Isolation and Culture of Lichen Photobionts and Mycobionts

ISAO YOSHIMURA, YOSHIKAZU YAMAMOTO, TAKETO NAKANO, and JEFFREY FINNIE

Introduction

Lichens are dual organisms formed from a symbiotic association of a fungus, the mycobiont, and an alga and/or cyanobacterium, the photobiont, in which numerous photosynthetic cells are intertwined in a matrix of fungal hyphae. Such definitions raise the question as to whether lichens are technically individual organisms. Many aspects of lichen biology are concerned with the interactions of these different organisms. The separation, isolation and culture of the symbionts offers the scientist a fascinating opportunity to study the components and contribute to the understanding of the nature of the symbiosis in lichens. The culture of mycobionts, photobionts and lichen thalli is central for the establishment of experimental systems for lichens, needed to solve questions associated with symbiosis biology. In addition, they are essential to solving the many fundamental problems of lichen physiology, morphogenesis and molecular biology.

Cultures of lichen symbionts were thought to be too difficult to study mainly because of the time consuming, long-term techniques necessary for successful culture of the symbionts. However, Ahmadjian (1967b) in his ground-breaking research stimulated interest in the culture of mycobionts and photobionts for many lichenologists. In the past two or three

✉ Isao Yoshimura, Kochi Gakuen College, Biology Laboratory, 292 Asahitenjincho, Kochi, 780-0955, Japan (*phone* +81-88-840-1121, ext. 432; *fax* +81-88-840-1124; *e-mail* isaoyosh@inforyoma.or.jp)
Yoshikazu Yamamoto, Akita Prefectural University, Department of Biological Production, Faculty of Bioresource Sciences, Laboratory of Advanced Bio-production Science, 241-7, Kaidobata-nishi, Shimoshinjyo-nakano, Akita, 010-0195, Japan
Taketo Nakano, Hiroshima Institute of Technology, Department of Environment and Information, 2-1-1, Miyake, Saiki-ku, Hiroshima, 731-5193, Japan
Jeffrey Finnie, University of Natal, Department of Botany and Zoology, Pietermaritzburg, Private Bag X01, Scottville, 3209, Republic of South Africa

I. C. Kranner et al. (eds.), *Protocols in Lichenology*

decades, research on mycobionts, photobionts, and on resynthesis of lichens from isolated symbionts has made considerable progress. Cultures of lichen mycobionts and photobionts can be obtained by different methods as outlined in Fig. 1.

Lichen symbionts (mycobionts and photobionts) are usually very difficult to culture using nutritionally rich media. This is because contaminating bacteria, foreign algae, and fungi may grow more rapidly than the slowly growing lichen symbionts. Contaminating organisms such as moulds, yeasts, and bacteria are located not only on the surface of the lichen thallus, but also in the medulla, and the gelatinous sheath of the photobionts, especially of cyanobacteria. To obtain sterile lichen symbionts several aseptic steps are necessary. After obtaining both symbionts in axenic condition, one may make an association of the two symbionts to reform a lichen thallus. Unfortunately, only a few examples of the reformation of new lichen thalli have been reported (see Chapter 3).

The aim of this chapter is to describe protocols for the isolation and culture of mycobionts and photobionts from lichen thalli. Subprotocol 1 describes alternative methods for culturing mycobionts, and Subprotocol 2 provides several alternative techniques for culturing photobionts. These culture techniques have been reported in the literature, but we have added additional notes based on our own observations. Comprehensive reviews of the techniques that can be used for the isolation of symbionts have been described by Ahmadjian (1967a,b, 1973) and Galun (1988).

Outline

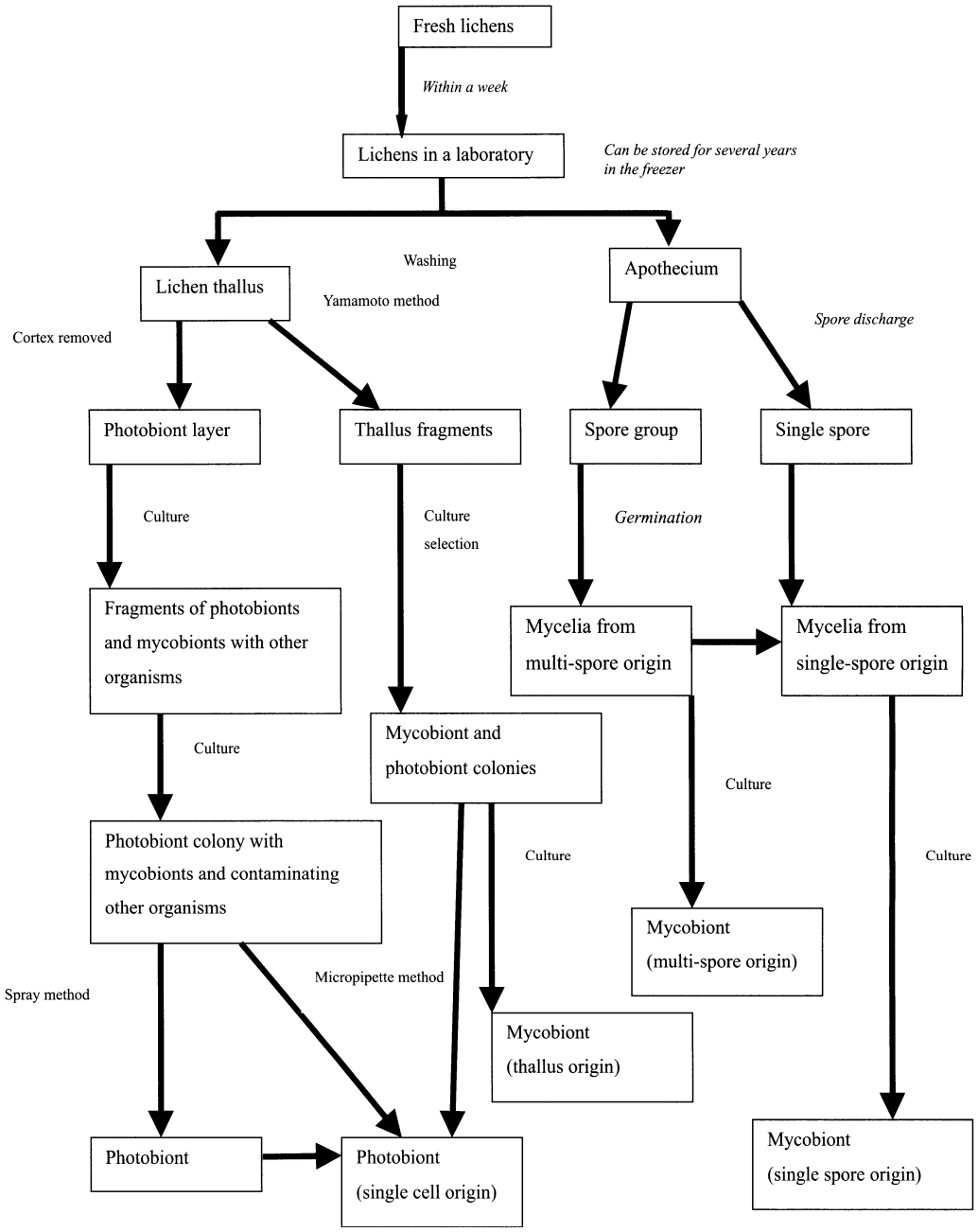

Fig. 1. Flow chart outlining the origin of mycobiont and photobiont cultures from lichen thalli.

