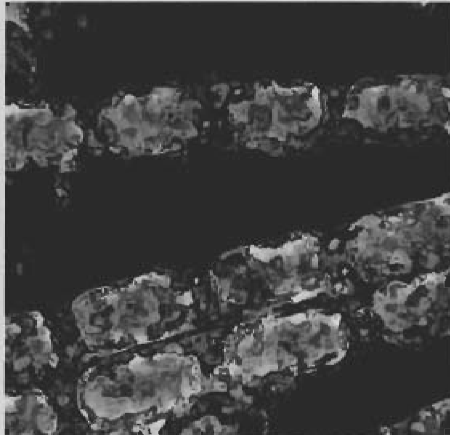

CHAPTER 9

Isolation and Purification Techniques for Macroalgae

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1.0. Introduction

Macroalgae is a general term for the algae that form a multicellular thallus at least in one stage of the life history, with the exception of siphonous ulvophycean algae that lack septa (e.g., *Caulerpa*, *Valonia*, and so on). In most cases they show differentiation between vegetative tissues and reproductive structures that release unicellular reproductive cells, as well as an alternation of generations. Therefore, to observe the development from unicellular reproductive cells to multicellular thallus, or to elucidate the whole life history that often cannot be observed from field-collected specimens, culture studies are necessary. Such studies began in the nineteenth century; however, it was often difficult to complete life histories using natural seawater and culturing at ambient temperatures. Gradually, because of

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the improvement of culture media and the use of controlled-temperature incubation chambers, unialgal culture techniques became well established, and by the 1960s culture studies of macroalgae became popular (Bold 1942, Tatewaki 1966).

In addition to their use in studies of early development and life histories, unialgal, clonal, and axenic cultures of macroalgae have become essential for many studies of morphogenesis, morphological development, nutritional physiology, responses to various chemicals, crossing experiments, extracting various compounds without contamination (including for molecular biology such as genomic DNA, cDNA libraries, Northern blotting, etc.), long-term strain preservation, exchange of research materials, mass culture and preparation of mariculture seed-stock, and so on.

In this chapter, a unialgal culture is a culture that includes only one species of alga (bacteria may be present). An axenic culture is unialgal and free of bacteria. A clonal culture is a culture of a single genome set (e.g., cultures derived from a single vegetative cell or tissue or from a reproductive cell) and propagated vegetatively. This chapter introduces the techniques for establishing and maintaining unialgal and axenic cultures of macroalgae.

2.0. Sampling

Collected specimens are transported in plastic bags, bottles, or containers suitable to their size, avoiding excess irradiation and temperature shocks relative to the prevailing habitat conditions. In general, temperature conditions 5–10°C cooler than the water temperature of the habitat (5–10°C for cold-water taxa and 20–25°C for tropical taxa) are preferable for transport. Most intertidal macroalgae (seaweeds) are tolerant of stresses such as desiccation and rapid temperature changes, compared with subtidal macroalgae. Fertile specimens collected under desiccating conditions (e.g., intertidal taxa collected during low tides) release reproductive cells (zooids, eggs, spores, etc.) as soon as they are reimmersed in seawater, such as in the containers used for transportation. Therefore, they may preferably be transported moist in plastic bags (specimens may be loosely wrapped with paper towels or newspaper) or plastic containers, instead of immersing in seawater. In contrast, subtidal macroalgae, especially those growing in deep habitats (below 5–10 m) are more sensitive to environmental changes such as desiccation and temperature shock, so they should be immediately transferred to containers filled with seawater, minimizing the exposure to air or temperature fluctuations. Disposable pipettes, the kind with the bulb and pipette molded in one piece of thin polyethylene (pastette), can be used for sampling very small specimens in the field (see Chapter 10). They are cheap, unbreakable, and sterile when packaged individually (bulk-packaged pipettes are nearly sterile and are suitable for field collecting). One draws the sample into the pipette by suction. For transport it is best to fill the pipette almost completely with seawater, shake down the liquid into the bulb, and then heat-seal by carefully melting the opening at the edge of a small flame, and squeeze the molten end together with forceps. To open, just snip off the tip.

