Jpn. J. Phycol. (Sôrui) 37: 150–152, June 20, 1989

Masakazu TATEWAKI, WANG Xiao-Yang and Isamu WAKANA: A simple method of red seaweed axenic culture by spore-washing*

Key Index Words: Antibiotic mixture—axenic culture—red seaweed—spore-washing medium
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The axenic culture is an essential of biological research including tissue culture, protoplast isolation and fusion, and gene transfer. In green and brown seaweeds, it is generally possible to obtain axenic cultures by washing the motile reproductive cells in a series of sterile medium (CHAPMAN 1973). However, establishing of axenic cultures in red seaweeds is hard because of the absence of motile cells throughout the life cycles. Earlier methods usually used small pieces of vegetative thalli for sterilization. The wounded part, resulting from cutting into small pieces, is contaminated by microorganisms, which also makes it difficult to obtain axenic cultures. Exceptionally, the sterilization, using the dip and drag on agar plates containing antibiotics (TATEWAKI and PROVASOLI 1964), is only successful for thalli with smooth surfaces or simple thallus organization, but not with rough and complex thalli.

Although the surface of red seaweeds have luxuriant microbial growth, the spores are generally produced under aseptic conditions in tetrasporangia and carposporangia. Thus, if freshly liberated spores are axenic while mechanically isolating by capillary pipettes, it should be possible to obtain axenic cultures. However, normally spores of red seaweeds settle and attach to the substratum soon after liberation, and a few times of serial washing of spores in plain seawater lower the percentage of spore germination. In this paper, a new and simple method of axenic culture, in conjunction with impeding spore settlement, the mechanical washing and the antibiotic treatment of spores in red seaweeds is reported.

* This research was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture (58540442 and 60480013).

Neorhodomela aculeata (PEREST.) MASUDA, *Symphyocladia latiuscula* (HARV.) YAMADA, *Ceramium japonicum* OKAMURA, *Ptilota serrata* KEUTZING, *Palmaria palmata* (L.) KUNTZE, *Chondrus yendoi* YAMADA *et* MIKAMI and *Dumontia simplex* COTTON were used in this study.

Small pieces of fertile branches (2–4 cm) or blades (2–4 cm^2) bearing tetrasporangia or carposporangia were excised from freshly collected thalli. They were cleaned by brushing with a soft paint-brush, rinsed several times in sterile (autoclaved) seawater, and placed in a Petri dish (9×2 cm) containing 30 m*l* of sterile seawater. Most pieces liberated spores within 30 min (Fig. 1A). Newly liberated spores were taken up with a capillary pipette under a stereo-microscope ($\times$40–80) and washed 3–5 times in a depression glass slide containing 1–2 m*l* of the spore-washing medium (Fig. 1B). This medium, containing extracellular substances of spore-producing thalli, was prepared as follows: 1) 10 g of fresh thalli was soaked in 50–100 m*l* of seawater for 1 hr and then the seawater was filter-sterilized through a 0.22 μm pore size membrane filter, or 2) 100 mg of fresh thalli was placed in a test tube containing 10 m*l* of seawater and autoclaved for a few minutes, then 1–2 drops of the decoction were diluted with 10 m*l* of sterile seawater. In the spore-washing medium, the rapid settling and attachment to substratum of spores in most species were delayed for a few hours without any adverse effects by washing and isolation. However, normal attachment occurred soon after

