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Antibiotic-agar Plate Method for Establishment of Axenic Primary Cultures in *Porphyra* (Bangiales, Rhodophyta)

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Porphyra is one of the most important edible macro-algae commercially cultivated in Japan, Korea, and China. Also, it has recently received great attention as a laboratory organism for fundamental and applied investigation of marine plants. *Porphyra* has a dimorphic life cycle which consists of macroscopic foliaceous thalli as the gametophytic generation and microscopic filamentous thalli as the sporophytic generation. It completes its life cycle in laboratory culture within a few months and has a small number of chromosomes (2-7) in the haploid phase [1]. The haploid genome sizes in the genus *Porphyra* ($2.7\text{-}5.3\times10^8$ bp) are of the same order of magnitude as those of *Arabidopsis thaliana* [2, 3]. The pure lines of *Porphyra* have been established [4] and maintained by cryopreservation [5, 6].

Axenic tissue cultures of algae are needed for studies of algal biotechnology in many areas. The development of sophisticated tissue culture methods making marine algae utilizable as laboratory organisms has been anticipated but few studies are known to date [7]. Since marine macro-algal surfaces are densely covered by a wide range of bacterial species [8] and each alga collected from the field has its own unique bacterial flora [9], obtaining axenic cultures is more difficult in algae than in higher plants. The purification methods of macro-algae were summarized by Chapman [10]. The pipette washing method is usually used as a basic technique for isolation of axenic strains, but a skilled technique is required for this method and it is troublesome to obtain large amounts of axenic strains. On the other hand, several methods employing chemical sterilants such as antibiotics [11], iodine [12], potassium tellurite [13], sulfa drugs [14], and combinations of antibiotics and sodium hypochlorite [15] have been reported for obtaining axenic cultures. These methods were convenient, but were considered insufficient in regard to efficacy of sterilization and toxicity of chemicals [16].

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In this study, we developed a simple and secure method for obtaining axenic primary cultures using antibiotic-agar plates.

A sterilization protocol for *Porphyra yezoensis* is shown in **Fig. 1.** First, antibiotic sensitivity tests of *P. yezoensis* and symbiotic bacteria were performed separately. The algal sources used in this study were filamentous thalli (sporophytic phase) which were derived from leafy thalli (gametophytic phase) of *P. yezoensis* strain no. TU-1 [4] maintained in ESS_2 medium [17] at 20°C, at a photon fluence density (PFD) of 13 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} and a photoperiod of 14/10 hr light/dark (LD). The following antibiotics were used for this study ; penicillin G, ampicillin, carbenicillin Na, erythromycin, tetracycline, neomycin, gentamicin, hygromycin B, kanamycin, streptomycin, geneticin, chloramphenicol, phosphomycin and polymyxin B. The effects of these antibiotics were tested at four concentrations (0.1, 1, 10 and 100 mg/*l*). These antibiotics were sterilized by filtration with 0.2 μm Nuclepore filter (Costar Co.). The filamentous thalli used for this test were cultured in the medium containing the appropriate concentration of each antibiotic by using 24-well plastic tissue culture plates at 20°C with the same PFD and photoperiod. After two weeks, the degree of sensitivity to antibiotics was evaluated by observing the growth of the plant thalli by an optical microscope. Antibiotic sensitivity of symbiotic bacteria was tested by inoculating bacteria isolated from culture medium of *P. yezoensis* to marine broth 2216 (Difco) containing each antibiotic in test tubes. The degree of sensitivity was evaluated by measuring the optical density (OD) of bacterial growth using a spectrophotometer. Mortality rates (M) were calculated from the following equation:

$$M=100-\{100\times(A'-A/B'-B)\}$$

where A′ is OD measurements after 1 week cultivation in Marine Broth 2216 (Difco) containing antibiotics, A is OD measurements before 1 week cultivation in Marine Broth 2216 (Difco) containing antibiotics, B′ is OD measurements after 1 week cultivation in Marine Broth 2216 (Difco) without antibiotics, and B is OD measurements before 1 week cultivation in Marine Broth 2216 (Difco) without antibiotics. According to these results, the antibiotics to which the

