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Axenic clonal cultures of filamentous brown algae: initiation and maintenance

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Abstract: The increasing number of molecular and biotechnological studies involving brown algae has led to a growing demand for bacteria-free cultures. We describe here simple methods to establish axenic clonal cultures of filamentous marine brown algae. Thallus fragments are exposed on agar plates to commercially available paper disks loaded with defined concentrations of antibiotics. Regenerating fragments are isolated and used to initiate axenic algal cultures in liquid medium or on agar. We found the following agents to be tolerated by algae and active against marine bacteria: ciprofloxacin, chloramphenicol, rifampicin, polymyxin B, and kanamycin. Axenic agar cultures are maintained by serial transfer on agar, or re-introduced into liquid medium. Although we recommend transfer of agar cultures in 2-3 month intervals, we have observed survival times on agar of up to 2 years without attention.

Résumé : *Cultures clonales axéniques d'algues brunes filamenteuses.* La multiplication des travaux de biologie moléculaire et des applications biotechnologiques sur les algues brunes entraîne une augmentation parallèle de la demande en cultures axéniques. Nous décrivons ici une technique simplifiée par rapport aux méthodes existantes permettant d'éliminer les contaminants bactériens de cultures d'algues brunes filamenteuses. Des morceaux de thalle cultivés sur milieu gélosé sont mis en présence de disques imprégnés d'antibiotiques achetés dans le commerce. Les filaments en cours de régénération sont ensuite transférés en milieu liquide ou sur un nouveau milieu gélosé afin d'initier la culture axénique. Nos observations indiquent que les antibiotiques suivants sont à la fois bien tolérés par les algues et actifs contre les bactéries marines : ciprofloxacine, chloramphénicol, rifampicine, polymyxine B et kanamycine. Les cultures axéniques sont ensuite maintenues par transfert régulier sur un nouveau milieu gélosé, ou en milieu liquide. Bien que nous recommendions de transférer les cultures sur gélose tous les 2-3 mois, nous avons observé que celles-ci peuvent survivre jusqu'à deux ans sans attention particulière.

Keywords: Antibiotics ● Axenic ● Clonal ● Culture ● *Ectocarpus* ● Phaeophyceae

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Introduction

Laboratory studies on the biology and biochemistry of multicellular marine algae are traditionally based on clonal unialgal cultures. These provide genetically well-defined material useful for various types of studies, such as taxonomy, reproduction, life histories or biochemistry. In general understanding the status "unialgal" describes an algal culture free from foreign eukaryotic organisms and Cyanophytes, while bacteria are still present.

However, contaminating bacteria and their products cannot be tolerated in many fundamental and biotechnological studies, where axenic cultures are an essential prerequisite. Presence of foreign DNA is unacceptable for studies on nuclear, mitochondrial or plastid genomes in algae (Oudot-Le Secq et al., 2001; Peters et al., 2004a & b). Likewise, many questions in algal chemical ecology such as those relating to bacterial biofilms on algal surfaces can only be investigated if axenic cultures are available (Küpper et al., 2002), and bacterial metabolites are often undesirable in biotechnological applications. This results in an increased demand for simple methods to establish and maintain axenic cultures of multicellular algae.

Axenic cultures of *Ectocarpus* were first described in the early 1960s (Boalch 1961a & b). This technique, like others of that period (Loiseaux & Rozier, 1978), however, is based on antibiotic treatment in liquid medium, and did not receive widespread acceptance. A more simple and rapid method to establish axenic algal cultures used antibiotic disks available for medical diagnostics on agar plates (Bradley et al., 1988).

We have modified this method and describe here our protocol, which we routinely use to produce axenic clonal cultures of filamentous brown algae, in particular *Ectocarpus* sp. and gametophytes of Laminariales.