Subprotocol 1
Mycobiont Culture

Note: You must carry out all treatments on a clean laminar flow bench or under sterile conditions, except for the pre-washing stage. All equipment should be autoclaved (15 - 20 min, 121°C, 1 atm) or oven-dried (30 min, 180°C) before use.

Materials

Equipment

- Compound microscope
- Dissecting microscope
- Inverted microscope
- Autoclave
- Incubator
- Sonicator
- Centrifuge
- Laminar flow bench or Clean box

Sources of mycobionts

We recommend that lichens should be freshly collected from the field and used within one week. However, lichens may be stored in the desiccated state for a few weeks, or in a freezer for several years (Yoshimura et al. 1990).

Mycobionts can be isolated from ascospores, conidia, isidia, soredia, and thallus fragments (Ahmadjian 1993). For laboratory cultures the most usual method of isolating mycobionts is to start from discharged spores, primarily ascospores. Another useful method for obtaining mycobionts and photobionts is to dissect out thallus fragments. This may result in large quantities of purified mycobiont. The use of thallus fragments for isolation of mycobionts or photobionts is described in detail in Chapter 2.

Mycobiont cultures stored at Akita Prefectural University and Kochi Gakuen College are listed in Table 1.

Table 1. Mycobiont cultures stored at Akita Prefectural University and Kochi Gakuen College

Acarospora fuscata (Nyl.) Arnold
Alectoria lata Tayl.
Alectoria ochloreuca (Hoffm.) Massal.
Amygdalia panaeola (Ach.) Hertel & Brodo
Anaptychia palmurata (Michux) Vain.
Anzia colpota Vain.
Anzia gregoriana Muell. Arg.
Anzia hypoleucoides Muell. Arg.
Anzia japonica (Tuck.) Muell. Arg.
Anzia leucobatoides Zahlbr.
Anzia opuntiella Muell. Arg.
Arthonia tumidula (Ach.) Ach.
Asahinea chrysantha (Tuck.) Culb. & Culb.
Asahinea kurodakensis (Assah.) Culb. & Culb.
Baeomyces absolutes Tuck.
Baeomyces placophyllus (Lamb.) Ach.
Bryocaulon divergens (Ach.) Kaernef.
Bryoria furcellota (Fr.) Brodo & Hawksworth
Calicium japonicum Asah.
Caloplaca leptopisma Zahlbr.
Caloplaca scopularis (Nyl.) Lettau
Candelariella vitellina (Hoffm.) Muell. Arg.
Cetraria delisei (Bory) Th. Fr.
Cetraria islandica (L.) Hoffm.
Cetraria islandica subsp. orientalis (Asah.) Koernef.
Cetraria nivalis (L.) Ach.
Cetraria ondontella (Ach.) Ach.
Cetrelia japonica (Zahlbr.) Culb. & Culb.
Cetrelia nuda (Hue) Culb. & Culb.
Chaenotheca brunnaola (Ach.) Muell. Arg.
Cladia aggregata (Sw.) Nyl.

Table 1. Continuous

Cladina arbuscula (Wallr.) Hale & Culb.
Cladina mitis (Sandst.) Hustich
Cladina portensoa (Dufour.) Follm.
Cladina rangiferina (L.) Nyl.
Cladina stellaris (Opiz.) Brodo
Cladonia bacilioformis (Nyl.) Glueck
Cladonia bellidiflora (Ach.) Schaerer
Cladonia boryi Tuck.
Cladonia cristatella Tuck.
Cladonia cyathomorpha Wats.
Cladonia graciliformis Zahlbr.
Cladonia humilis (Wirth) Laundon
Cladonia merochlorophaea Asah.
Cladonia pityrea (Floerke) Fr.
Cladonia poccillum (Ach.) O. Rich
Cladonia ramulosa (Wirth) Laundon
Cladonia subpityrea Sandst.
Cladonia vulcani Savicz.
Cornicularia aculeatum (Schreber) Link.
Cyphellium tigirare Ach.
Dactylina ramulosa (Hook.) Tayl.
Dermatocarpon miniatum (L.) Mann.
Dermatocarpon reticulatum Magnusson
Diploschistes scuruposus (Schreber) Norm.
Durietzia crenulata (Hook.) Yoshim.
Eriooderma velligerum Tuck.
Evernia divaricata (L.) Ach.
Evernia esosrediosa (Muell. Arg.) Du Rietz
Evernia prunastri (L.) Ach.
Everniastrum cirrhatum (Fr.) Sipman
Faraminella ambigua (Wulf. In Jaeg.) Fricke Meyer
Flavoparmelia caperata (L.) Hale

Table 1. Continuous

Graphis cervina Muell. Arg.
Graphis connectans Zahlbr.
Graphis proserpens Vain.
Graphis scripta (L.) Ach.
Graphis tenella Ach.
Gymnoderma lineare Yoshim. & Sharp
Haematomma ochrophaeum (Tuck.) Massal.
Haematomma ventosum (L.) Massal.
Heterodermia diademata (Tayl.) Awasthi
Heterodermia obscurata (Nyl.) Trev.
Heterodermia pandurata (Kurok.)
Heterodermia pseudospeciosa (Kurok.) Culb.
Hypogymnia physodes (L.) Nyl.
Icmadophila ericetorum (L.) Zahlbr.
Lasalia papulosa (Ach.) Llano
Lasallia asiae-orientalis Asah.
Lasallia papulosa (Ach.) Llano
Lasallia pensylvanica (Hoffm.) Llano
Lecanora argopholis (Ach.) Ach.
Lecanora expectans Darb.
Lecanora fuscata Nyl.
Lecanora muralis (Schreber) Rabenh.
Lecanora pulverulenta Muell. Arg.
Lecanora stenospora Stiz.
Lecidea confluens (Weber) Ach.
Lecidea inopsis Th. Fr.
Leprocaulon arbuscula (Nyl.) Nyl.
Letharia columbiana (Nutt.) Thomson
Letharia vulpina (L.) Hue
Lobaria adscripturiens (Nyl.) Hue
Lobaria linita (Ach.) Rabenh.
Lobaria spathulata (Inum.) Yoshim.