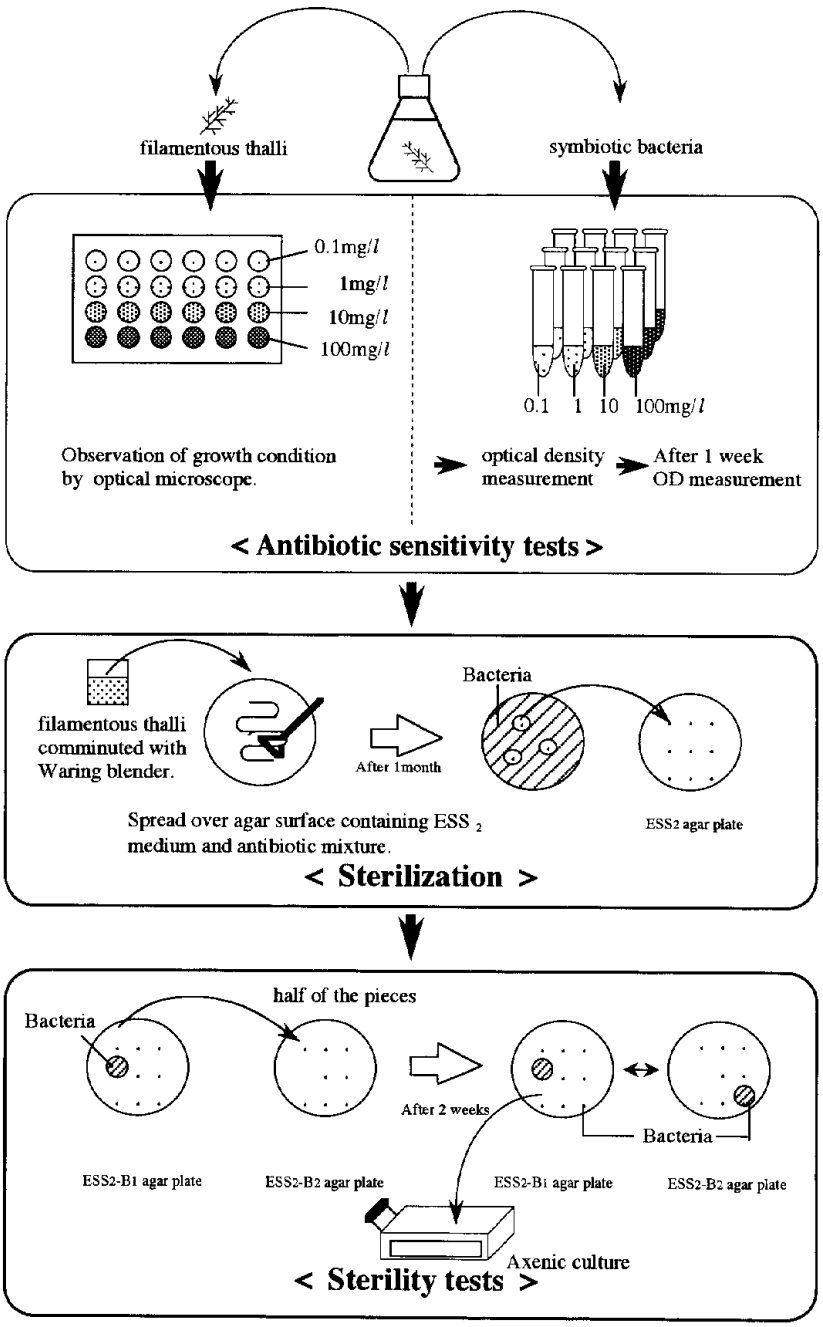

Fig. 1 Schematic diagram of sterilization protocol for obtaining axenic culture.

plant thalli were resistant and symbiotic bacteria were sensitive, were selected.