Some acidic macroalgae (e.g., some *Desmarestia* spp., *Dictyopteris* spp., *Plocamium* spp., and so on) need special care, similar to sensitive subtidal taxa. It is also important to protect other specimens from those acidic taxa at the time of collection and transportation (i.e., avoid putting both types in the same container, because even a small amount of damaged acidic algae can ruin the other specimens). The maturation of some taxa (e.g., *Dictyota*) is reported to be synchronized with lunar rhythms (Phillips et al. 1990), so special attention should be paid in scheduling collections of these taxa.

Either vegetative tissue or the cells released from reproductive structures may be used to establish cultures (see Section 4.0). In either case, cleaner plants with few epiphytes and epizoa, and fertile plants in the latter case, should be selected in the field. Fertile portions (parts of the plants bearing reproductive structures) often can be detected from the gross appearance; only those portions need be cut and transported to the laboratory.

3.0. Tools and Facilities

For unialgal cultures, glass and plastic petri dishes, beakers with glass covers, test tubes with screw caps, and clear thin polystyrene cups (so-called ice cream cups) are commonly used (Fig. 9.1). For small plants (less than several centimeters), plastic (polystyrene) petri dishes of 60 or 90 mm in diameter are convenient and also cheap, because their flat bottom and top are suitable for observation using an inverted microscope or stereomicroscope. They also save space, because the dishes may be stacked. For larger plants, beakers, ice cream cups, or various glass containers are used. The lids of the petri

FIGURE 9.1. Tools used for macroalgal cultures (glassware, multiwell dishes, small vials for stocks, plastic containers for medium, Pasteur pipettes with silicone bulbs and tubing, polystyrene transfer pipettes, depression slides, glass homogenizer, forceps, scalpel, needles, paint brushes, syringe with membrane filter cartridge, razor, alcohol lamp, watch glass, and glass and plastic petri dishes).

dishes should be sealed with sealing films (e.g., Parafilm, American National Can) to avoid evaporation of water and accidental leakage that contaminate other cultures. Culture media are changed every 2–4 weeks, although this interval varies depending on the material and temperature.

Cultures are normally maintained in climate-controlled culture chambers or incubators that can regulate the temperature and are illuminated by daylight-type, white fluorescent tubes. Plant growth chambers designed for higher plants generally provide illumination that is too intense, and they may not provide stable temperature control at lower ranges. Generally incubators converted from cooling incubators by adding lighting units (fluorescent tubes and a timer controlling the lighting; the ballasts should be mounted outside the chamber) are cheaper and more reliable (Fig. 9.2) (e.g., MIR-552HK, Sanyo). Because mechanical problems can occur that will cause extreme temperature fluctuations, it is important that the lights turn off when the cooling unit of the incubator fails. A temperature-gradient incubation chamber (Fig. 9.3) (e.g., TG180–5L, Nihon Ika), having several closed chambers whose temperatures can be independently controlled, is especially convenient for life history studies or to compare growth and differentiation under different temperature conditions. In many macroalgae, reproduction is controlled by a combination of temperature and daylength (photoregime) conditions (see Chapter 21 for details).

FIGURE 9.2. Incubators converted from cooling incubators by adding lighting units (fluorescent tubes and a timer controlling the lighting; the ballasts should be mounted outside the chamber).

FIGURE 9.3. Temperature-gradient incubation chamber having several closed chambers whose temperatures can be independently controlled.

For long-day conditions, intervals of 14–16 hours lighting and 8–10 hours darkness (e.g., 16-hr light: 8-hr dark) are commonly used, and for short-day conditions, 6–8(–10) hours lighting and (14–)16–18 hours darkness. Light intensities of 10–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$ are commonly used for macroalgal cultures. The light intensity can be reduced by placing smoked transparent glass (or plastic panel) between the illumination and the cultures or by covering the culture containers with gray plastic window screens. Colored fluorescent tubes (e.g., FL40S-R-F, National) or photodiodes (e.g., MIL-R18, Sanyo) are available for red-light illumination (for photomorphogenesis experiments or to suppress fertilization in laminarialean gametophytes), but shading by colored plastic filters is also effective.