Table 1. Continuous

Lopadium ferrugineum Muell. Arg.
Megalospora sulphurea Meyen
Melanelia stygia (L.) Esslinger
Menegazia terebrata (Hoffm.) Massal.
Nephroma arcticum (L.) Torss.
Nephroma helveticum Ach.
Nephromopsis endocrocea Asah.
Nephromopsis ornata (Muell. Arg.) Hue
Niebla homalea (Ach.) Rundel & Bowler
Normandina pulchella (Borr.) Nyl.
Ochrolechia parellula Muell. Arg.
Ochrolechia trochophora (Vain.) Oshio
Ochrolechia yasudae Vain.
Parmotrema austrosinense (Zahlbr.) Hale
Parmotrema tinctorum (Delise ex Nyl.) Hale
Peltigera aphthosa (L.) Willd.
Peltigera canina (L.) Willd.
Peltigera neckelli Hepp ex Muell. Arg.
Peltigera polydactyla (Necker) Hoffm.
Peltigera ponijensis Gyelnik
Pertusaria corallina (L.) Arn.
Pertusaria laeviganda Nyl.
Pertusaria ophthamaliza Nyl.
Phaeographina pseudomontagnei Nakanishi
Phaeophyscia endococcina (Koerber) Moberg
Physcia adscendens (Fr.) H. Olivier
Physcia phaea (Tuck.) Thomson
Pilophorus clavatus Th. Fr.
Platismatia interrupta Culb. et Culb.
Platismatia lacunosa (Ach.) Culb. & Culb.
Porpidia albocaerulescens (Wulfen) Hertel & Knoph
Porpidia macrocarpa (DC. In Lam. & DC.) Hertel & Schweb..

Table 1. Continuous

Pseudevernia intensa (Nyl.) Hale & Culb.
Pseudocyphellaria aurata (Ach.) Vain.
Pseudoephebe pubescens (L.) M.
Pseudoevernia furfurcea (L.) Zopf
Pseudoevernia olivetorum Zopf.
Puncteria rudecta (Ach.) Krog.
Pyrenula japonica Kurok.
Ramalina exilis Asah.
Ramalina leiodea (Nyl.) Nyl.
Ramalina litoralis Asah.
Ramalina menziesii Tayl.
Ramalina pacifica Asah.
Ramalina roesleri (Hochst.) Nyl.
Ramalina subbreviuscula Asah.
Ramalina subfraxinea var. *leiodea*
Ramalina subgeniculata Nyl.
Rhizocarpon flavum Dodge & Baker
Rhizocarpon geographicum (L.) DC.
Rimelea reticulata (Tayl.) Hale & Fletcher
Roccella fusiformis DC.
Solorina crocea (L.) Ach.
Solorina saccata (L.) Ach.
Sphaerophorus meiophorus (Nyl.) Vain.
Stereocaulon alpinum Laurer ex Funck
Stereocaulon azureum Yoshim. & Weber
Stereocaulon curtatum Nyl.
Stereocaulon dactylophyllum Floerke
Stereocaulon grande (Magn.) Magn.
Stereocaulon paschale (L.) Hoffm.
Stereocaulon sorediiferum Hue
Stereocaulon subcoralloides (Nyl.) Nyl.
Stereocaulon tomentosum Fr.

Table 1. Continuous

Stereocaulon vesuvianum Pers.
Sulcaria sulcata (Lev.) Bystr.
Teloschistes flavicans Norm.
Thamnolia subuliformis (Ehrh.) Lamb.
Thamnolia vermicularis (Swartz) Ach. Ex Schaerer
Thelotrema lepadium (Ach.) Ach.
Thelotrema subtile Tuck.
Trapeliopsis granulosa (Hoffm.) Lumbsch
Tremolechia atrata (Ach.) Ach.
Trypetheliopsis boninensis Asah.
Tuckermannopsis sepincola (Ehrh.) Hale
Umbilicaria aprina Nyl.
Umbilicaria caroliniana Tuck.
Umbilicaria cylindrica (L.) Delise ex Duby
Umbilicaria decusata (Vill.) Zahlbr.
Umbilicaria deusta (L.) Baumg.
Umbilicaria esculenta (Miyoshi) Mink.
Umbilicaria hyperborean (Ach.) Hoffm.
Umbilicaria kisovana Kurok.
Umbilicaria mammulata (Ach.) Tuck.
Umbilicaria muhlenbergii (Ach.) Tuck.
Umbilicaria polyphylla (L.) Baumg.
Umbilicaria proboscidea (L.) Schrader
Umbilicaria torrefacta (Lightf.) Schrader
Umbilicaria vellea (L.) Ach.
Umbilicaria virginis Schaerer
Usnea arizonica Mot.
Usnea bismolliuscula Zahlbr.
Usnea diffracta Vain.
Usnea flexillis Stirt.
Usnea hirta (L.) Weber ex Wigg.
Usnea longissima Ach.

Table 1. Continuous

Usnea misamiensis (Vain.) Mont.
Usnea montis-fuji Mot.
Usnea roseola Vain.
Usnea rubescens Stirt.
Usnea strigosa (Ach.) A. Eaton
Usnea sulphurea (Koenig) Th. Fr.
Vermilacinia combeoides (Nyl.) Spjut & Hale
Vulpicida juniperinus (L.) J. E. Mattsson & M. J. Lai
Vulpicida pinastri (Scop.) J. E. Mattsson & M. J. Lai
Xanthoparmelia subpolyphyloides (Geyln.) Kurok.
Xanthoria elegans (Link.) Th. Fr.
Xanthoria mandsculica (Zahlbr.) Asah.
Xanthoria mawsonii Dodge

Culture media for mycobionts

Ahmadjan (1993) and Pyatt (1973) recommend that the culture media used for spore collection and germination should have a low nutrient content (i.e. should be plain or mineral agar). We have obtained good results using 4 % water agar media at 15°C in the dark. Media for culture of mycobionts include the following:

WA4 medium

4% distilled water agar medium	
Agar	4 g
Distilled water	make up to 100 ml

MY medium

Malt /Yeast extract medium (Ahmadjian 1967a)	
Malt extract	20 g
Yeast extract	2 g
Agar	20 g
Distilled water	make up to 1000 ml

LB Medium

Lilly and Barnett's medium (Lilly and Barnett 1951)

Glucose	10.0 g
Asparagine	2.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$Fe(NO_3)_3 \cdot 9H_2O$	0.2 mg
$ZnSO_4 \cdot 7H_2O$	0.2 mg
$MnSO_4 \cdot 4H_2O$	0.1 mg
Thiamine	0.1 mg
Biotin	5 µg
Distilled water	make up to 1 l

For a solid medium, add 15-20 g of agar to the above ingredients and make up to 1 l

LBG medium

Lilly and Barnett's Gelrite medium (Yamamoto et al. 1998)
Lilly and Barnett's Medium containing 1% w/v Gelrite instead of agar.

Note: Autoclave all media before use and pour in Petri dishes (5 mm thick) or test tubes (5 ml) in the laminar flow bench.

Procedure

Isolation of mycobionts from spores

Spore discharge

1. Clean thalli collected from the field, then leave for a few days after collection to equilibrate with the environment. Alternatively, clean and freeze material, and before use allow a few hours for equilibration.
2. Remove the apothecia or perithecia from the thallus and place into dishes containing distilled water and allow them to soak for about 4 h, or alternatively wash them in running water. Blot dry the spore bearing structures to remove excess water.
3. Fix these structures to the bottom of a plastic petri dish using petroleum jelly and place 4 % water agar medium on the top cover of the Petri dish (Fig. 2). Placing the media in the upper lid limits contamination of the agar.