Culture media, chemicals, tools and culture conditions

For all procedures described below, natural seawater enriched according to Provasoli (PES) is used as described by Starr & Zeikus (1987). Agar plates in 60 mm diameter polystyrene Petri dishes are prepared by autoclaving 1% agar (microbiology quality) with PES medium. They must be stored for 2 to 3 weeks before use until all condensed liquid water has disappeared. Paper disks impregnated with the following antibiotics are obtained from Becton Dickinson GmbH (Heidelberg, Germany; www.bd.com): rifampicin (RA, 30 µg), chloramphenicol (C, 30 µg), kanamycin (K, 30 µg), polymyxin B (PB, 300 IU), and ciprofloxacin (CIP, 5 µg). Penicillin G (P), neomycin sulfate (N) and rifampicin in powder form are purchased from Sigma (www.sigmaaldrich.com). P and N stock solutions (10 $mg.mL^{-1}$) are made in autoclaved sea water and sterilized using 0.2 µm cellulose acetate syringe filters (Orange Scientific, www.orangesci.com). RA (50 $mg.mL^{-1}$) is dissolved in DMSO.

We use a dissecting microscope with variable magnification up to 63 x and a laminar flow unit for aseptic manipulations.

The unialgal cultures to be axenized must be pre-incubated for optimum growth. The following conditions are suitable for many marine algae: temperature range between 12 and 15°C, illumination by 20 to 30 $\mu E.m^{-2}.s^{-1}$ from daylight type fluorescent lamps for 16 $h.day^{-1}$ and weekly transfers to fresh culture medium. Our tool kit contains the following items:

Surgical blade with holder, or razor blade; standard stainless steel dissection needles; extra-fine straight inoculation needles; stainless steel forceps; a supply of cm-size pieces of sterile filter paper, sterile polystyrene Petri dishes, heat-sterilized Pasteur pipettes, and a pencil-style diamond glass cutter. We use 96% ethanol dispensed from a dropper bottle to sterilize our steel forceps, dissection and inoculation needles by wiping with ethanol-soaked paper tissue. Traditionally, tools like stainless-steel dissection needles are flame-sterilized, which however causes destructive corrosion. Having abandoned this step, in years of work we have encountered no case of contamination with this simplified practice using 96% ethanol.

To confirm axenicity, we routinely use ZMA10 agar medium (Green et al., 2004) or half-strength Provasoli medium enriched with 1% glucose and tryptone. An incubation of several weeks is necessary to confirm the absence of bacteria. Bacterial contamination can also be directly detected under the fluorescence microscope (Axioscope 2, Zeiss), using a double DAPI (4',6-diamidino-2-phenylindole)-CFSE (carboxyfluorescein diacetate succinimidyl ester) staining. The latter is a vital stain accumulating in the cytoplasm of living cells. The culture is incubated in a mixture of 50 $ng.mL^{-1}$ DAPI and 10 $\mu mol.mL^{-1}$ CFSE for 15 min in the dark, then washed in sterile seawater. It is then observed under UV light illumination using the following filtersets: (DAPI) Exc: 365 nm, FT 395 nm, Em: LP 420 nm; (CFSE) Exc: BP 450-490 nm, FT 510 nm, Em: 515 nm. If present, bacteria exhibit both a blue and green fluorescence at the algal surface (Fig. 2). In case of very low contamination levels, this double staining is preferred to a classical DAPI staining, because it enables to distinguish with increased confidence between bacteria and inert particles such as dust.