FIGURE 9.4. Cleaning of algal tissue with a paintbrush.

4.0. Unialgal Isolation Techniques

Most species of macroalgae have high potentials for regeneration and totipotency, so that theoretically unialgal cultures can be established for most species by cutting off vegetative tissues (cells) and cleaning them in the course of their growth. However, in practice epiphytic algae or cyanobacteria, which may be very difficult to remove from the surface of the tissues, grow faster and more vigorously than the desired algae. Therefore, isolation from vegetative tissues is usually restricted to taxa with apical meristematic growth (e.g., Dictyotales, Sphacelariales in brown algae, red algae with apical cells), siphonous green algae, and some taxa with rapid cell division (e.g., *Ectocarpus*, *Ulva*, and so on). In other cases, unialgal cultures are established from zooids such as zoospores and planogametes, or zygotes, carpospores, tetraspores, or aplanospores.

4.1. Crude Cultures

When the collected specimens do not immediately release reproductive cells, or as a preliminary step in isolation from vegetative tissues, the whole plant or a part of the plant may be cultured (maintained) in the laboratory. These are often called crude cultures. To clean the surface of the plants, fine paintbrushes are helpful before starting the culture (Fig. 9.4). Enriched seawater media are used, as for unialgal cultures, but to avoid overgrowth of epiphytes, plain sterilized seawater or reduced enrichment may be used. To suppress the growth of diatoms and cyanobacteria, germanium dioxide (GeO_2) and antibiotics may be added to the media (see the following sections). Because the reproduction of many macroalgae is controlled by temperature and daylength conditions, to induce reproduction of the plant (formation or maturation of reproductive structures), experimentation may be required to discover the necessary temperature and daylength conditions (e.g., some brown algal taxa form reproductive structures only under low- temperature and short-day conditions).

The vegetative thalli of *Ulva* spp. (Ulvophyceae) normally do not become reproductive in crude cultures, but small pieces (a few millimeters across), cut with a razor or punched with a cork borer, become fertile within a few days (Norby and Hoxmark 1972). Various ulvophycean algae (e.g., *Bryopsis*, *Chaetomorpha*, and so on) form reproductive cells within several days to several weeks after collection and can be used for unialgal isolations; however, for some taxa, extended crude culture (up to one month or longer) is required (e.g., *Caulerpa* in Enomoto and Ohba 1987, *Polyphysa* in Berger and Kaever 1992). Field-collected vegetative plants should be cleaned and maintained in suitable culture conditions simulating the temperature and daylength of the original habitat. When the formation of reproductive cells is noticed from the change in the external appearance (especially color), the culture should be transferred into a dark box, and the release of reproductive cells can be induced the following morning by transferring the plant to a new dish filled with fresh medium and stimulating with high-intensity lighting.

4.2. Isolation from Vegetative Cells

If the species has obvious apical or marginal meristematic cells, isolation from these cells to establish unialgal culture is easy. In such cases, first cut out a small fragment including the apical cell(s) using a razor blade or scalpel, and place it in a 60-mm–diameter petri dish

FIGURE 9.5. Cutting off clean apical (distal) portion with a scalpel.

FIGURE 9.6. Cleaning of algal tissue by dragging the fragment through an agar plate.

FIGURE 9.7. Cleaning of algal tissue by dragging the fragment through an agar plate.

filled with filtered seawater. Under observation by stereomicroscopy, cut out clean, smaller pieces of tissue including intact apical cells (the smaller the better, but care is required to avoid damage to the apical cells) (Fig. 9.5). Transfer the excised pieces one by one into individual wells of a multiwell plate, petri dishes, or test tubes filled with culture medium, using a clean fine forceps, a mechanical pipettor (e.g., Pipetman, Gilson) with disposable tips, or capillary pipettes (see the following sections). Excised apical tissues may be pipetted into successive well plates of sterilized seawater, or for big apices put in a tube or vial and agitated in several changes of sterilized seawater using a vortex mixer, before putting them into growth medium. GeO_2 or antibiotics may be added to the medium (see the following sections). Culture the isolates for 1–2 weeks (or longer) in appropriate temperature conditions, and then observe with a stereomicroscope or an inverted microscope and select clean cultures. If contaminants are still present (epiphytes or cells on the bottom of the dishes), repeat the isolation processes (cutting off clean apical cells and isolating into new wells of a multiwell plate or individual dishes), until the culture becomes unialgal. For some taxa, culture of the primary isolate in dim light (5–10% of normal culture conditions) is effective to suppress the overgrowth of contaminants. Brief immersion in a dilute nonionic detergent (e.g., Triton X-100) or fresh water is effective for eliminating protozoa or diatoms, if the desired alga is tolerant of such treatment.

