Studies on Microbial Participation to Food Cycle in the Sea-III*

Trial Cultivation of Brine Shrimp to Adult in a Chemostat (1)

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Abstract: A trial culture of brine shrimps in a chemostat was made, starting from dissolved organic matter through bacteria and protozoa. Only if we regulate the redox potential of the system, the organisms employed in this experiment were manifested to be cultured. From the results obtained in this experiment, the possibility of the cultivation of brine shrimps for many generations in this system was secured.

1. Introduction

As the importance of the cultivation of aquatic animals in the field of fisheries increases, sufficient supply of food for the animals becomes more and more important. It has been concluded by many investigators that live food is the most suitable form for the culture of larval stage of any aquatic animal compared to other forms of foods, because it does not vitiate the water and the environmental condition does not become harmful for the animals. These live food, used today in the field of fisheries in Japan, originate mostly in the form of phytoplankton, and they are successfully used in cultivation. MATSUÉ was the first to study scientifically in this field in Japan and his success for the mass culture of marine phytoplankton (MATSUÉ, 1954) is now applied for the cultivation of many kinds of animals. On the other hand, there is a possibility of cultivation of marine animals starting from dissolved organic matter, through bacteria and protozoa.

This report presents the results of a trial mass culture of a kind of crustacean, which is often used as a bait, in the food chain starting from dissolved organic matter.

2. Experimental procedures

Marine bacterium, *Achromobacter* sp., proto-

zoa, *Oxyrrhis* sp. and *Uronchia transfuga* (O. F. MÜLLER), and brine shrimp, *Artemia salina* LEACH, were selected for study and the experiments were started. All the microorganisms except *Uronchia* were already used for the series of this study. *Uronchia transfuga* (O. F. MÜLLER) was also isolated in June at the Misaki Marine Biological Station of the University of Tokyo from sea water of Aburatsubo Inlet.

The chemostat used for the experiment was as shown in Fig. 1. In the cotton wool plugged glass bottle, 2 l of sea water medium was prepared. The supply of the medium to the next glass bottle is regulated at the middle of the connecting glass tube between the bottles. The medium is first supplied to the small glass bottle with cotton wool plug, in which the bacterium is mass cultured. As the bottle has an outlet near the center, the interface of the culture has a spacious area, which enables gaseous exchange easily. The overflow of the bacterial culture pours into the bottle, in which the protozoa are cultured. The protozoa culture is aerated with an air pump, and this culture is next poured into the water tank. As the water tank is used for the culture of brine shrimps, the quality of droplets of the bacterial and protozoa culture that is poured into the tank should be monitored. The water tank is also aerated with an air pump. Brine shrimps were cultured at the concentration of one individual in 10 ml medium. All the equipments were placed in a room controlled at the temperature

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Fig. 1. Chemostat apparatus.

at 20°C.

The pH and redox potential of the medium were measured using pH Meter Model DM-1A (Toa Electronics Ltd.) and Redox Meter Model RM-1 (Toa Electronics Ltd.) respectively.

Conversion of organic nitrogen was traced by the Kjeldahl method, using mercuric sulfate as a catalyst (Standard Methods, 1960). Details of the techniques of separation of creatures for the determination of organic nitrogen are given in SEKI (1964).

The medium used for the experiment had the following composition:

Polypeptone (Daigo eiyo kagaku):	0.1 g
Aged sea water:	1 l
pH adjusted to 7.6~7.8.	

From the results obtained by SEKI (1964, 1965), the above composition of the medium is considered to be most satisfactory for this purpose.

3. Results

1) Regulation of the environmental conditions

As already mentioned in the series of this experiment, of the creatures employed, brine

shrimp is the most sensitive to environmental conditions. In the system of peptone added sea water, exposure to Eh 260 mV has no lethal effect on brine shrimp in the pH values between 7.0 and 8.0 (SEKI, 1964). Therefore, the velocity of the supply of the medium in the chemostat was controlled to maintain at this condition. The monitoring was made every 2 days using the samples, which were collected from the glass bottle of bacterial and protozoa culture (indicated in Fig. 1 as III CULTURE). The controlled environmental condition is presented in Fig. 2. At the well regulated condition, a droplet of the medium from the glass tube having diameter of 4 mm was observed every 2 minutes. The main purpose of the regulation was to maintain the Eh values at more than 300 mV. Although a little variation in Eh values of the medium occurred in the system, it had no lethal action on brine shrimps and other microorganisms.