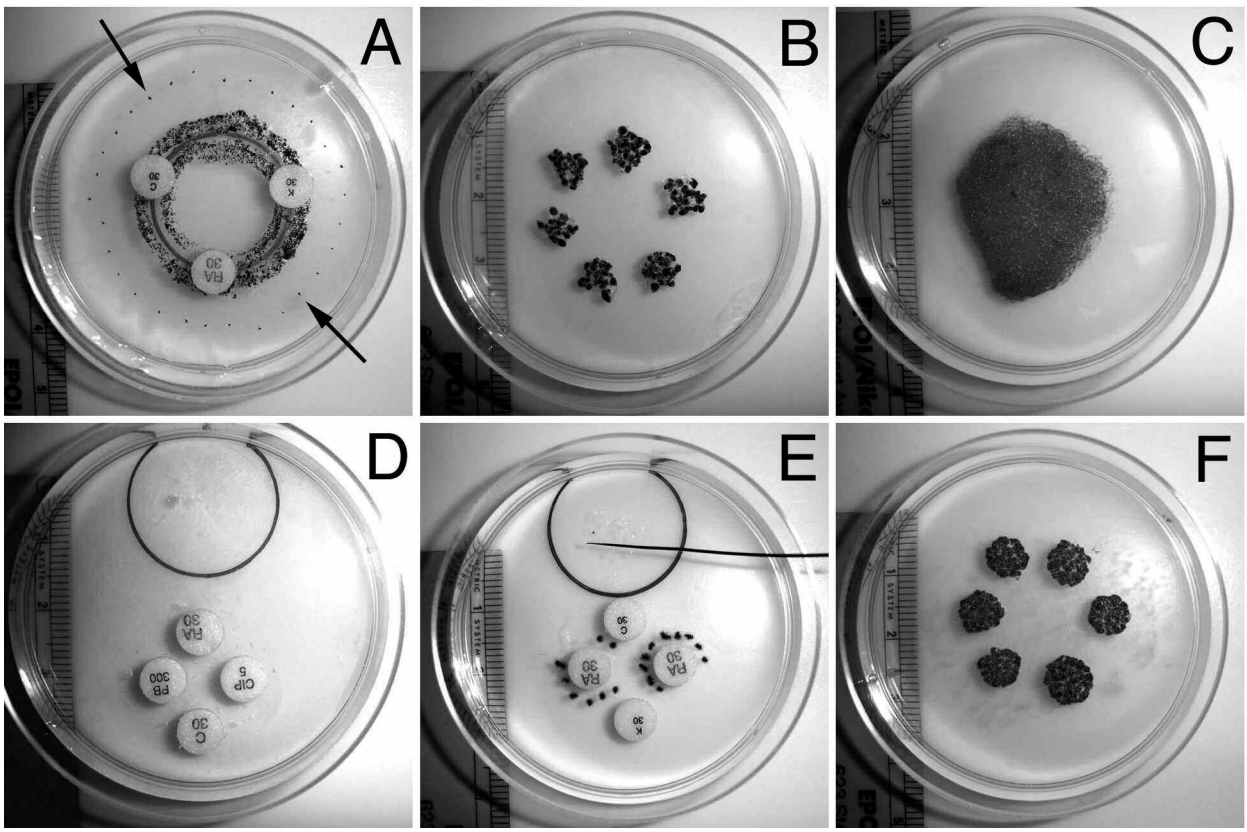

Figure 1. Establishment of axenic cultures. **A.** *Macrocystis pyrifera*. Fragments of unialgal male gametophyte clone spread on agar surface and exposed to antibiotic disks. Selected clean regenerates had been transferred from original location to periphery of agar plate (arrows) for confirmation of growth potential and absence of bacteria. **B.** *Ectocarpus siliculosus*. Female partheno-sporophyte, axenic strain in agar-to-agar maintenance. **C.** *Ectocarpus siliculosus*. Axenic male gametophyte, returned from liquid culture medium to agar culture. **D.** Modified method. *Choristocarpus tenellus*. Algal fragments with apical cells were distributed individually from liquid medium (circular ink mark) with steel needle (as shown in E) along the circumference of four antibiotic disks. One week after start of experiment, algal fragments not visible. **E.** Same arrangement as D, with virus-infected fragments of *Hincksia hincksiae*. Two months after start of experiment; several clean regenerates have already been removed to establish axenic clonal cultures. **F.** *Macrocystis integrifolia*. Axenic female gametophyte, inoculum from liquid culture onto agar.