For some taxa, cleaning using agar plates is effective. Cut off a small fragment including the apical cells, and drag the fragment through an agar plate (1–2% agar in seawater) holding the proximal end with forceps or a fine needle (Figs. 9.6 and 9.7). Transfer the cleaned fragment to a petri dish filled with sterilized seawater, cut off a smaller fragment including the apex (distal fragment), and isolate it into a well of a multiwell plate filled with medium.

Certain multinucleate siphonous algae (Ulvophyceae [e.g., *Bryopsis* and *Valonia*]) show healing responses in wounded cells, forming protoplasts (Tatewaki and Nagata 1970, La Claire 1982). Protoplasts artificially induced by cutting the siphonous thallus or by puncturing the wall by a fine needle, and which are formed several hours after wounding, can be suspended in sterilized seawater by agitating the wounded thallus. Clean the protoplasts by transferring them into new petri dishes filled with sterilized seawater using fine Pasteur pipettes (or a mechanical pipettor), and then isolate them into individual wells of a multiwell plate. The protoplasts regenerate cell walls and eventually develop into thalli.

4.3. Isolation from Reproductive Cells

4.3.1. Isolation from Swimming Zooids (Zoospores and Gametes)

Many species release zooids or eggs/aplanospores more vigorously and synchronously on the day(s) following rather than immediately after collection, if properly

stored in a dark (and cool for cold water and temperate taxa) place. Those specimens stored under cool and dark conditions release reproductive cells immediately after immersion in seawater, stimulated by the temperature rise and lighting, so they should be transferred just before isolation.

Prepare a plastic multiwell plate, test tubes, or petri dishes (see Fig. 9.1) for culturing the isolates, and fill the wells with liquid medium. Prepare a sterilized Pasteur pipette with fine tip, pulled in a flame (see Chapter 6 for the method), and attach a segment of silicone tubing with a mouthpiece (or a rubber bulb) to the wide end; a mechanical pipettor with disposable sterile tips may also be used. Fill a depression slide, small petri dish, or watch glass with liquid culture medium and place on the stage of a stereomicroscope. The temperature of the medium should be adjusted depending on the specimen (5–10°C for cold-water species, because the zooids soon settle or stop swimming in warm medium). Clean the surface of the fertile portion of the plants with a paper towel, gauze, or paintbrush, and cut off a small piece of tissue (1–3 mm in length) with reproductive structures. Gently place the tissue on the bottom of the depression slide. Expose to strong unilateral lighting, preferably with fiber-optic illuminations to avoid rapid temperature rise of the sample and medium (Figs. 9.8 and 9.9).

When swimming zooids are released, observe their phototactic behavior to determine the orientation of the taxis. Dark-field (or semi-dark-field) illumination is helpful for observing swimming zooids. If they are phototactic, adjust the direction of illumination to cause the zooids to accumulate at the surface of the medium (illumination from the upper side when they are positively phototactic, and from the lower side when negatively phototactic), and distant from the algal tissue to avoid contaminants (Fig. 9.10a,b). When the zooids are ready for isolation, dip the tip of the fine pipette into the medium of the well into which the zooids are to be isolated, and allow medium to enter by capillary action (if the tip of an empty capillary is dipped into the liquid surface of the depression slides, medium including con-