Fig. 2. Controlled environmental condition.

pH value does not fluctuate after the 2nd day, which is favourable for all the creatures in the system. Eh value during the incubation of 30 days is always 50 mV higher than the critical point of 260 mV (SEKI, 1964), which is also favourable for the creatures.

2) Bacterial culture in the chemostat

In order to avoid bacterial contamination, the supply of the medium and the culture of the marine bacterium were carried out aseptically in a closed system. Therefore, all the bacteria that overflowed to the next glass bottle were from the pure culture of the bacterium and the bacteria reached stationary phase in the biological space.

Fig. 3. Organic nitrogen in bacterial culture in chemostat.

The regulated bacterial amount in the bottle can be seen in Fig. 3. More than $250 \mu\text{g N}$ of organic nitrogen in 100 ml of the medium was always present as bacterial cells in the cultural medium, which results in the omnipresence of the sufficient level of the bacteria in the supplied medium for protozoa culture. There is also about $250 \mu\text{g N}$ in 100 ml of the medium as dissolved organic matter, which is also poured into the bottle of protozoa culture with bacteria. The overflowing was carried out aseptically through a glass tube into the bottle, in which the protozoa are present.

3) Protozoa culture in the chemostat

Uronchia transfuga (O. F. MÜLLER) appears in Aburatsubo Inlet commonly, and its collection

is easily made. Its body length is in the range of 50 to 150μ . Though the protozoan is killed by contact with a medium having Eh levels less than 200 mV , it has greater resistance to the reduced condition than brine shrimps. As will be discussed later, brine shrimp cannot become mature with only a supply of the bacterium and *Oxyrrhis*, and so, *Uronchia* was employed as an additional food supply for brine shrimps.

The regulated amount of protozoa in the bottle can be seen in Fig. 4. Little decrease in the bacterial amount was observed in the protozoa culture bottle, compared to the bacterial concentration in the medium of bacterial culture. As the dissolved organic nitrogen also decreases in the bottle, the uptake of the bacteria by the

Fig. 4. Organic nitrogen in protozoa culture in chemostat.

protozoa is considered to be very rapid in the system and bacterial multiplication must also be occurring in this bottle. Although aeration of the medium was made in this bottle, almost all the bacteria in the bottle may be the inoculated bacteria in the bottle of the bacterial culture, and the effect of the contaminated bacteria may be negligible, because the pure culture of inoculated bacteria in the stationary phase in the biological space is supplied into this bottle. The decreased population of the bacteria eaten by the protozoa, moreover, is always supplied by the multiplication of the bacteria which are already dominant in the bottle. In this regulated condition, the amount of protozoa in this bottle is nearly constant at the concentration of $135 \mu\text{g N}$ in 100 ml of the medium. The protozoa together with bacteria and dissolved organic matter are overflowed into the tank in which brine shrimps are cultured. Amount of each of the species of the protozoa cannot be measured by the method employed here, and the amount is presented as the total amount of both species. Therefore, the composition ratio of the two species is unknown here.

The environmental condition of the medium in this bottle was measured and the supply of the original medium to the glass bottle of bacterial culture was regulated as already stated. The regulation was made as follows: A decreasing of the supply of the medium was done as the redox potential decreased to 300 mV and *vice versa*.

4) Conversion to brine shrimps in the chemostat

100 individuals of brine shrimps soon after hatching were let into the water tank and the experiment was started. The medium used in the tank was about 1 l and, therefore, the concentration of brine shrimps in the tank was about one brine shrimp in 10 ml medium. After about 30 days incubation from 85 to 95 individuals survived in the tank. All of them became adults and most of them copulated, and, as a result, small nauplii were actively swimming in the tank in about 30 days. After about 60 days incubation these young also became adults, as the quantitative analysis was stopped after 30 days incubation. The reason why the analysis was stopped then was due to the difficulty of

the estimation of the nauplii and their mothers' relationship.

Fig. 5 shows the growth of the brine shrimps in the system as indicated by organic nitrogen of a brine shrimp. From 10 to 200 individuals of brine shrimps at each of the same cultural condition were used for the quantitative analysis according to the size of brine shrimps, and each of the obtained results is indicated as the amount of organic nitrogen per individual at each condition. For example, the brine shrimp soon after hatching was used for the estimation at the mean value of 200 individuals, whereas that after 30 days was indicated as the mean value of 10 individuals.

