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# Microbiological Studies of *Porphyra* Plants-VI.

## An Investigation of Bacteria-Free culture of *Porphyra* with a Shaking Culture Apparatus

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A bacteria-free culture was carried out to study the effects of organic nutrients on the growth of *Porphyra*. *Porphyra* did not grow well in a bacteria-free batch culture with a defined medium.* Many vitamins and amino acids were tried to make it grow better but without success. Unlike one-celled or uniseriate algae, ones of more complicated shape usually do not grow without an aeration or shaking even in the bacterized culture.** Therefore, a bacteria-free shaking culture was designed to investigate whether *Porphyra* grows or not, in the first place, with the defined medium.

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## Materials and Methods

The materials used in the experiments were *Porphyra leucosticta* from a laboratory culture, and *Porphyra yezoensis* and *Porphyra tenera* from a natural field.

### 1. Methods of purification of algae

Many algae from one-celled to more complicated shape have been grown in a bacteria-free culture.[1)—23)] A number of reviews were also reported on the purification of algae.[24)—34)] From all these reports, the purification methods could be summarized as follow. 1) Washing. 2) Dilution. 3) Sterilization by antibiotics or detergents. 4) Elimination by shaking or sonication. A washing is applied for a one-celled alga or spores of a multi-celled alga. Cells are picked up and transferred into a test tube with a sterilized artificial seawater under the microscope. An axenic culture is obtained by the repetition of this procedure. The washing can be also applied to a filamentous or a foliaceous alga for the purification. Algae are placed onto the agar plate for dipping and dragging. The better results come out with an addition of antibiotics in the agar plate. A dilution is applicable to a one-celled alga or spores of a multi-celled alga. Algae are put into a test tube with a sterilized artificial seawater and shaked

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in it. After that, a small amount of mixtures is transferred to another test tube with the sterilized artificial seawater and again shaked in it. These procedures are repeated. A small amount of mixtures is then brought into a Petri dish with a bacterial medium and mixed gently. The Petri dish was kept for some days in the incubator. An axenic culture can be established by picking up a clean alga among bacterial colonies. A sterilization of algae is effectively done by using antibiotics, or detergents like Jodopax, sulfa drugs, or ultraviolet rays. The point of this method is to keep algae alive and at the same time to make accompanying microorganisms kill. In order to do the sterilization successfully, it should de determined at the beginning that the kind of antibiotics or sulfa drugs and their balance and the duration to be chosen depending upon the sorts of algae. An elimination of microorganisms from algae can be also done effectively by using a sonication or a centrifuge. As this method alone does not always come out good results, it should be followed with other methods.

**2. Methods of purification of *Porphyra***

Since *Porphyra* is a multi-celled alga and has a mucilaginous layer on the surface of the cell, and spores are non-motive and relatively weak, a sterilization by the antibiotics was mainly practised with some other techniques. A thallus was cut off about 25 $mm^2$. This piece of thallus was brought onto a 1 % agar plate in artificial seawater (Table 1) to dip and drag for 30–35 cm with a Pasteur pipette. This procedure is to get microorganisms off *Porphyra* thallus physically. After that, the piece was brought to another 1 % agar plate

Table 1. Chemically defined medium used in the presnt research.

| | |
|---|---|
| NaCl | 2.4 % |
| $MgSO_4$ $7H_2O$ | 0.8 % |
| KCl | 70 mg % |
| $CaCl_2$ $2H_2O$ | 55 mg % |
| $NaHCO_3$ | 16.8 mg % |
| $NaNO_3$ | 20 mg % |
| $Na_2$glycero$PO_4$ | 2.5 mg % |
| Metal solution | 1 mℓ/100 mℓ |
| Metal solution 1 mℓ= | |
| $H_3BO_3$ | 0.2 mg |
| $FeCl_3$ $6H_2O$ | 0.048 mg |
| $MnCl_2$ $4H_2O$ | 0.144 mg |
| $CoCl_2$ $6H_2O$ | 0.04 mg |
| $ZnCl_2$ | 0.01 mg |
| $Na_2$ EDTA | 1 mg |

in 50 m$\ell$ artificial seawater with a penicillin G of 40,000 units and a streptomycin sulfate of 20 mg (Pot.) to dip and drag for about 30 cm. Then it is preserved as it is for 2 weeks. Besides this process of purification, another methods of eliminating microorganisms were used. The piece was placed for overnight in the seawater of 50m$\ell$ with the penicillin G of 20,000 units and the streptomycin sulfate of 10 mg (Pot.) added ES solution (Table 2) of 2 m$\ell$, after

Table 2. Component of the enrichment used in the present research.