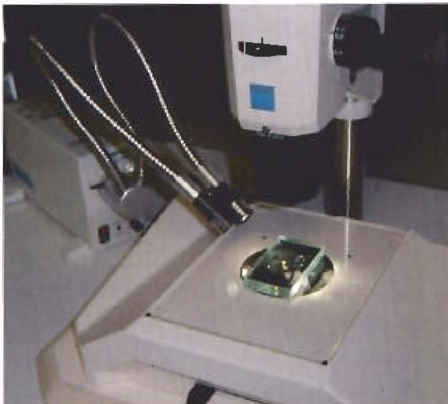
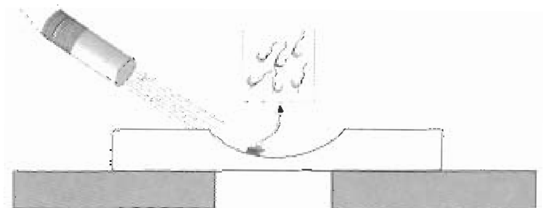


FIGURE 9.8. Isolation of phototactic zooids under observation with a stereomicroscope: Fertile algal tissue is placed at the bottom of a depression slide and the release of zooids is induced by intense illumination from a fiber-optic light source. Released zooids are accumulated at the surface of the medium (lighting from the upper side when they are positively phototactic, and from the lower side when negatively phototactic), and distant from the algal tissue.

FIGURE 9.9. Enlargement of depression slide and illuminator.

a



b

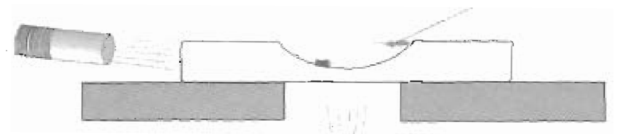


FIGURE 9.10. Isolation of swimming zooids using phototaxis. **(a)** Unilateral illumination from a fiber-optic light source to stimulate zooid release; **(b)** simultaneous lateral and bottom illumination to accumulate zooids at one upper edge of the depression slide well.

taminants such as diatoms floating on the surface by surface tension would automatically flow in by capillary action). Change the lighting direction or reduce the intensity for a short time to make the zooid suspension somewhat diffuse, or let them start to swim downward. Then manipulate the tip of the fine pipette to a position just above the liquid surface over the accumulated zooids, and gently dip the tip of the pipette into the liquid and let the medium including the zooids flow underneath the liquid surface (to avoid inflow of contaminants on the surface). If pipetting by mouth, control the air pressure by using the tip of the tongue. Place the tip of the pipette in the liquid of the isolation well, test tube, or petri dish, and discharge the medium by breathing out gently until an air bubble is released. Then dip the tip of the empty pipette into the liquid of a new isolation well, and repeat this procedure several times.

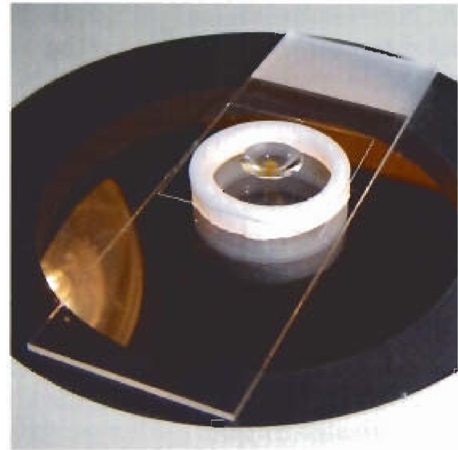


FIGURE 9.11. Hanging drop method: Fertile algal tissue is suspended in the drop of medium under the cover glass that is placed on the plastic spacer ring on a slide glass. The space between the cover and slide glasses and plastic spacer is sealed with petroleum jelly.

4.3.2. Isolation from Zygotes and Aplanospores

The isolation procedure for zygotes and aplanospores (e.g., carpospores, tetraspores, monospores) of red and brown algae is similar to that for swimming zooids in the brown and green algae and can be easier, because they are larger and nonmotile. Spores of various red algae adhere to the substratum during the washing process, probably by the secretion of sticky polysaccharides and proteins. Detaching the settled spores from the substratum by force may damage the spores, and as a result, they do not develop. Tatewaki et al. (1989) found that attachment of red algal spores on the substratum is delayed in seawater conditioned by the spore-producing thallus, and they therefore recommended washing spores with filter-sterilized seawater in which the mother thallus had been immersed (10 g mother thalli in 50–100 mL seawater for 1 hour). Finally, spores are inoculated into sterilized seawater or enriched seawater medium on slides or cover glasses. After settlement of the spores, these substrates are transferred into enriched seawater medium (e.g., Provasoli's ES medium; see Chapter 3) in petri dishes or test tubes.