4. Ensure that the agar is within the discharge range of the spores (ca. 5 - 10 mm). Discharged spores attach to the agar surface either singly or in groups. If you want to carry out single spore isolation, it may be necessary to reduce the discharge time or increase the distance between the ascocarp and the water agar, because multiple spore discharge may occur. Replace the top cover of the Petri dish with new covers containing fresh medium several times at the appropriate interval (according to the discharge time, normally one day).
   Alternatively, spores can be discharged onto glass slides or onto sterilised Parafilm in a damp environment, and the spores washed off with distilled water. Then, transfer the spores to a medium immediately.
5. Seal Petri dishes containing apothecia with Parafilm and store in an incubator at 15°C in the dark.
6. To monitor germination, observe spores discharged onto the water agar medium under an inverted microscope. Remove discharged spores onto a glass microscope "well slide" containing agar medium. Keep the slides in a Petri dish in humid conditions and observe continuously. Monitor germination of spores and mycelial growth either in water or stain using lactic-glycerol-cotton blue.

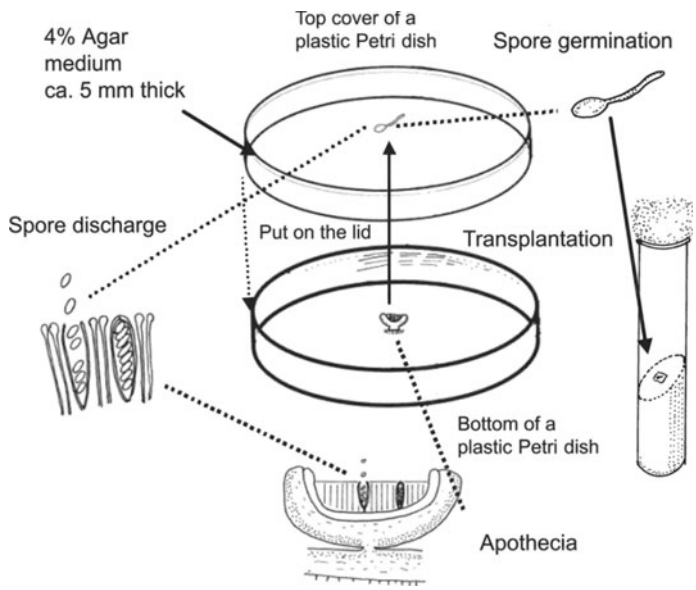

Fig. 2. Isolation of spores after discharge from an apothecium.

Spore germination and mycelial growth

In some lichens, spores germinate within one day after dispersal.

- After germination, excise and transfer to culture tubes or Petri dishes (containing nutrient culture media) small blocks of agar containing the spores.
- Malt-Yeast Extract Medium (Ahmadjian 1967a) and Lilly and Barnett's Medium (Lilly and Barnett 1951) are the culture media most frequently used.

Isolation of mycobionts from the thallus

Where mycobionts cannot be obtained from the spores because of a lack of apothecia, mature spores or poor spore germination, other mycobiont sources may be applicable, e.g. conidia (Vobis 1977) and soredia (Honegger and Bartnicki-Garcia 1991; Honegger et al. 1993). In our experience, isidia are usually too heavily contaminated by epiphytic micro-organisms to be used for isolating lichen mycobionts. Yamamoto et al. (1985) have outlined a method using thallus fragments, see Chapter 2.

- Cut pieces from a fresh thallus using a sterilised razor blade and store in small test tubes containing water (without nutrients) or on wet filter paper in humid conditions at about 15°C. New medullary hyphae usually elongate after a couple of weeks. Excise a portion of the newly elongated hyphae using aseptic technique and transfer to fresh culture media in test tubes. We have found this method useful for obtaining mycobionts of *Cladia rangiferina* and *Parmotrema tinctorum*.
- Sufficient numbers of replicates should be prepared to ensure that the fungal growth obtained is likely to be that of the mycobiont and not a foreign fungus growing on or in the thallus.

Maintaining mycobiont cultures

Mycobionts can be stored for long periods (about one year). However, we recommend that you subculture every 2 to 3 months as follows.

Cut cultured mycobiont colonies into several segments (usually about 5 mg) with a scalpel. Place segments on either MY or LB medium in petri dishes and culture for 2 to 3 months at 15°C in the dark. Repeat this procedure every 2 to 3 months.

Mycobionts in general show maximum growth between 15 and 20°C. The pH of the medium has a significant effect on the development of my-

cobiont cultures. Each species has an optimum, usually in the range of pH 5-6; significantly higher or lower pHs will retard growth. Light is not necessary for maintaining mycobiont cultures.

Lichen mycobiont cultures can be cryopreserved under liquid nitrogen where they remain viable for extended periods of time.

Comments

Spore Discharge

In most lichens, spore discharge can be observed within one day of placing an apothecium in the culture dish. However, in some lichens, spore discharge may be observed only after 2-3 weeks (Table 2). The time after which the first spores discharge after positioning apothecia in the petri dish varies widely and depends on the species, the developmental and metabolic condition of the apothecia at the time of collection, treatment of the thalli after collection and the age of the individual ascocarps. Similarly, the amount and duration of spore discharge also varies widely depending on the above conditions. Maximum spore discharge occurs from ascocarps that are soaked in water for 15 min to 24 h, blotted dry, and then placed in a humid atmosphere (90% RH) where they will dry slowly (Ahmadjian 1993).

In some lichens, e.g. *Porpidia albocaulescens*, *Graphis cervina* (Table 2), the spores separate rather easily after their discharge from the ascocarp, and fall onto the agar surface. In such cases, single-spore culture can be easily carried out. However, in *Umbilicaria vellea* and other species (Table 2), spores remain together in packets of eight (or less). In these species, single-spore isolations are rather difficult. Ahmadjian (1993) recommends further techniques for isolation (e.g. the micropipette method described below).

Yamamoto et al. (1998) found that spore discharge from the apothecia of many lichens was influenced by collecting seasons, storage temperatures and storage periods. Tested species had more or less an endogenous rhythm of sporulation. Winter and spring were good seasons for spore discharge in temperate lichens, although we have found that *Umbilicaria* spores collected in Canada in summer germinated well at this time.

Spore germination

Pyatt (1968) reported that while some spores may germinate as quickly as 2-4 h after discharge, others might take up to 4 or 5 days. In most lichens, spores germinate a few days after their dispersal. In our experience, the germination period varies from 1 to 21 days after dispersal (Table 2). Some spores germinate at the same time as spore dispersal. Lawrey (1984) re-

Table 2. Time required after setting apothecia for spore discharge and germination to occur in a range of lichen species. Culture: Lilly and Barnett's Gelrite Medium at 15 °C under dark conditions.