| | |
|---|---|
| Pure water | 100 m$\ell$ |
| $NaNO_3$ | 350 mg |
| $Na_2$ glycero$PO_4$ | 50 mg |
| $FeCl_3$ $6H_2O$ | 12.1 mg |
| Metal solution | 25 m$\ell$ |
| Vitamin $B_{12}$ | 10 $\gamma$ |
| TRIS | 500 mg |

dipping and dragging for 30−35 cm. Sometimes these processes were repeated several times. A young thallus of 1 mm long was obtained in axenic culture by the following techniques. The young thallus was put into the artificial seawater of 30 m$\ell$ with the penicillin G of 200,000 units and the streptomycin sulfate of 1 g (Pot.) for 2 days to kill the accompanying organisms. After that, it was transferred into a test tube and shaked violently to eliminate the organisms. This procedure was repeated three times. The young thallus was, then, taken out from the test tube and spread in the Petri dish with the bacterial medium. A cleaned thallus was successfully picked up among the bacterial colonies in 5 to 7 days.

**3. Sterility test**

This is to check if there is any microorganism on the cleaned thallus. The most widely accepted way of checking it, is to grow purified pieces of thallus with nutrient-rich medium of microorganisms to examine whether microorganisms exist. The sterility test media used were $E_6$[20] and $ST_3$[32].

**4. Culture methods and culture conditions**

As for the experiments 1, 2, 3, and 4, an axenic thallus of 25 $mm^2$ was put into a 20 m$\ell$ L test tube with 10 m$\ell$ of medium in the culture apparatus of Monad. The thallus grew at 16°C+0.5°C with the light intensity of 5,000 lux at the surface of the test tube on a 10−14 light-dark cycle. As for the experiments 5 and 6, an axenic thallus of 1 mm long was put into a 125 m$\ell$ Erlenmeyer's flask with 50 m$\ell$ of medium in the shaking culture apparatus. The thallus grew at 17°C+2°C with the light intensity of 3,000 lux at the

surface of the flask on a 9—15 light-dark cyle.

**5. Observation of thallus**

Colour, shape and growth were observed by the naked eyes and measured a length when the experiments were finished.

## Results

Results are shown in Tables 3~8. In experiment 1 (Table 3), the media were replaced aseptically in 2 weeks. *Porphyra* grew better with tris (hydroxymethyl) aminomethane (TRIS) than without. In experiment 2 (Table 4), the media were not replaced. On the 19 th day after the experiment started, *Porphyra* grew better and had more normal colour with TRIS than without. In experiment 3 (Table 5), the media were replaced in 3 weeks. On the 30 th day after the experiment started, *Porphyra* grew better with TRIS and 2 times concentration of carbonic acid substances than artificial seawater alone. Carbonic acid substances refer to as dissolved carbon dioxide, carbonate ion, bicarbonate ion and carbonic acid in this paper. In experiment 4 (Table 6), the media were not replaced. On the 21 th day after the experiment started, *Porphyra* grew better with TRIS and 2 times concentration of carbonic acid substances than artificial seawater alone. In experiment 5 (Table 7), the media were not replaced. On the 44 th day after the experiment started,

Table 3. Growth of *Porphyra* with addition of TRIS on a bacteria-free culture.

| Addition mg % | Remarks on growth | |
|---|---|---|
| | 14 days | 30 days |
| None | Reddish but growing | Reddish but growing |
| TRIS 50 | Normal and growing | Normal and growing |

Table 4. Growth of *Porphyra* with addition of TRIS on a bacteria-free culture.

| Addition mg % | Remarks on growth | | |
|---|---|---|---|
| | 9 days | 16 days | 19 days |
| None | Reddish but growing | Reddish but growing | Pale 5 mm long |
| TRIS 50 | Normal and growing | Normal and growing faster | Normal 1 cm long |

Table 5. Growth of *Porphyra* with additions of TRIS and sodium bicarbonate on a bacteria-free culture.

| Addition mg % | Remarks on growth | | |
|---|---|---|---|
| | 3 days | 21 days | 30 days |
| None | Growing | Growing | Not growing but alive |
| TRIS 50<br>$NaHCO_3$ 16.8 | Growing | Growing faster | Normal and growing 3 cm long |

Table 6. Growth of *Porphyra* with additions of TRIS and sodium bicarbonate on a bactria-free culture.

| Addition mg % | Remarks on growth | | |
|---|---|---|---|
| | 3 days | 9 days | 21 days |
| None | Growing | Normal and growing | Growing |
| TRIS 50<br>$NaHCO_3$ 16.8 | Growing | Normal and growing | Growing faster 5 mm long |

Table 7. Growth of *Porphyra* on a bacteria-free culture.

| Addition mg % | Remarks on growth | |
|---|---|---|
| | 39 days | 44 days |
| ASW | Growing | Growing 5 mm long |

Table 8. Growth of *Porphra* on a bacteria-free culture.

| Addition mg % | Remarks on growth | | |
|---|---|---|---|
| | 10 days | 14 days | 22 days |
| ASW | Growing | Growing | Growing 5 mm long |

*Porphyra* did not grow well and reached only 5 mm in length.In experiment 6 (Table 8), the media were not replaced. On the 22 th day after the experiment started, *Porphyra* attained 5 mm in length.