4.3.3. Isolation from Reproductive Tissues

When fertile reproductive structures (e.g., sporangia, gametangia, and so on) are found in the collected algae, but actual release of reproductive cells (spores, zooids, gametes, etc.) does not take place when observed under stereomicroscopy, the fertile reproductive structures (e.g., sporangia) or small fragments including reproductive structures may be excised and maintained (crude-cultured) in individual wells of multiwell plates until they release reproductive cells during incubation and germlings become apparent in the culture dishes. To minimize contamination, the tissue should be removed as soon as release of reproductive cells can be detected. Transfer the clean, individual germlings attached on the bottom or walls of wells into new wells using fine forceps, capillary pipettes, or mechanical pipettors. The so-called hanging-drop method is useful to isolate algae forming flagellate reproductive cells, avoiding contamination by diatoms and other nonflagellate algae: Drop several drops of sterilized seawater onto a clean cover glass; place a small fragment of fertile tissue in the seawater; invert the cover glass quickly to leave the seawater drop, including the algal tissue, hanging, and place the cover glass on a plastic ring that is attached by petroleum jelly on a slide glass (Fig. 9.11); place the slide glass in a 90-mm–diameter petri dish and incubate in the culture chamber; observe under compound microscopy, and when the settlement of the released reproductive cells on the surface of the cover glass is noticed (often at the periphery of the drop), remove the algal tissue and contaminants by removing the drop and washing the cover glass in sterilized seawater, and then place in petri dishes filled with culture medium.

4.3.4. Elimination of Diatoms and Bacteria from Algal Cultures

Establishing unialgal cultures preferably starts from isolating reproductive cells, but sometimes other

organisms contaminate the culture vessel. Protozoa, fungi, and diatoms are common contaminants. These contaminants can grow more rapidly than the desired alga and attach firmly; as a result, it is very difficult to eliminate these organisms. In particular, contamination by protozoa and fungi stunt the growth of unialgal cultures, although some kinds of antibiotics may be able to inhibit their growth for a time. Although diatom contamination may be eliminated by adding GeO_2, which inhibits silicon-metabolism in diatoms (Lewin 1966, McLachlan et al. 1971, Chapman 1973; see Chapter 11). Tatewaki and Mizuno (1979) showed that 2.5–5 $mg \cdot L^{-1}$ GeO_2 in the medium inhibits the growth of brown algae, although it does not affect growth of green and red algae. Because diatoms could be eliminated at a GeO_2 concentration of 1–5 $mg \cdot L^{-1}$, they recommended that the maximum concentration of GeO_2 should be 1 $mg \cdot L^{-1}$ in the case of brown algal cultures (Markham and Hagmeier 1982).

To eliminate bacteria or inhibit the growth of bacteria in cultures, antibiotics have been used singly or in combination (Table 9.1) (Spencer 1952, Provasoli 1958, Tatewaki and Provasoli 1964). Although penicillin G (potassium or sodium salt), streptomycin sulfate, and chloramphenicol were originally used by the pioneers (Table 9.1), gentamycin is preferably used in place of chloramphenicol recently (see Chapter 8). After the antibiotic mixture is sterilized by filtration (0.22 μm), 1 mL aliquots in microtubes are stored at −20°C. The antibiotic treatment procedure is described in Section 5.0. However, it must be cautioned that antibiotics may also affect the growth of the algae, so this treatment must be kept to a minimum (a few days; see Chapter 8).

TABLE 9.1 Antibiotic mixtures used by Provasoli (1958) and Tatewaki et al. (1989). Quantities are added to 10 mL dH_2O.