Fig. 5. Organic nitrogen gained by one brine shrimp in chemostat.

From these results, the possibility of the cultivation of brine shrimps, starting with dissolved organic matter through bacteria and protozoa, for many generations in the chemostat is secured. The converted ratio of organic nitrogen in various creatures in the water tank are not presented here and the most effective condition for the conversion to brine shrimps is the present task for the author.

4. Discussions

A trial culture of brine shrimps was made in the chemostat, based on the basic results of the previous investigations (SEKI; 1964, 1965). Only if we controlled the Eh values of the system, the organisms employed here were manifested to be easily cultured.

Although many trials were made in the same chemostat using the same organisms presented here except *Uronchia*, the brine shrimps did not become adults. As it was considered that the nutrients contained in the bacterium and protozoa together with peptone sustain the growth of brine shrimp to a limited extent, the addition of *Uronchia* to the system was employed. As this species of protozoa is often encountered in the *Zostera* region of Aburatsubo Inlet, it is considered to be an important food source for protozoa feeders in this region. This is the chief reason why this kind of protozoan was employed here. The brine shrimps could be cultured successfully with newly employed microorganisms and they became adults after about 20 days incubation.

As is shown in the previous reports, brine shrimps are nourished chiefly with protozoa, although bacteria and dissolved organic matter sustain the growth of brine shrimps to a limited extent. As for the culture of the marine heterotrophic bacteria, sufficient amount of bacteria can be obtained by culture in a medium of peptone added sea water adjusted pH values between 7.6 and 7.8. The marine protozoa that feed on the bacteria can be cultured when they are let in the bacterial culture medium. These protozoa which are considered to be most important for the culture of brine shrimps should also be cultured in the medium in order to obtain the favourable growth of brine shrimps. To obtain the most favourable condition for this purpose is the most important task.

In the condition that is employed in this experiment, maintenance of Eh values of the medium of the culture for brine shrimps to be more than 300mV was essential. In such a condition almost all the environmental factors are balanced in every culture bottle and, as the result, the quantitative balance of organic matter

in each organisms is also observed. In the glass bottle for the bacterial culture 250 μg N of organic nitrogen was observed as bacterial cells in 100 ml of the medium. These bacterial cells reduced in the next bottle of the protozoa culture to be about 240 μg N of organic nitrogen in 100 ml of the medium. The protozoa feed on these bacteria and their amount in the medium was nearly constant at the concentration of 135 μg N in 100 ml of the medium. From these observations it is concluded that the amount of bacteria suitable for the nutrition of protozoa should always be supplied. If the difference of the bacterial amount between the two culture bottles is too large, the proliferation of the bacteria in the protozoa culture becomes intense, which results in the decrease of redox potential of the medium. The reduced condition is not favourable for the survival of the protozoa, especially for *Uronchia*.

Dissolved organic matter reduced from about 250 μg N in bacterial culture to about 180 μg N in protozoa culture in 100 ml of the medium, which indicates the utilization of dissolved matter in the medium of the bottle of protozoa culture by the microorganisms. From these points of view, the amount of dissolved organic matter is sufficient for the culture of these microorganisms in the chemostat.

The concentration of brine shrimps in the water tank of the chemostat was one individual per 10 ml of the medium, which was the concentration of the best growth for brine shrimps in the microcosm employed here (SEKI, 1964) at the static condition. As aeration of the medium in the tank was made in this chemostat and therefore, the water movement of the medium was caused by the bubbling, the condition for the culture of brine shrimps in this system is much more favourable. From this point of view, the more concentrated condition of brine shrimp population may be still at the best population density, which results in the convenient mass culture of brine shrimps.

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海産微生物による溶存有機物質の食物連鎖への転移に関する研究 III

関 文 威

要旨 バクテリアを、水産有用動物の飼育に使用することは、水産微生物学上の一つの大きな問題である。

本報は、前報で得られた基礎的研究の資料をもとにして、溶存有機物を利用するバクテリアに始まり、有用動物までの食物連鎖を、物質環境制御装置を用いて転移を容易に行なわしめた一例である。

酸化還元電位を指標として監査することによって、急速な菌の増殖に伴う溶存酸素の急速な消費と著しい還元状態の形成を防止し転移を行なわしめた結果、溶存有機物質→バクテリア→原生動物→ブラインシュリンプの連鎖が滞りなく行なわれた。