## Discussion

A sterilization of *Porphyra* was not always an easy work. It was certain, however, that better results came out with using a thallus less than 1 mm long. In most cases, *Flavobacterium* was the last to disappear in the process of sterilization. In the previous experiment*, it was found that *Flavobacterium* disappeared from the bacterial flora and *Pseudomonas* or *Vibrio* predominated on the surface of *Porphyra* when it was grown with casamino acids. Therefore it might be a better way to apply some sterilization after the bacterial flora is changed.

As the culture vessels were not large enough, *the Porphyra* grew only 3 cm long at most. The growth was also observed to retard, and colour of the plant was paled in less amount of carbonic acid substances. However it could be said that the *Porphyra* in bacteria-free culture grows healthy with shaking and replacing media during the culture. Shaking is assumed to play an important role in getting carbonic acid substances to the surface of *Porphyra*. The carbonic acid substances are certainly one of the indispensable factors for the growth of *Porphyra* in bacteria-free culture. This is confirmed in the experiment of an amino acids analysis* and a tracer experment using $^{14}C$** that the bacteria take great part in producing $CO^2$ production. The calculation from a continuous culture of *Porphyra**** shows that 10 m$\ell$ of artificial seawater with casamino acids generates 0.06 mg of carbon as pH changes from 8.0 to 8.4. This amount of carbon keeps growing 0.9 mg of the *Porphyra* in dry for a day if the content of carbon is 40 % of total weight and if the growth rate is 16 % a day. At the end of the experiment 3, the *Porphyra* reached 3 cm long, weighing 1.8 mg in dry. Since the artificial seawater of 10 m$\ell$ can only sustain 0.9 mg, the calculation says that the *Porphyra* of 3 cm long is not able to keep the growth of 16 %, even a day. A bacteria-free culture could be brought up the same growth rate as a bacterized one if there is enough carbonic acid substances in the medium. However, the *Porphyra* cultured in the laboratory does not look so healthy as the one grown in the field, though the growth rate does not differ much. A bacteria-free culture should be further carried out to elucidate this problem.

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## Summary

*Porphyra* did not grow well in a bacteria-free batch culture with a defined medium. From the facts that unlike one-celled or unisriate algae, ones of more complicated shape usually do not grow without an aeration or shaking even in a bacterized state, a bacteria-free shaking culture was designed to investigate whether *Porphyra* grows or not, in the first place, with the defined medium.

The following results were obtained from six experiments. 1. *Porphyra* grew better in the shaking culture. 2. It grew better with TRIS than without. 3. It grew better with 2 times concentrations of carbonic acid substances than the concentration in the defined medium. 4. It grew better with repetition of medium.

It is assumed that the main reason for bad growth of *Porphyra* in bacteria-free culture is the deficiency of carbonic acid substances from the present experiments and another experiments of an amino acids analysis, of a tracer experment using $^{14}C$, and of a continuous culture of *Porphyra*. Therefore, it is possible that *Porphyra* grows normally in bacteria-free culture to an adult thallus, if there is sufficient carbonic acid substances.


## References


1) Lewin, R. A., 1951: Isolation of sexual strains of *Chlamydomonas*. J. Gen. Microbiol., 5, 926-929.

2) Provasoli, L., 1958: Effect of plant hormones on *Ulva*. Biol. Bull., 114 (3), 375-384.

3) Fries, L., 1959: *Goniotrichum elegans*. A marine red alga requiring vitamin $B_{12}$. Nature, 183, 558-559.

4) ————————, 1960: The influence of different $B_{12}$ analogues on the growth of *Goniotrichum elegans* (Chauv.). Physiol. Plant., 13, 264-275.

5) ————————, 1961: Vitamin requirements of *Nemalion multifidum*. Experientia 17, 75.

6) ————————, 1963: On the cultivation of axenic red algae. Physiol. Plant., 16, 695-708.

7) ————————, 1964: *Polysiphonia urceolata* in axenic culture. Nature, 202 (4927), 110.

8) ————————, 1964: Temperature optima of some red algae in axenic cultre. Botanica marina, 9, 12-14.

9) ————————, 1966: Influence of iodine and bromine on growth of some red algae in axenic culture. Physiol. Plant., 19, 800-808.