Figure 1. Obtention de cultures axéniques. **A.** *Macrocystis pyrifera*. Fragments clonaux d'un gamétophyte mâle. Certains fragments ont été transférés à la périphérie de la boîte (flèches) afin de les laisser se régénérer et de confirmer l'absence de croissance bactérienne à leur surface. **B.** *Ectocarpus siliculosus*. Parthéno-sporophyte femelle, souche axénique maintenue par transfert en série sur agar. **C.** *Ectocarpus siliculosus*. Gamétophyte mâle, souche axénique après transfert de milieu liquide à milieu gélosé. **D.** Méthode perfectionnée. *Choristocarpus tenellus*. Des filaments contenant des cellules apicales ont éte transférés d'une culture liquide sur la gélose à l'aide d'une aiguille montée (voir E) et disposés autour des disques imprégnés d'antibiotiques. Après une semaine d'incubation, les filaments ne sont toujours pas visibles à l'œil nu. **E.** Méthode perfectionnée. Même disposition qu'en D, avec des filament de *Hincksia hincksiae* infectés par un virus. Photographie prise après deux mois d'incubation, alors que plusieurs thalles en cours de régénration ont déjà été prélevés pour établir des cultures clonales axéniques. **F.** *Macrocystis integrifolia*. Gamétophyte femelle, souche axénique cultivée en milieu liquide et transférée sur agar.

Antibiotic treatment

Algal biomass corresponding to a few mm³ packed volume is washed in fresh culture medium and placed in the center of a dry polystyrene Petri dish. All liquid water is removed with a Pasteur pipette, which leaves the algal material with capillary water on the plastic surface. Now the algal mass is thoroughly chopped with a surgical blade or a razor blade into fragments of 10 to 20 cells in length. Immediately afterwards, the algal mass is scraped together to form a small ball on the bottom of the Petri dish. This mass should have a volume of 1 to 2 mm³. It is placed with dissecting needles onto sterile dry filter paper for about 10 to 20 seconds, until all free liquid water is removed from the algae.

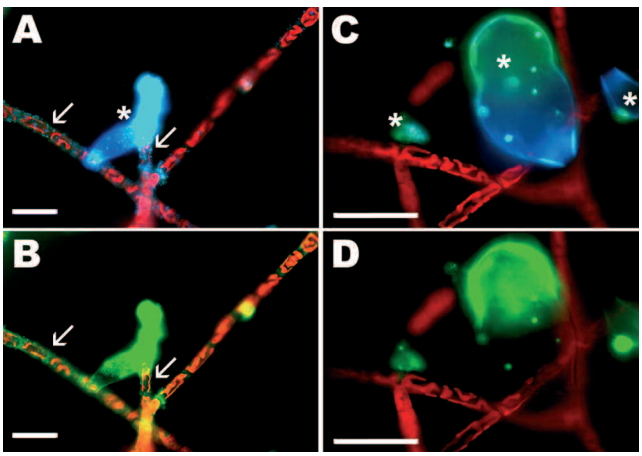

Figure 2. Double staining with DAPI and CFSE of a non axenic and an axenic culture of *Ectocarpus sp.* infected with the oomycete parasite *Eurychasma dicksonii*. **A & C.** DAPI staining. **B & D.** CFSE staining. The bacteria normally present at the algal surface (A, B, arrows) are absent in the axenic culture (C, D). *: mature sporangia of *E. dicksonii* containing spores. Scale bars: 50 µm.

Figure 2. Double coloration DAPI-CFSE d'une culture non axénique (A, B) et axénique (C, D) d'*Ectocarpus sp.* infecté par l'Oomycète parasite *Eurychasma dicksonii*. **A & C.** Coloration DAPI. **B & D.** Coloration CFSE. Les bactéries normalement présentes à la surface des filaments (A, B, flèches) ne sont jamais observées dans la culture axénique (C, D). *: sporocystes d'*E. dicksonii*, contenant quelques spores. Echelle : 50 µm.