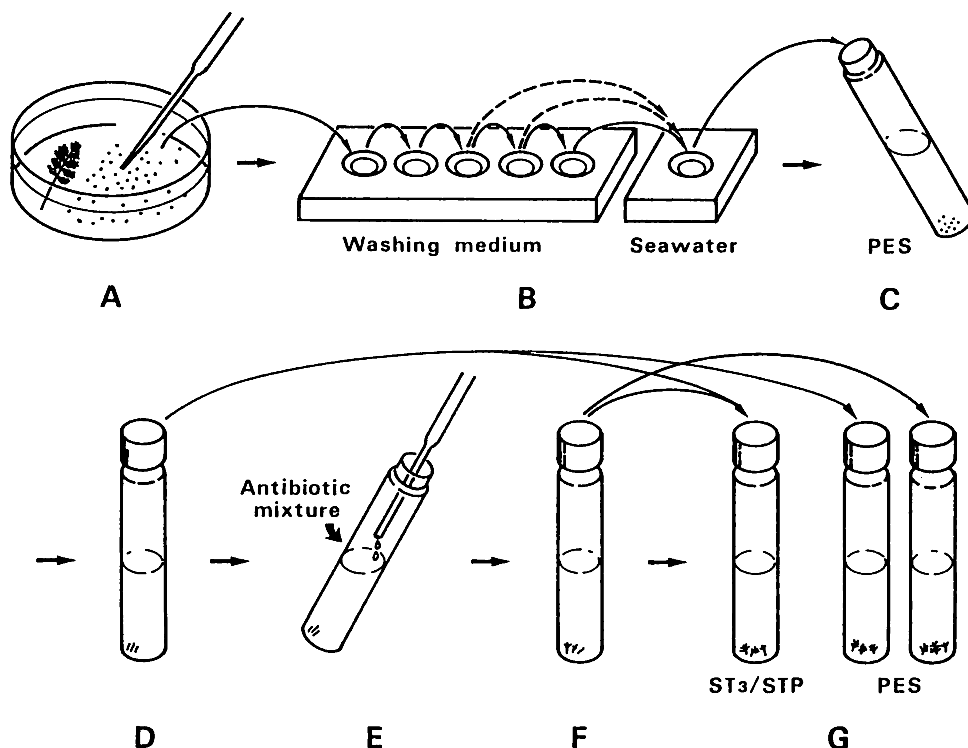

Fig. 1. Simple method of obtaining red seaweed axenic culture. A. Place a piece of cleaned fertile thallus in sterile seawater to get newly liberated spores. B. Wash spores 3–5 times in the spore-washing medium containing extracellular substances of the spore-producing thallus and once in seawater. C. Inoculate 10–20 spores to culture medium (PES). D. Culture under the optimum condition for 10–15 days until sporelings recognized with the naked eyes. E. Add 4–5 drops of antibiotic mixture to the medium. F. Culture for 7–14 days in the medium with antibiotics (replace with new culture medium). G. Inoculate young sporelings into sterility-test media and maintain them 20°C for 20 days—When confirmed axenic culture, inoculate them into desired culture media.

transfer to plain seawater or culture media.

Following the wash with the spore-washing medium, the spores were rinsed in plain seawater and then 10–20 spores were introduced into a screw cap test tube containing 10 m*l* of PES medium (PROVASOLI 1966) to establish unialgal culture. The above-mentioned isolation and washing procedures were done at low temperatures; sterile media or seawater used for washing were kept at 10–14°C. The isolated algae were maintained under optimum culture conditions for each species (usually 14–18°C, 12–18 W/m^2, 14 L: 10 D cycle) (Fig. 1D). Some of the sporelings which were developed from the serial washed spores, were axenic. For example, with tetraspores of *Palmaria palmata* and *Ptilota serrata,* axenic cultures were obtained at 10–20% of the trials. Sterility was tested by transferring a few sporelings into ST3 or STP sterility-test medium (TATEWAKI and PROVASOLI 1964) and maintaining them for at least 20 days at approximately 20°C. Confirmed axenic sporelings were transferred into the desired culture media (Fig. 1G).

In addition, 10–15 days after establishing unialgal cultures, when the sporelings first become visible to the naked eyes, 4–5 drops (75–100 μl) of the antibiotic mixture were added aseptically to each culture tube containing 10 m*l* of PES medium (Fig. 1E). The antibiotic mixture contained 100,000 units penicillin G potassium, 250 mg streptomycin sulfate, 25,000 units polymyxin B sulfate and 1 mg chloramphenicol per 10 m*l* of distilled water (modifying the formula of the mixture developed by PROVASOLI 1958, TATEWAKI and PROVASOLI 1964, and TATEWAKI 1981). The

mixture was filter-sterilized and could be preserved by freezing in 1-2 ml aliquots for one week, although it is best to prepare the mixture just before using. The sporelings were left in the medium with antibiotics for 7-14 days under optimal culture conditions and then the medium was replaced with fresh PES (Fig. 1F). Axenic cultures were thus obtained with high percentage (>90%) of success for the species examined. When spores and sporelings younger than 5 days were dosed with the aitibiotic mixture, they were killed or stunted even by 3-5 days antibiotic treatment. Thus, we recommend that 10-day or older sporelings are treated by antibiotics after the spore-washing procedure. Such sporelings which are visible to the naked eyes, survive and develop into healthy axenic cultures.

The spore-washing media presumably contain extracellular substances originating from spore-producing plants (tetrasporophytes or female gametophytes). These substances which delay spore settlement, may assure spore dispersal from the site of the sporulating plants. We are presently investigating the biological and chemical nature of these substances.

Acknowledgements

We wish to express our sincere thanks to Dr. L.D. DRUEHL (Simon Fraser University) for his kind reading and correcting of the manuscript. Anonymous reviewers also provided constructive criticism.

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舘脇正和・王　暁　陽・若菜　勇：胞子洗浄を利用した簡易な紅藻の無菌培養法

胞子の機械的洗浄と，それに続く発芽体の抗生物質処理の併用による，簡単かつ確実な紅藻の無菌化法を報告する。まず，放出直後の四分胞子及び果胞子を，胞子放出藻体の溶出液からなる無菌化済みの胞子洗浄液中で洗浄する。この洗浄液は胞子の基質着生時期を遅延させる働きを示し，洗浄操作に伴う胞子の損傷を軽減するのに役立つ。数回の洗浄によって，胞子は正常な発芽能を備えたまま単藻培養株として得られ，その一部はこの洗浄処理だけで無菌となる。さらに，生長した発芽体が肉眼で認められるようになる培養開始10-15日後に，洗浄だけでは無菌化されなかった培養に抗生物質を添加することによって，効果的に無菌株が得られた。(051 室蘭市母恋南町1-13　北海道大学理学部附属海藻研究施設)