10) ————————, 1967: The sporophyte of *Nemalion multifidum* (Weber et Mohr) J. AG., Sv. Bot. Tidskr., 61 (4), 457-462. 4.

11) ————————, 1969: The sporophyte of *Nemalion multifidum* (Weber et Mohr) J. AG., found on the swedish west coast. Sv. Bot. Tidskr., 63 (1), 139-141. 4.

12) Fries, L., & H. Pettersson, 1968: On the physiology of the red algae *Asterocytis ramosa* in axenic culture Br. Phycol. Bull., 3 (3), 417-422.

13) Fries, L., 1970: The influence of microamounts of organic substances other than vitamins on the growth of some red algae in axenic culture. Br. Phycol. J., 5 (1), 39-46.
14) Neujahr, H. Y., & L. Fries, 1966: On the occurrence of light-sensitive corrinoids in axenic cultures of unicelluar algae. Acta Chem. Scand., 20 (2), 347-360.
15) Iwasaki, H., 1961: Nutritional studies of the edible seaweed *Porphyra tenera*-I. The influence of different $B_{12}$ analogues, plant hormones, purines and pyrimdines on the growth of Conchocelis. Plant Cell Physiol., 6, 325-335.
16) ————————, 1967: ———————— II. Nutrition of Conchocelis. J. Phycol. 3 (1), 30-34.
17) Mcdaniel, H. R., J. B. M. Brook, & R. O. Bowman, 1961: Isolation of pure cultures of algae from contaminated cultures. Appl. Microbiol. 10 (3), 223.
18) Brown, R. M., & H. W. Bischoff, 1962: A new and useful method for obtaining axenic cultures of algae. Phycol. News Bull., 15 (4), 43-44.
19) Soli, G., 1964: A system for isolating phytoplankton organisms in unialgal and bacteria-free culture. Limnology and Oceanography, 9 (2), 265-268.
20) Tatewaki, M., & L. Provasoli, 1974: Vitamin requirements of three species of *Antithamnion*. Botanica Marina, 6, 193-203.
21) Tokuda, H., 1966: The spread agar plate method for isolation and purification of marine phytoplankton. Bull. Japan. Soc. Sci. Fish., 32 (2). 568-572.
22) Druehl, L., & S. I. C. Hsiao, 1969: Axenic culture of *Laminariales* in defined media. Phycologia, 8 (1), 47-49.
23) Kanazawa, A., 1968: Axenic culture of multicellular algae-I. Treatment of algae with sulfa drugs. Bull. Japan. Soc. Sci. Fish., 34 (7), 570-575,
24) Provasoli, L., 1958: Nutrition and ecology of protozoa and algae. Ann. Rev. Microbiol. 12, 279-308.
25) Provasoli, L., 1963: Growing marine seaweeds. Proceedings of the 4th International Seaweed Symposium, 9-17.
26) Hutner, S. H., & L. Provasoli, 1964: Nutrition of algae. Ann. Rev. Plant Physiol., 15, 37-56.
27) Lewin, R. A., 1959: The isolation of algae. Rev. Algol., 3, 181-198.
28) Iwasaki, H., 1965: Studies on the physiology and ecology of *Porphyra tenera*. J. Fac. Fish Anim. Husb., 6, 133-211.
29) Fries, L., 1971: Requirements of organic substances among the littoral seaweeds. Svensk Naturvetenskap, 151-155.
30) Pringsheim, E. G., 1964: Pure Culture of Algae. Their Preparation & Maintenance, xii+119 pp. Hafner Publishing Company, New York and London.
31) Stein, J. R., 1973: Handbook of Phycological Methods. Culture Methods & Growth Measurements, xii+448 pp, Cambridge University Press, London.
32) Provasoli, L., J. J. A. McLaughlin, & M. R. Droop, 1956: The development of artificial media for marine algae. Arch. f. Mikrobiol., 25, 392-428.

# ノリの微生物学的研究—VI

## ノリの無菌振とう培養

月　館　潤　一

ノリを無菌にして，無菌培養を止水で行うとあまり生長しない。一方混菌でも静止培養では生長が悪いので，培養液を動かしながら無菌培養を試みた。

6回の実験から，1．培養液を動かすと生長は良くなる。2．トリスを入れると生長は良くなる。3．炭酸を入れると生長は良くなる。4．培養液をとりかえた方が，生長は良い。などの結果を得た。

今回の実験結果及び培養液のアミノ酸分析，バクテリヤによる炭酸ガスの生成，及び連続培養の結果から，無菌培養すると生長が悪くなるのは炭酸欠乏がその原因と考えられた。従って炭酸を充分補給すると，ノリは無菌でも正常に成体まで生長すると考えられる。