Sterilization of *P. yezoensis* was then attempted by applying an antibiotic mixture containing the antibiotics selected. The procedures are as follows: the plant thalli were comminuted with a Waring blender, and spread over the surface of 12 g/*l* agar-solidified ESS_2 medium containing the appropriate antibiotic mixture. The agar plates were cultured at 20°C, at a PFD of 13 μmol • m^{-2} • s^{-1} and a photoperiod of 14/10 hr LD. After 1 month, we picked out conchocelis in clear zones where bacterial growth was inhibited. These plant thalli were placed on to another ESS_2 agar plate, which did not contain any antibiotics, and cultured under the same conditions for 2 weeks.

To assess the sterility of the pieces of plant thalli, ESS_2-B_1 and ESS_2-B_2 agar media (**Table1**) were used. The pieces were transferred to ESS_2-B_1 agar plates and were cultured at 20°C, at a PFD of 13 μmol • m^{-2} • s^{-1} and a photoperiod of 14/10 hr LD. After 2 weeks, half of the pieces without bacterial contamination were transferred to ESS_2-B_2 agar plates. The plates were cultured at 30°C with the same PFD and photoperiod. After 2 weeks, materials which were assessed to be axenic by applying both sterility test media at this stage were transferred to ESS_2 medium in a Tissue Culture Flask (Corning® 25 cm^2, one piece per flask with 30 m*l* ESS_2 medium).

Table 1.
Composition of ESS_2-B_1 and ESS_2-B_2 agar plates.

	ESS_2-B_1	ESS_2-B_2
For 1000 m*l* of ESS_2-B_1 or ESS_2-B_2 medium, add the following to distilled water.		
Natural seawater	900 m*l*	700 m*l*
ESS_2 stock solution*[1]	10 m*l*	10 m*l*
Bacto-peptone	5 mg	5 g
Casamino acid	5 mg	—
Yeast extract	5 mg	1 g
Malt extract	5 mg	—
Beef extract	5 mg	1 g
C-source mix II*[2]	5 m*l*	—
Agar	10 g	15 g
pH	8.0	8.0

*[1] See Kitade *et al.* (1996) [17]

*[2] C-source mix II (per m*l*) contains 1 mg glycine, 1 mg D,L-alanine, 1 mg L-asparagine, 2 mg sodium acetate•3 H_2O, 2 mg glucose, 2 mg L-glutamic acid.

Table 2.
Effect of antibiotics on *Porphyra yezoensis* TU-1 strain.

	Concentration (mg/*l*)			
	0.1	1.0	10	100
Penicillin G	−	−	−	−
Ampicillin	−	−	−	−
Carbenicillin Na	−	−	−	−
Erythromycin	−	−	−	−
Tetracycline	−	−	−	−
Neomycin	−	−	−	−
Gentamicin	−	−	−	−
Hygromycin B	−	−	−	−
Knanamycin	−	−	−	−
Streptomycin	−	−	−	−
Geneticin	−	−	−	−
Chloramphenicol	−	−	−	+
Phoshomycin	−	−	−	−
Polymyxin B	−	−	−	−

+ : mortal effect, − : no-mortal effect

Table 3.
Effect of antibiotics on symbiotic bacteria.

	Concentration (mg/*l*)			
	0.1	1.0	10	100
Penicillin G	−	−	−	−
Ampicillin	−	−	−	−
Carbenicillin Na	−	−	−	−
Erythromycin	−	−	−	−
Tetracycline	−	−	−	−
Neomycin	−	−	++	++
Gentamicin	−	−	−	++
Hygromycin B	−	−	−	−
Knanamycin	−	−	−	−
Streptomycin	−	−	−	−
Geneticin	−	−	−	++
Chloramphenicol	−	−	+	+
Phoshomycin	−	−	−	−
Polymyxin B	−	−	−	++

Mortality rates:
++ ≧ 90 %, 50 % ≦ + < 90 %, 10 % ≦ + < 50 %, − < 10 %

To regularly assess sterility of these axenic explants, DAPI (4',6-diamidine-2-phenylindole) staining was applied. With the addition of the dye, nuclear and chloroplast DNA of the alga could be observed using fluorescence optics. Fluorescences as brilliant blue cocci or rods on the algal cell wall occur if bacterial DNAs are present. The axenic primary cultures of *P. yezoensis* established in the present study were maintained in a Tissue Culture Flask (Corning® 25 cm^2) containing 30 m*l* ESS_2 medium at 20°C, 13μmol•m^{-2}•s^{-1} PFD, 24 hr photoperiod.