This semi-dry algal mass is now placed onto an agar surface in a petri dish. However, before that, we recommend to place a circular ink mark of about 20 mm in diameter onto the bottom outside of the agar dish (Fig. 1A). The algal material will take up traces of water from the agar. Under a dissecting microscope, at low magnification (ca. 10x), a sterile needle is used to roll the algal ball in a circular path along the mark on the bottom of the dish. The rolling movement of the algal ball leaves behind a trail of fragments on the surface of the agar. If the algal ball has drawn too much moisture from the agar, the rolling action can be interrupted by an additional drying step on filter paper.

The inoculation process is finished when a trail of algal fragments 6 to 8 mm wide has been spread in a circle following the ink mark (Fig. 1A). After a new drying step the rest of the algal ball can be used to inoculate a second agar plate, and the final remainder can be re-dispersed in liquid culture medium to continue unialgal culturing in order to save valuable material.

We start our antibiotic treatment as follows: Three to five antibiotic disks (RA, C, K, PB, CIP) are placed with a sterile forceps onto the algal trail on the agar in equidistant fashion, and gently pressed to the agar surface to facilitate contact for diffusion of antibiotics (Fig. 1A). Subsequently, the agar dish is sealed with Parafilm™ and exposed to culture conditions.

Occasionally, we encountered materials that needed increased attention and had to be treated more specifically for various reasons: small quantities of valuable cultures, or host-pathogen systems, where requirements of two partner organisms had to be considered. For such cases, we have modified our standard technique and obtained bacteria-free cultures of *Ectocarpus* hosts with plasmodiophoralean and heterokont parasites such as *Eurychasma dicksonii* (Müller et al., 1999; Küpper et al., 2006) and *Maullinia ectocarpii* (Maier et al., 2000), as well as various brown algal species including their virus infections (Müller et al., 1998): A circular ink mark of ca. 15 mm diameter is applied to the bottom outside of a 60 mm agar Petri dish. Inside the Petri dish, on the surface of the agar and opposite the mark, 4 antibiotic disks are placed at ca. 2 mm distance from each other (Fig. 1D). The algal specimen is introduced in a plastic Petri dish with culture medium. Suitable fragments of the sample are selected and cut to a size of about 500 µm under the dissecting microscope as follows:

The tip of a Pasteur pipette is cut with a diamond glass cutter. The edge of the pipette is then used as a cutting instrument by gentle pressure against the semi-soft bottom surface of the plastic Petri dish. The selected algal fragments are collected with the same pipette and transferred to a new Petri dish for a washing step with fresh culture medium. Then about 40 to 50 selected fragments are accumulated and placed with a small amount of liquid onto the agar within the circular area marked before. The next step is to use a fine steel needle to take up or push individual fragments from the culture liquid and deposit them around the circumference of the four antibiotic disks in 100 to 150 µm distance (Fig. 1E). The inocula must be located on the surface of the agar without liquid. The Petri dish is sealed and subjected to culture conditions.

Selection of bacteria-free algal fragments

After one to 3 weeks of antibiotic treatment the algal filaments have started to regenerate on the agar, and the dish is examined in dark field illumination at ca. 50 x magnification under a dissecting microscope. The Provasoli medium offers good growth conditions for bacteria, and colonies are easily identified as turbid areas, either well circumscribed or diffusely penetrating into the agar. In the vicinity of antibiotic disks, bacterial growth is retarded or totally suppressed, depending on the type of the antibiotic agent and the distance from the source.