Lichen names	Number of days required for first spore discharge after setting apothecia	Number of days for spore germination	Number / condition of discharged spores
Anzia hypoleucoides	1	6	single spores
Anzia opuntiella	1	7	single spores
Caloplaca sp.	1	8	single spores
Graphis cervina	1	1	single spores
Graphis cicatricosa	5	14	single or together
Heterodermia pandurata	1	7	single spores
Lasallia papulosa	1 or 2	6	single spores, but muriform
Ochrolechia parerulla	18	18	single spores
Peltigera praetextata	1	4	single spores
Porpidia albocaulescens	1	1	single spores
Ramalina boninensis	1	21	single spores
Sarcographa melanocarpa	4	ca. 30?	single spores
Trypethelium boninensis	3	7	8 spores together
Umbilicaria proboscidea	2	not germinated	8 spores together
Umbilicaria torefacta	2	ca. 4?	single or a few spores together
Umbilicaria vellea	2	6	8 spores together

ported that spores of *Cetraria ciliaris* only germinate six weeks after their dispersal. Rousssard (1969) and Mathey and Hoder (1978) reported that some spores germinate in their ascus. In general, single-cell spores of crustose lichens can germinate faster than two- or many-cells spores. Spores of foliose and fruticose lichens require more time for germination than those of crustose lichens. Muriform spores or single spores with many nuclei may require more time before they germinate.

Spore germination is influenced by medium composition, initial medium pH and culture temperature. Most spores of the species tested germinated on plain agar medium at pH 6 at 15°C. Spores of *Letharia* species could not germinate on agar-medium, but did on Gelrite-medium. Malt-yeast extract medium prevented spore germination of a few species.

Spores of some *Peltigera* species germinated on media supplemented with resins that can absorb phenols that inhibit spore germination.

Modifications of culture media

Studies on the utilisation of nitrogen, either as amino acids or other nitrogenous substances, have yielded such a diversity of results that no general conclusions can be drawn. Addition of most amino acids permit good growth. Only cysteine, cystine, phenylalanine, and tryptophane fail consistently to sustain good growth. Most of the hexoses used as a carbohydrate source permit satisfactory growth. Mannitol, maltose and lactose allow good growth, while citrate, acetate, erythritol and trisaccharides are poor carbohydrate sources (Ahmadjian 1967b; Hale 1983). Both nitrogen and carbohydrate sources can change or modify the morphological and physiological characteristics of the mycobiont. Mycobionts from a variety of species have a requirement for both thiamine and biotin, while some have a requirement for biotin or thiamine alone.

Use of filters

The mycobiont may be cultured directly on agar or supported on filters. Filters can be made from cellulose, esters of cellulose acetate and nitrate, or glass fibre (Oliver et al. 1989, Honegger and Kutasi 1990). The advantage of using filters is that they can be transferred to new media.

Liquid media

Submerged culturing in liquid media necessitates regular replacement of the culture fluid by fresh medium (Honegger and Kutasi 1990, Honegger et al. 1993). Because most taxa tend to form hard, cartilaginous colonies with only marginal growth, it is advantageous to grind the material at regular intervals with a sterile homogeniser (Honegger and Kutasi 1990, Armaleo 1991). It is difficult to generalise about suitable growth media for culture of mycobionts, as different species appear somewhat individualistic in their nutrient requirements (Bubrick 1988).

Effects of pH and light

The effects of the pH of the medium and of light on the morphology or physiology of the mycobionts have received little attention but are probably important. As heterotrophic organisms, lichen mycobionts would not be expected to respond to differences in irradiance or duration of light. However, in non-lichenized fungi light has a greater effect on reproduction than on vegetative growth.

Subprotocol 2
Photobiont Culture

Early investigators placed thin slices of a lichen into an illuminated damp chamber and waited for the moisture and light conditions to cause outgrowths of the algal symbiont and disintegration of the fungal tissue (Ahmadjian 1967b).

The simplest method, in terms of equipment and time, for obtaining photobiont culture was described by Ahmadjian (1967a,b). Nakano modified Ahmadjian's method and has described it in detail (Nakano 1987). Nakano and his co-workers have obtained many photobionts from Japanese lichens (Nakano 1988, Takeshita et al. 1989).

Note: You must carry out all treatments on a clean laminar flow bench or under sterile conditions, except for the pre-washing stage. All equipment should be autoclaved or oven-dried before use.

Materials

Equipment

- Compound microscope
- Dissecting microscope
- Autoclave
- Incubator
- Sonicator
- Centrifuge
- Laminar flow bench or Clean box
- Capillary tube
- Micropipettes

Preparation of a special micropipette (Fig. 3)

1. Heat a glass tube (4-5 mm inside diameter, 20 cm long) at the centre and stretch from both ends, and divide at the centre (a Pasteur pipette can be used).
2. Place a cotton stopper at the wider end of the pipette, and connect a long rubber tube.

3. Wrap the micropipettes in aluminium foil and sterilise. Before use, in the laminar flow bench, heat the small end of pipette and stretch to make a capillary tube by grasping with forceps. Make the diameter of the capillary several times wider than a typical algal cell (about 50 to 75 µm).

Culture media for photobionts

Most photobionts grow easily in culture. While few photobionts have absolute requirements for organic carbon or nitrogen sources, some green algal photobionts grow much faster after the addition of glucose and / or proteose peptone to the culture medium. Media for culturing photobionts include the following.

BBM

BBM Bold's Basal Medium (Deason and Bold 1960; Bischoff and Bold 1963)

$NaNO_3$	250 mg
KH_2PO_4	175 mg
K_2HPO_4	75 mg
$MgSO_4 \cdot 7H_2O$	75 mg
$CaCl_2 \cdot 2H_2O$	25 mg
NaCl	25 mg
EDTA	50 mg
KOH	31 mg
$FeSO_4 \cdot 7H_2O$	4.98 mg
H_3BO_3	11.42 mg
$ZnSO_4 \cdot 7H_2O$	8.82 mg
$MnCl_2 \cdot 7H_2O$	1.44 mg
MoO_3	0.71 mg
$CuSO_4 \cdot 5H_2O$	1.57 mg
$Co(NO_3)_2 \cdot 6H_2O$	0.49 mg
Make up to 1 l with distilled water	

For a solid medium, add 15-20 g of agar to the above ingredients and make up to 1 l with distilled water.

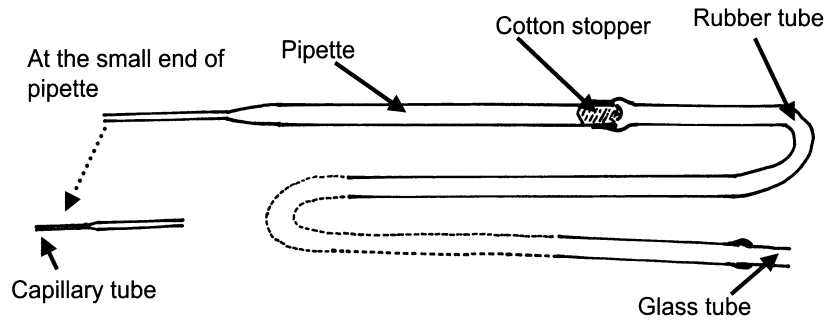

Fig. 3. Micropipette used for a single cell separation of photobionts (after Nakano 1987).

