacterial dose was 10⁷–10¹⁰ CFU/ml.

Table 6. Vitamin B₁₂ productivity of 57 bacterial strains isolated from semicontinuous culture in Kanagawa and promotion in rotifer growth*^{1,2}

Strain	No. of rotifers	B ₁₂ productivity	Strain	No. of rotifers	B ₁₂ productivity
1-1	19	—	3-1	12	+
1-2	10	+	3-2	22	—
1-3	12	—	3-3	12	—
1-4	15	—	3-5	12	—
1-5	11	—	3-6	10	—
1-6	10	—	3-8	18	+
1-7	178	+	3-9	12	—
1-8	12	—	3-10	450	+
2-2	17	—	3-12	18	+
2-3	11	—	3-13	14	—
2-4	10	—	3-14	10	—
2-5	248	+	4-2	102	+
2-7	17	—	4-5	202	+
2-8	10	—	4-6	13	—
2-9	11	—	4-7	14	—
2-10	20	—	4-8	10	—
2-11	11	—	4-9	123	+
2-13	14	+	4-11	10	+
2-14	10	—	4-12	12	+
2-16	10	—	4-13	27	+
2-17	14	—	4-14	11	+
2-18	10	—	4-16	73	+
2-19	25	—	4-17	130	+
2-20	35	—	4-19	76	+
2-21	17	—	4-20	49	+
2-22	10	—	4-21	25	—
2-23	15	—	4-22	17	—
2-24	10	—	C-1	390	+
SW-1	18	—	Control	25	

*¹ Yeast, 200 µg/ml; Temperature, 25°C.

*² 10 individuals of rotifers were received in 5 ml of yeast suspensin with one bacterial strain for 7 days. Bacterial dose was 10⁷–10¹⁰ CFU/ml.

raxella (4), *Vibrio* (3), *Bacillus* (3), *Acinetobacter* (1) and *Staphylococcus* (1). B₁₂ productivity had no concern with bacterial genus.

Nutritive Complementarity of Those B₁₂ Producers on Rotifer Growth

The results of nutritive complementarity test of B₁₂ producing bacteria are given in Tables 5–6. Total 88 strains were tested, and the final number of the rotifer in a culture tube ranged from 10 to 450 individuals after 7 days, and 19 strains of them were found to have a complementary effect on rotifer growth (>49 individuals/tube), which coincided with B₁₂ producers. On the other hand, those strains without B₁₂ productivity did not promote rotifer growth.

Discussion

Burkholder* examined vitamin B₁₂ productivity of 344 marine bacterial strains and found that 27% of them could produce the vitamin. Ericson and Lewis¹⁸⁾ isolated 34 bacterial strains and found that 24 had B₁₂ productivity, and identified them as *Pseudomonas* (11), *Achromobacter* (8), *Flavobacterium* (2), *Bacillus* (2) and *Ervinia* (1). Kurata and Kimata¹⁹⁾ reported approximate 10³–10⁴ cells of B₁₂ producing bacteria existed in one gram of mud.

Those reports mean that B₁₂ producers exist very popularly in natural bacterial flora, which coincide with our results (Table 4). Considering that 35 percent of bacterial strains had B₁₂ productivity and 19 strains had the potential to promote rotifer growth, bacteria would play

* P. R. Burkholder: Preprints Intl. Oceangr. Congress, 912–913 (1959).

an important role in rotifer culture providing essential nutritional elements such as B₁₂.

The vitamin B₁₂ contents of *Nannochloropsis* cultured in three mass culture tanks (2.2×10^{-8} pg/cell) are higher than the values obtained from the axenic one ($3.2-7.5 \times 10^{-8}$ pg/ml) shown in the previous report.¹¹ Vitamin B₁₂ is considered to be produced generally in the mass culture of *Nannochloropsis* as well as rotifer, and stored inside *Nannochloropsis* cells. This may give a promising explanation to the empirical techniques that *Nannochloropsis* must be introduced into the rotifer culture at least at the initiation, because, in the early stage of rotifer culture, bacterial flora may not still be stable and constant B₁₂ provision cannot be expected.

The calculation of the B₁₂ balance, that the total output of B₁₂ was much higher than the total input, suggests that the increment of B₁₂ during the course of rotifer culture must be caused by bacterial production, for only a small amount of B₁₂ is contained in *Nannochloropsis*, yeast or water as compared with bacteria.

From the previous report,¹¹ it can be inferred that the B₁₂-producing bacteria reproduce schizogeneously every 2-3 h in a pure culture, and each cell contains 8×10^{-17} g of B₁₂. Also in a rotifer tank, those bacteria are thought to reproduce vigorously and to be taken in one after another by the feeding of the rotifer. On that occasion, bacteria may be ingested as bacterial flocks after agglutination, because free living bacteria pass through the masticatory organ of the rotifer.²⁰ Accordingly, a large amount of B₁₂-containing particulate matters, which were mostly free living and agglutinated bacteria, should have been produced in a rotifer culture tank, than the values observed. The standing crops of bacteria, in other words, are the remainder of production and consumption in a food web.

The intake model of B₁₂ was discussed. Calculating from the report of Hino *et al.*²¹ in which an individual rotifer filtrated 3.37×10^{-8} ml of bacterial suspension per hour, when no food other than bacteria was available. Meanwhile, in semicontinuous culture, the introduced food, such as *Nannochloropsis* or yeast is generally eaten up within a few hours, and afterwards the rotifer can intake nothing but bacteria. As to the data from Kanagawa, B₁₂ producers existed over 10^6 through the culture

(Fig. 3), which means that over 3.37×10^8 ($3.37 \times 10^{-8} \times 10^6$) cells of B₁₂ producers may have been ingested per hour. An individual rotifer could intake at least 2.7×10^{-18} g ($8 \times 10^{-17} \times 3.37 \times 10^8$) of B₁₂ per hour. Considering that the B₁₂ content evaluated for masscultured rotifer represents the total B₁₂ demand in a lifetime, a few hours' filtration may have satisfied the B₁₂ demand.

As for batch culture of the rotifer, it is well known that bacterial density in a culture fluid is higher than in semicontinuous culture because of the higher feeding rate, which can be supported by the difference in particulate B₁₂ between culture methods (Fig. 2 and Fig. 3). On the other hand, B₁₂-producing bacteria promote rotifer growth more effectively when supplied with yeast,¹¹ which may be applied to the case in semicontinuous culture of Tamano where yeast suspension was continuously poured into a rotifer tank. Accordingly, sufficient B₁₂ was thought to be supplied from bacteria to the rotifer in batch culture as well as in semicontinuous culture.

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