As a next step, we make intermediate transfers of regenerating algal fragments, which appear to be free of

bacteria: We use a very finely pointed steel needle (Fig. 1E), sterilized by wiping with 96% ethanol, to displace individual algal fragments out of the inoculation trail towards the clean sterile periphery of the agar dish. In this way, a series of potentially clean algal fragments is arranged at the periphery of the dish (Fig. 1A, arrows). Two to 3 weeks later, these selected sub-inocula are again screened for the presence of bacteria under the stereomicroscope, and isolates, in which contaminants had passed through the first selection step are easily recognized. Clean and well growing algal isolates are then transferred one by one to new Petri dishes containing sterile liquid culture medium. Several weeks later, after the clean isolates have grown to full thalli, algal tufts are transferred to a Petri dish with agar. Adhering liquid is completely removed with a Pasteur pipette, leaving the alga on the surface of the agar with minimum capillary water only. Most filamentous brown algal thalli tolerate this treatment and continue to grow axenically in a semi-aerial manner, partly penetrating into the agar, partly extending into the air head space (Fig. 1B & C). This type of culture can also serve as an additional test for the presence of bacteria.

In a different technical approach we also use antibiotics incorporated in agar plates, in modification of a published protocol (Aguirre-Lipperheide & Evans, 1992). We found that a cocktail of RA, N and P (at a final concentration of 100 $\mu g.mL^{-1}$ each) is very efficient against marine bacteria. This mixture is usually more effective than RA alone, although neomycin or penicillin G by themselves have little or no effect on bacterial growth and abundance. This latter method has the advantage of increasing the agar surface available for antibiotic treatment, therefore increasing the probability of success. In difficult materials, this treatment is combined with the use of antibiotic discs as described above.

Long-term maintenance of axenic cultures

We prefer to maintain most of our axenic stock cultures on agar, since serial agar-to-agar passages are convenient, and tolerate longer transfer intervals. Furthermore, the invasion of contaminants, although occurring very rarely, can be detected easily and at an early stage. Cultures are maintained in 60 mm Petri dishes with Provasoli agar by serial transfers of half the biomass into a new dish with a sterile inoculation needle. Our preferred style is to inoculate 6 spots as seen in Fig. 1B & F in intervals of 2-3 months. Shorter or longer intervals are possible. Although we have not attempted to determine maximum survival times, we found axenic cultures to survive considerable time spans on agar without attention (Table 1).

For various purposes, we occasionally inoculate axenic agar cultures into liquid culture medium. Subsequently, they can be returned to agar again by depositing algal material with some culture liquid on an agar plate, and removing all liquid with a Pasteur pipette. Such cultures appear slightly different from direct agar-to-agar inocula (Fig. 1C).

Discussion

Our main motive to eliminate microbial contaminants from macroalgal cultures was to standardize and simplify the long-term maintenance of important stock materials for experimental scientific and mariculture use. The techniques described here are simple and based on standard laboratory equipment. Reference collections of axenic *Ectocarpus* strains for genomic and post-genomic studies (Gachon et al., 2007) and *Macrocystis* stock for mariculture projects (Westermeier et al., 2006 & 2007) were established and are presently maintained in time- and space-saving manner on agar with the methodology described here. Although we routinely use transfer intervals of 2 - 3 months, we have found axenic agar cultures to survive for up to two years without attention, indicating that transfer intervals may be expanded for stock cultures. An additional advantage of agar cultures is that invasion by contaminants can be easily recognized at an early stage, in contrast to liquid cultures.

In particular, it should be highlighted that the techniques presented here have worked well and reliably with the model organism *Ectocarpus* sp. and a number of virus-infected filamentous brown algae, as well as gametophytes of ecologically and biotechnologically important kelp species such as *Laminaria* sp. and *Macrocystis* sp. The techniques described here can also be successfully applied to other groups of algae. With slight modifications, fragments of multicellular thalli that are capable to regenerate, or propagules seeded on agar can be exposed to antibiotic treatment followed by selection of clean isolates.