Antibiotic	*Provasoli*	*Tatewaki et al.*
Potassium penicillin G	120,000 U	100,000 U
Chloramphenicol	500 μg	1 mg
Polymyxin B sulfate	500 μg	
Neomycin	600 μg	
Streptomycin sulfate		250 mg
Polymyxin B sulfate		25,000 U

4.3.5. *Clonal Cultures*

Unialgal cultures established from the vegetative tissue of a single individual and propagated vegetatively are generally regarded as clones, although gene mutations might occur during the course of long-term maintenance. Cultures established from the tissues of more than one individual, or the reproductive cells formed after meiosis (e.g., zoospores), include genetic variations and are not clonal. To establish clonal cultures from these nonclonal (mixed) cultures derived from reproductive cells, at an early stage of germination of reproductive cells in petri dishes (it is recommended to prepare low-density isolates for this purpose), isolate individual germlings (or young plants) into individual petri dishes or separate wells of multiwell dishes using fine pipettes or forceps under observation by stereomicroscopy. When the cultures are already overgrown and hard to distinguish and separate from each other, homogenize a small part of the mixed culture into fragments as small as one to several cells using a glass homogenizer (1 mL volume type), spread the fragments in a petri dish filled with medium, select appropriate cells under stereomicroscopy (single, healthy cells are best, but if these cells are not available, a few-celled fragment, apparently of a single individual), and isolate them into individual wells of a multiwell dish.

4.4. Stock Cultures

In general, unialgal cultures of macroalgae can be maintained without changing the medium for several months under lower temperatures and light intensity conditions than normal culture conditions for the alga. Generally 5–10°C is suitable for cold-water and cool-temperate species, 15°C for warm-temperate species, and 20°C for subtropical to tropical species, illuminated by 1–10% of the light intensity of normal culture conditions. A convenient way to create dim conditions is to shade the area using a smoked transparent plastic panel or to wrap the culture containers with a plastic window screen. Small vials (Fig. 9.12), test tubes with screw caps, or plastic petri dishes tightly sealed with sealing film are used for these stocks. Disposable pipettes (e.g., pastettes) can also be used for long-term storage of isolates. Because polyethylene is sufficiently permeable to oxygen and carbon dioxide, the cultures can live indefinitely in the sealed pipette. Agar plates (0.5–1% agar in seawater medium) can also be used for stock cultures, especially for filamentous algae (e.g., *Ectocarpus*). The interval required for change of the medium depends on the

FIGURE 9.12. Stock cultures of unialgal strains in small vials.

species and light intensity/temperature; some brown algae can survive for more than two years without any medium change in sealed 4-mL plastic vials.

5.0. Axenic Cultures

5.1. Purification

Axenic cultures of unicellular algae are quite common; however, their use in marine macroalgae is still limited. During the 1960s–1980s, several reports were published on axenic cultures, focusing on growth regulators, effects of nutrition on morphogenesis, and the improvement of methods for establishing axenic cultures (Provasoli and Pintner 1964, 1980; Provasoli and Carlucci 1974; Fries 1975; Tatewaki et al. 1983). Obtaining axenic strains of macroalgae is difficult; however, it has merits, because the preservation of these strains is not difficult, and the interval of reinoculation into new medium can be prolonged six months or more in axenic strains compared with unialgal strains.

In this section, an outline of methods to obtain axenic cultures of macroalgae is described. To establish an axenic culture, isolation from unicellular reproductive cells (e.g., zoospores, gametes, tetraspores, and carpospores) is a standard procedure. These reproductive cells are aseptically formed in the characteristic reproductive structure, although numerous bacteria, fungi, and epiphytes are attached to the surfaces of the reproductive structures and to the vegetative thalli. Although these contaminants might be killed and removed by various antibiotics, it may be impossible to completely eliminate them, because they can penetrate into the algal cell wall.