3xN BBM

3xN BBM Modified Bold's Basal Medium with three times more nitrogen (Brown and Bold 1964)	
As above except	
$NaNO_3$	750 mg

For a solid medium add 15-20 g of agar to the above ingredients and make up to 1 l with distilled water.

***Trebouxia* Organic Nutrient Medium**

Trebouxia Organic Nutrient Medium (Ahmadjian 1967a)	
1 x N BBM	970 ml
Proteose peptone	10 g
Glucose	20 g

For a solid medium add 15-20 g of agar to the above ingredients and make up to 1 l with distilled water.

MDM medium

MDM medium for cyanobacteria instead of BBM (Watanabe 1960)	
KNO_3	1 g
$MgSO_4 \cdot 7H_2O$	250 mg
K_2HPO_4	250 mg
NaCl	100 mg
$CaCl_2 \cdot 2H_2O$	10 mg
Fe solution	1 ml
A5 solution	1 ml
Make up to 1 l with distilled water	

For a solid medium add 15-20 g of agar to the above ingredients and make up to 1 litre with distilled water.

Fe Solution	
$FeSO_4$	1 g
Distilled water	500 ml
Concentrated H_2SO_4	2 drops

A5 solution	
H_3BO_3	286 mg
$MnSO_4 \cdot 7H_2O$	250 mg
$ZnSO_4 \cdot 7H_2O$	22.2 mg
$CuSO_4 \cdot 5H_2O$	7.9 mg
Na_2MoO_4	2.1 mg
Distilled water	100 ml

Other media or media constituents have been recommended by Kratz and Myers (1955), Stanier et al. (1971), Starr (1980), Nichols (1973), Allen (1968; 1973), Archibald (1975; 1977) and Carr et al. (1973).

Note: Autoclave all media before use and pour into Petri dishes (5 mm thick) or test tubes (5 ml) in the laminar flow bench.

Procedure

Pre-treatments

- For macrolichens (foliose and fruticose):
  Cut off about 1 cm^2 from the apex of the thallus, then place into tap water for about 5 to 10 min. Brush the surface of the thallus in running tap water using a paintbrush, and then wash in sterilised water.
- For microlichens
  If the lichen thallus is small (most crustose lichen thalli), place it into a small test tube with 1-2 ml sterilised water and one drop of Tween 20 and ultrasonicate for about 3 min. Centrifuge (2000 rpm) to separate the epiphytes detached from the surface of lichen thalli.

Producing a thallus homogenate

Note: You must carry out all treatments on a clean laminar flow bench or under sterile conditions, except for the pre-washing stage. All equipment should be autoclaved (15 -20 min, 121°C, 1 atm) or oven-dried (30 min, 180°C) before use. Sterilise all glassware and implements before use, clean slides (if dirty) with detergent or acid and wash well in distilled water.

Macrolichens (foliose and fruticose species)

1. After the washing procedure mount the lichen thallus on a sterilised glass microscope slide.
2. Under a dissecting-microscope, carefully scrape or shave away the surface of the thallus (cortex) using a small knife made by filing a small needle.
3. Under the microscope, remove the photobiont layer and transfer it onto a new, sterilised glass slide.
4. Add one drop of sterilised water to the slide, cover the excised portions of photobiont layers with another glass slide and grind into smaller fragments using light pressure. Photobionts are mechanically separated from mycobionts, although some mycobiont hyphae may remain attached. Both symbionts are suspended in the liquid.

Microlichens

After washing, place small portions of the thallus on a sterilised microscope slide and grind between two microscope slides by applying light pressure. Unlike the above protocol for macrolichens, the cortex is still present, so apply more pressure. The resulting suspension contains both photobionts and mycobionts. Break up larger pieces using a blender, or mortar and pestle (Yamamoto 1987). However, fragile cells may be destroyed in blenders, and thus it is best to use less "violent" techniques such as grinding between two glass slides (Nakano 1987).

Variations in the method how to wash thalli, and recommendations for best equipment have been reported by several researchers e.g. a wooden board by Ahmadjian (1967a) and a small washing chamber, cup and filter by Yoshimura et al. (1993). With cyanolichens, use thin slices (up to 40 μm thick), obtained with a freezing microtome for inoculation.

Isolation of photobionts

1. Add a few drops of solution containing a suspension of photobionts and mycobionts onto solid agar media in petri dishes. For green algae use 1 x N BBM culture medium. For cyanobacteria use MDM culture media. Alternatively, thinly spray (see spray method) a solution containing a suspension of symbionts over the surface of an agar medium in a petri dish.
2. Culture at 15°C in an incubator. Generally the cultures should be kept cool at 15-20°C and a light intensity of 10-27 μmol $m^{-2}s^{-1}$ (PPFD, photosynthesis photon flux density). We recommend that initially you expose the cultures to lower light intensities.
3. After about 1 month (depending on the photobiont species) small photobiont colonies appear on the surface of the agar medium. If you have done enough washing of thalli previously, almost all of the algal colonies that appear are true photobionts.
4. It is important to confirm that the isolated algae are the true photobiont. For filamentous algae, trace the algae back to a cell that is affiliated with fungal tissue within the fragment. For single-celled algae, make a search for cells that retain fragments of the fungal hyphae on their walls.

Obtaining axenic photobiont cultures

Amongst the photobiont colonies developing on an agar medium, many of the colonies may be contaminated. In order to obtain axenic photobiont cultures, the following alternatives are given.

Direct method

1. Under the stereo-microscope, photobiont colonies without contamination are selected and are transplanted onto suitable solid agar (either petri dish or test tube) media. *Trebouxia* Organic Nutrient Medium (Ahmadjian 1967a) is used for green algae, MDM medium containing 1% glucose for cyanobacteria (Watanabe 1960).
2. If uncontaminated, a true axenic photobiont culture is obtained. However, photobiont colonies are usually contaminated with bacteria or mycobionts, or some other organisms. Additional spray regimes or micropipette methods are necessary for obtaining true axenic photobionts cultures.

Spray method

The spray method (Fig. 4) can be useful for single cell green algae isolation. This technique was developed by Wiedeman et al. (1964) and can be used to both isolate single cell green algae and produce axenic cultures of algae (without contamination of bacteria, yeast etc. and without mycobiont hyphae attached to the photobiont surface).

1. To obtain cultures derived from a single cell, select colonies containing low levels of contamination (or no contamination), from the photobiont colonies growing on the agar plate, and transplant onto 1 x N BBM slant agar media in test tubes.
2. Culture the colonies for several weeks.
3. Transfer the colonies into 10 ml centrifugation tubes with 1 ml of sterilized water and one drop of Tween 20.
4. Ultrasonicate. This results in the photobiont colonies dissociating from contaminating bacteria and mycobionts attached to the surface of their cell walls.
5. Centrifuge (1000 rpm, 5-10 min) the mixture of dissociated photobiont cells and other organisms.
6. Remove the supernatant and add 1 ml of sterilised water and a drop of Tween 20 to the photobiont cells remaining in the centrifuged tube. Repeat this treatment about 10 times.
7. Insert a capillary tube in the bottom of the centrifuge tube and hold in place.
8. Direct compressed air through a small opening across the top of the capillary tube extending from the centrifuge tube.
9. The algal suspension is drawn up the microtube and atomised into a fine spray.
10. Quickly pass a Petri dish containing medium (usually *Trebouxia* Organic Nutrient Medium) through the spray. The dish will become coated with a suspension of algal cells.
11. After 1 or 2 weeks, remove non-contaminated algal colonies and transplant them onto suitable culture media.