Most of our experimental materials, mainly uniseriate filamentous gametophytes and microthalli of heteromorphic brown algae such as various Ectocarpales, *Laminaria* and *Macrocystis* grow well and with normal habitus in total absence of microbes. Nevertheless, although useful for conservation and experimentation, axenic cultures are clearly artificial, protected laboratory constructs. This view is confirmed by recent studies, which indicate that in more natural and complex habitats, eukaryotic multicellular algal hosts may be strongly influenced by their microbial epiflora. Marine bacteria have been shown to modulate settlement of spores in *Ulva* (Wheeler et al., 2006) and subsequent morphological differentiation in *Ulva*, *Enteromorpha* and *Monostroma*, with significant impact on host algal taxonomy (Hayden et al., 2003; Matsuo et al., 2003 & 2005; Marshall et al., 2006). Quorum sensing and chemical signalling between

Table 1. List of strains used in this study. Strain codes - CCAP: Culture Collection of Algae and Protozoa, Oban, Scotland (http://www.ccap.ac.uk); DGM: private collection of D.G. Müller, Konstanz, Germany; KU: Macroalgal Culture Collection, Kobe University Japan. Some confirmed survival times on agar plates in months are listed in parentheses under Remarks.

Tableau 1. Liste des souches utilisées dans cette étude. Code des souches – CCAP : collection de cultures d'algues et de protozoaires, Oban, Ecosse (http://www.ccap.ac.uk) ; DGM : collection privée de D.G. Müller, Constance, Allemagne ; KU : collection de cultures de macroalgues, Kobe University Japan. Certains temps de survie confirmés sur les plaques d'agar (en mois) sont indiqués entre parenthèses.

Species	Strain code	Remarks	References
Choristocarpus tenellus	KU-1152	unialgal and axenic	
Ectocarpus siliculosus	CCAP 1310/332	female parthenosporophyte axenic on agar	
Ectocarpus siliculosus	KU-1796	male gametophyte axenic on agar	
Ectocarpus siliculosus	CCAP 1310/49	(16) EsV-1 virus type culture axenic on agar	(Müller & Knippers, 2001)
Ectocarpus host with *Eurychasma dicksonii*	CCAP 4018/2	With oomycete infection	(Müller et al., 1999; Küpper et al., 2006)
Hincksia hincksiae	DGM	Hinc-V1 virus type culture axenic on agar	(Parodi & Müller, 1994)
Laminaria digitata	DGM	(24) female gametophyte, axenic on agar	
Lessonia nigrescens	DGM	(20) male gametophyte on agar	
Macrocystis integrifolia	DGM	female gametophyte axenic on agar	(Westermeier et al., 2007)
Macrocystis pyrifera	DGM	(22) male and (23) female gametophyte axenic on agar	(Westermeier et al., 2006)
Maullinia ectocarpii	CCAP 1538/1	axenic, alternating agar and liquid, in *Ectocarpus* host	(Maier et al., 2000)
Myriotrichia clavaeformis	CCAP 1325/2 KU-1298	(16) axenic on agar	(Müller et al., 1996)
Pylaiella littoralis	DGM	(16) axenic on agar	

host and pro- and eukaryotic epiflora may determine the composition of biofilms and the degree of fouling (Tait et al., 2005; Qian et al., 2007). Even apparently autonomous algal activities such as spore release in epiphytic red algae may be controlled by chemical signals from bacterial communities (Weinberger et al., 2007). Analysis of these and related phenomena in chemical ecology will be another important use for axenic algal cultures.

The methods described here offer an easy way to obtain bacteria-free algal cultures with a simple straightforward method and minimum effort. Only standard laboratory equipment and inexpensive materials are required. The commercial market of antibiotic disks for medical diagnostics represents a reliable source for a great variety of standardized antibiotic agents. The seven antibiotics used in our study are tolerated by brown algae and highly active against marine bacteria, but additional compounds may be similarly useful and may be substituted or combined with these.

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