The method for isolating reproductive cells, such as zoospores and gametes of the brown and green algae, is identical to that for unialgal cultures. The liberated cells are quickly washed or diluted several times in sterilized seawater or an artificial medium. Use of glass capillary pipettes with a fine tip is convenient for isolating these cells under the stereomicroscope or inverted microscope. Then, 5–10 reproductive cells are inoculated into each screw-capped test tube containing 10 mL autoclaved artificial seawater medium. It is important to transfer several cells into each test tube, because often not all cells develop. Moreover, in some algal groups (e.g., laminarialean plants), the zoospores develop into either male or female gametophytes, and if these gametophytes grow densely, then afterward it becomes difficult to separate them. The isolation procedure for reproductive cells can be carried out in a room of normal cleanliness, so the clean bench is not always necessary during this process, because bacteria commonly found in air rarely grow in seawater.

To establish axenic cultures by washing the zoospores and gametes, a minimum of four to five washes is necessary. During sequential washes the number of swimming zooids decreases considerably, and the final isolation becomes difficult. Therefore, liberation of numerous swimming zoospores and gametes is an important condition for axenic culture. Tatewaki et al. (1989) reported a simple method using an antibiotic mixture for axenic cultures. After washing zooids 2–3 times, they are inoculated into autoclaved artificial culture medium in screw-capped glass test tubes. The next day, two to five drops of the antibiotic mixture are added to each test tube containing 10 mL culture medium, using a sterile capillary pipette in a clean bench. After antibiotic treatment for about three days, the medium in the tubes is replaced with new autoclaved artificial medium using a clean bench. Normally growing zoospores or gametes firmly attach to the glass wall, so they do not detach during the medium exchange. After about one month of culture under appropriate temperature and light conditions, several thalli of the desired algae can be detected by the naked eye. The thalli in each test tube must be checked using a sterility test for axenic cultures. When establishing axenic culture strains, it is customary to prepare 20–40 test tubes in each sample because only some of them prove axenic.

In axenic culture, many workers prefer to use artificial seawater media such as the ASP series developed by Provasoli and his co-workers (Provasoli et al. 1957, Provasoli 1958, 1963), rather than enriched seawater media (e.g., Provasoli's ES; see Chapter 3 and Appendix A).

5.2. Sterility Tests for Axenic Cultures

Established cultures can be tested for axenicity by several methods. The most common sterility tests use general media for marine bacteria such as ST3 medium and STP medium (Tatewaki and Provasoli 1964), commercially available Bacto Marine Broth 2216 (Difco), or DAPI (4′,6-diamidino-2-phenylindole) for directly staining bacteria on the algal thallus. Five to 10 mL of marine bacterial medium containing 0.5% agar is put into test tubes and autoclaved. One thallus or germling from each test tube obtained through the axenic culturing process is inoculated into each sterility test tube containing bacterial medium, using sterile capillary pipettes in a clean bench. The sterile capillary pipette must be changed for each sample. It is better to embed the sample into the agar medium rather than depositing it on the surface. Controls (with no inoculum, and unialgal culture samples with bacterized or nonautoclaved seawater) must be prepared simultaneously. These test tubes are maintained at 15–25°C for 2 weeks (it is noteworthy that optimum growth temperatures are different among bacterial strains). If bacteria are present, their growth is evident as a white or light yellow cloud around the algal thallus. Axenic strains of the algae are maintained in appropriate artificial culture media (e.g., ASP_7 or $ASP_{12}NTA$) (Provasoli 1963). Fluorescent dyes (e.g., DAPI) are useful for revealing bacteria attached on the surface of cultured thalli. Algal samples are fixed and stained in autoclaved (or preferably, sterile-filtered) seawater containing 1% formalin and 0.5 μg/mL DAPI, and observed under a fluorescence microscope.

6.0. CULTURE MEDIA

Various types of enriched seawater media have been used for culturing macroalgae, such as Erdschreiber medium (Schreiber 1927, Føyn 1934, Gross 1937), Grund medium (von Stosch 1963, 1969), ES medium (Provasoli 1968), PESI medium (Tatewaki 1966), or SWM (McLachlan 1964, Chen et al. 1969). Artificial seawater media such as the ASP series (Provasoli et al. 1957, Provasoli 1963, Iwasaki 1967) are also frequently used. Several media are described in Chapter 3 and recipes are listed in Appendix A.

7.0. ACKNOWLEDGMENT

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8.0. REFERENCES

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