In antibiotic sensitivity tests, monospores from gametophytic leafy thalli of *P. yezoensis* were sensitive to chloramphenicol (100 mg/*l*) (**Table 2**). The contaminating bacteria were sensitive to five antibiotics; neomycin (10 and 100 mg/*l*), gentamicin (100 mg/*l*), geneticin (100 mg/*l*), chloramphenicol (10 and 100 mg/*l*), and polymyxin B (100 mg/*l*). All of these five antibiotics, except for chloramphenicol gave 90 % or more lethality of symbiotic bacteria at 100 mg/*l* (**Table 3**). Chloramphenicol was not so toxic to the bacteria but showed toxicity to *P. yezoensis* at 100 mg/*l*. From these antibiotic sensitivity tests, the following four antibiotics, neomycin, gentamicin, geneticin, and polymyxin B were regarded as effective antibiotics for obtaining an axenic culture.

We could repeatedly obtain axenic primary cultures in *P. yezoensis* at frequencies of more than 90% each time when applying the antibiotic mixture which contained neomycin, gentamicin, geneticin and polymyxin B (**Fig. 2**). The present antibiotic-agar plate method is an efficient and secure method for

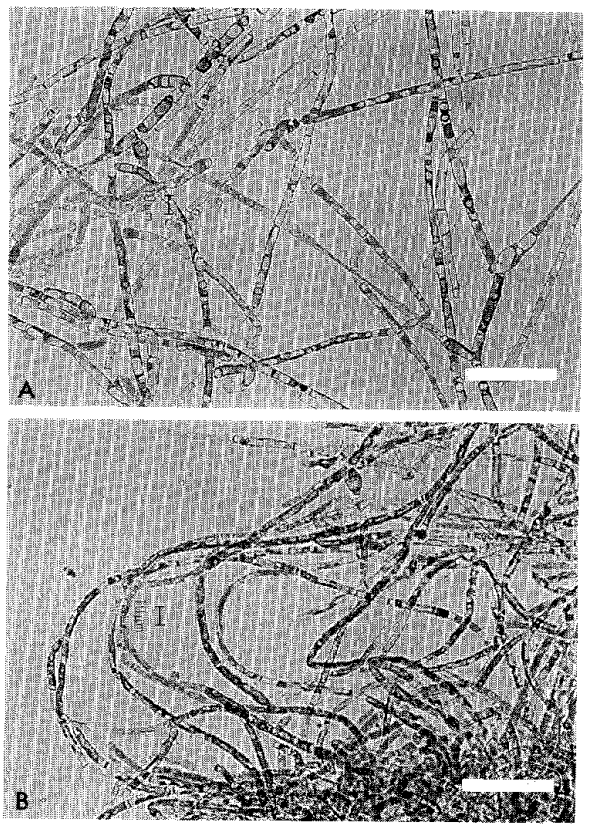

Fig. 2 Observations of filamentous thalli under a light microscope.
A: The axenic primary culture of *P. yezoensis* unialgal culture established in this study (white scale bar=50μm).
B: *P. yezoensis* unialgal culture contaminated by bacteria (white scale bar=50 μm).
These cultures (A, B) were maintained in a Tissue Culture Flask (Corning® 25 cm^2) containing 30 m*l* ESS_2 medium at 20°C.

establishing axenic cell lines. These results suggest that the present method will be useful in obtaining axenic primary culture of members of the genus *Porphyra* and should be applicable to other macroscopic algae as well.

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