Micropipette method

The micropipette method is a useful and reliable way to isolate green algae, although you cannot use it for long filamentous algae.

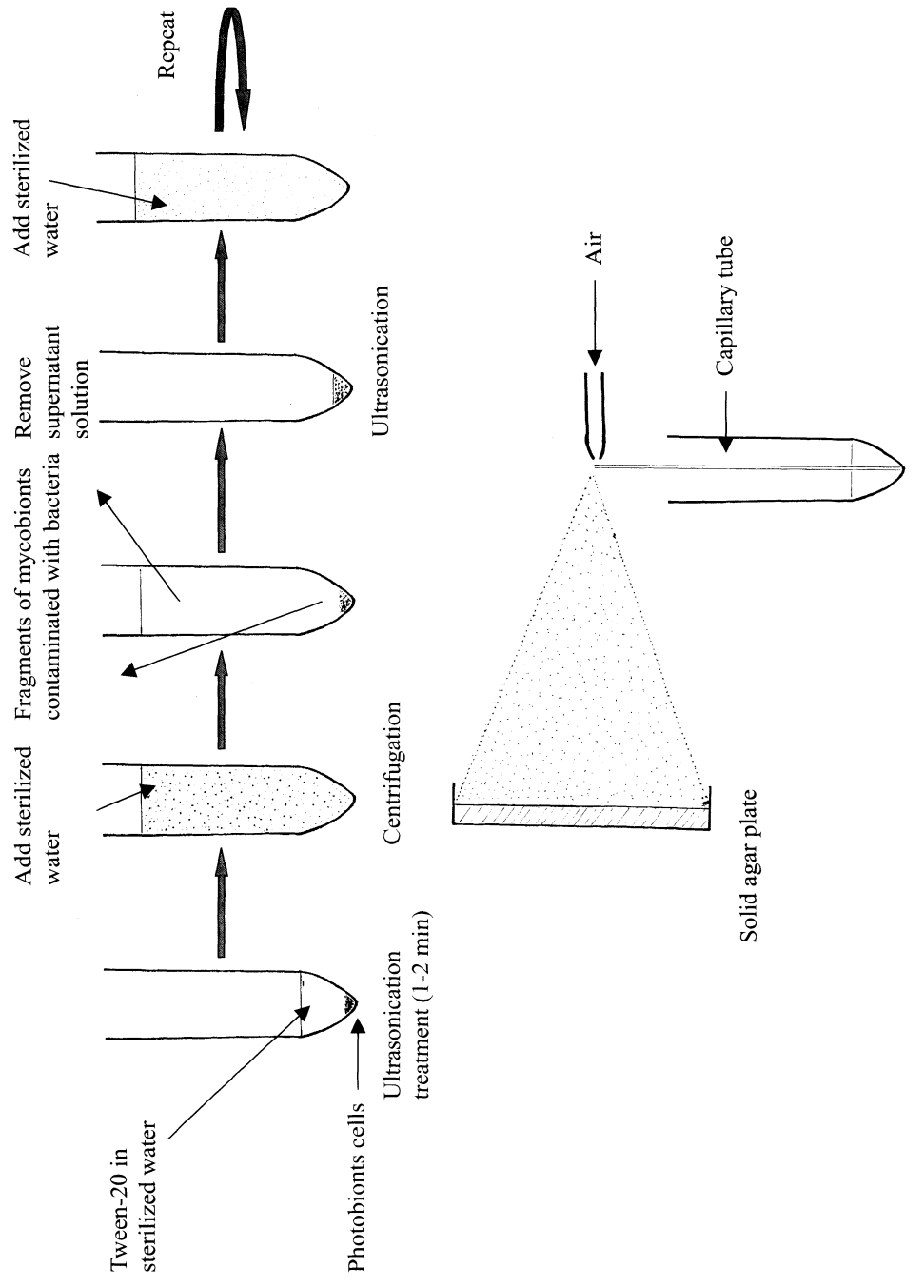

Fig. 4. Isolation protocol of photobionts using the spray method (after Nakano 1987).

1. Add a drop of sterilised water or sterilised culture medium to each well of a glass microscope slide with 5 wells. Remove a developed algal colony from the nutrient medium and place into the first well. If algal colonies are compact then they can be diffused by ultrasonication.
2. Suck up about 5 to 10 algal cells using the micropipette under the stereo-microscope.
3. Expel the cells into the next well, by blowing through the glass tubing attached to the rubber end of the micropipette.
4. Repeat the technique more than 5 times. The micropipette tip can be steamed to reduce contamination. Sometimes, when the algal cell is introduced into a new drop of water, it floats on the surface. It is impossible to suck up the cell in this position with a micropipette. Generally, most cells sink after a few minutes (Ahmadjian 1967b).
5. If contamination is serious, the procedure must be repeated again using more sterilised water or sterilised culture medium.
6. After the final wash, pick up a single algal cell, and transplant it to a new culture medium (transplant about 10 to 50 algal cells).
7. The following culture media should be used to test that your culture is successful: *Trebouxia* Organic Nutrient Medium for green algae, and MDM media containing glucose for cyanobacteria. When contaminating bacteria or yeast are present, they rapidly grow on rich nutrient culture medium. It is very difficult to remove some bacteria in the gelatinous sheath of cyanobacteria; however, they grow in harmony with cyanobacteria. Probably the complete purification of cyanobacteria can be made by using micro-manipulation methods, although we have never successfully achieved this in our laboratory.
   Make an average of fifteen single-cell isolates for each lichen species.

Cutting method

It is difficult to obtain axenic cultures of filamentous green algae, e.g. *Trentepohlia*, using only the methods described above. The cutting method may help you to obtain axenic cultures of these algae.

1. Cut the newly grown algal filaments off at their apices using sterilised forceps, and transplant them onto new culture media.
2. Apply Yamamoto's method (see Chapter 2), and after the second filtration select greenish pieces under a stereo-microscope, then transfer them to test tubes with solid nutrient or plain agar.

3. Maintain many (ca. 50) test tubes with agar slants for a few weeks, and remove any contaminated test tubes. Contamination of culture tubes depends on the part and condition of the thallus, and the species.
4. In the uncontaminated tubes, new growth of the symbionts (photobionts and mycobionts) occurs after about 4 weeks of incubation. The photobiont can be further isolated using additional treatments (the spray method or micropipette method) to get a genetically pure photobiont colony derived from a single cell.

Centrifugation methods

Protocols and guidelines for centrifugation have been published by Richardson (1971).

1. Use suspension derived from the procedure "Producing a thallus homogenate" (Subprotocol 2), and centrifuge (100 - 400 g, about 10 min). In general, most small photobionts remain in the supernatant after low speed centrifugation (100 - 200 g, 10 min). For larger photobionts you must test various combinations of speed and time. Use low-speed centrifugation (100 g) to remove large fragments of thalli or clumps of tissue, and higher speeds (400 g) to separate intact cells from cell fragments and debris.
2. Filter your sample through a nylon sieve mesh 10 - 30 μm, or through other large-pored filters to remove larger size debris before centrifugation (Bubrick 1988).

Maintaining photobiont cultures

Under low illumination conditions, photobionts can be cultured for long periods (about one year). However, we recommend that you subculture every 2 to 3 months. Most lichen algae have an optimum temperature range between 15-20°C. However, the temperature in which the lichen naturally grows should be considered when selecting culture conditions. The optimum pH range is 4.0 - 7.0

The optimum light intensity range lies between 16 - 27 μmol m^{-2} s^{-1} (PPFD) (Ahmadjian 1967a; b). *Trebouxia* from pigmented lichens are more sensitive to high irradiances whereas those from lichens with a non-pigmented cortex are more light tolerant.

1. Cut cultured photobiont colonies with agar media into several segments (about 5 mm^2) with a scalpel. Place colonies on a plate with appropriate medium in Petri dishes and culture for 2 to 3 months.

2. Every 2 to 3 months, transfer growing colonies to fresh medium of the same composition and culture under the same conditions.

Lichen photobiont cultures can also be cryopreserved under liquid nitrogen where they remain viable for extended periods of time.

Comments

Some *Trebouxia* strains lose their colour when cultured at light intensities above 11 μmol m^{-2} s^{-1} (Ahmadjian 1967a). We recommend culturing *Trebouxia* at about 5 μmol m^{-2} s^{-1} illumination to preserve strains for a long time. However, for taxonomical observations, *Trebouxia* strains must be cultured on 3 x N BBM under 22°C, 33 μmol m^{-2} s^{-1}, and a light-dark cycle of 12 h light and 12 h dark (Archibald 1975).

Troubleshooting

- Ascospores do not germinate
  In the absence of compatible photobiont cells, the ascospores of some taxa (e.g. the Peltigerales) may germinate, but fail to grow (Lallemant and Bernard 1977; Ahmadjian 1989). In these cases, germination can be stimulated by including photobiont extracts and growth may be stimulated by adding whole photobiont cells to the agar (Bubrick 1988). See also Chapter 3.
  Various conditions that influence lichen spore germination have been well summarised by Pyatt (1973), Ahmadjian (1993) and Yamamoto et al. (1998). Spore germination may be affected by various environmental factors. These factors include collection season (Pyatt 1969, Ostrofsky and Denison 1980), culture humidity (Garrett 1971), medium pH (Pyatt 1968, Chrismas 1980, Ostrofsky and Denison 1980), culture temperature (Ostrofsky and Denison 1980), alternation of light and dark (Pyatt 1968), salinity (Ramkær 1978), natural extracts (Ostrofsky and Denison 1980), the presence of glucose (Belandria et al. 1989), secondary lichen compounds (Whiton and Lawrey 1982, 1984), air pollutants (Pyatt 1969, Belandria et al. 1989), heavy metals (Pyatt 1976), low oxygen concentration (Kofler 1970), and influence of bark extract (Ostrofsky and Denison 1980).

- Contamination
  To rid cyanobacterial photobionts from bacteria that are normally present in the gelatinous sheaths enveloping the algal cells, irradiate with ultraviolet light (sufficient to destroy bacteria but not the algal cells) or treat them with antibiotics (Ahmadjian 1967b). During the culture, mites often eat cultured algae or mycobionts. Vinyl tape shields are often effective against mites.

Acknowledgements. We thank Prof. V. Ahmadjian of Clark University for his encouragement and advice during our studies on lichen culture. Thanks are extended to Prof. M. Nakanishi of Hiroshima University for kindly helping us to establish our spore-derived cultures of lichen mycobionts.

References

Ahmadjian V (1967a) The Lichen Symbiosis. Blaisdell Publishing Company, Massachusetts

Ahmadjian V (1967b) A guide to the algae occurring as lichen symbionts: isolation, culture, cultural physiology and identification. Phycologia 6: 127-160

Ahmadjian V (1973) Methods of isolation and culturing lichen symbionts and thalli. In: Ahmadjian V, Hale ME (eds) The Lichens. Academic Press, New York, pp 653-660

Ahmadjian V (1989) Studies on the isolation and synthesis of bionts of the cyanolichen *Peltigeria canina* (Peltigeraceae). Pl Syst Evol 165:29-38

Ahmadjian V (1993) The Lichen symbiosis. John Wiley and Sons, New York

Allen MM (1968) Simple conditions for growth of unicellular blue-green algae. J Phycol 4: 1

Allen MM (1973) Methods for Cyanophyceae. In: Stein JR (ed) Handbook of Phycological Methods. Culture Methods and Growth Measurements. Cambridge University Press London, pp 127-138.

Archibald PA (1975) *Trebouxia* de Puymalay (Chlorophyceae, Chlorococales) and *Pseudotrebouxia* gen. nov. (Chlorophycee, Chlorosarcinales). Phycologia 14: 125-137

Archibald PA (1977) Physiological characteristics of *Trebouxia* (Chlorophyceae, Chlorococcales) and *Pseudotrebouxia* (Chlorophyceae, Chlorosarcinales), Phycologia 16: 295

Armaleo D (1991) Experimental microbiology of lichens: mycelia fragmentation, a noval grow chamber, and the origins of thallus differentiation. Symbiosis 11: 163-177

Belandria G, Asta J, Nurit F (1989) Effects of sulfur dioxide and fluoride on ascospore germination of several lichens. Lichenologist 21: 79-86

Bischoff HW, Bold HC (1963) Some soil algae from enhanced rock and related algal species. Phycological Studies IV. Univ. Texas Publ. No. 6318, Texas, 95 pp

Bubrick P (1988) Methods for cultivating lichens and isolated bionts. In: Galun M (ed) Handbook of Lichenology, vol 3. CRC Press, Boca Raton, pp 127-138.

Brown RM Jr, Bold HC (1964) Comparative studies of the algal genera *Tetracystis* and *Chlorococcum*. Phcological Studies V. Univ. Texas Publ. No. 6417, Texas, 213 pp

Carr NG, Komarek J, Whitton BA (1973) Notes on isolation and laboratory culture, In: Carr NG, Whitton, BA, (eds) The Biology of Blue-Green Algae, University of California Press, Berkeley, pp 525-530.

Chrismas M (1980) Ascospore discharge and germination in *Xanthoria parietina*. Lichenologist 12: 403-406

Deason TR, Bold HC (1960) Phycological studies I. Exploratory studies of Texas soil algae. Univ. of Texas Publication Nr. 60022

Galun M (1988) Handbook of Lichenology Vol 3. CRC Press, Boca Raton.

Garrett RM (1971) Studies on some aspects of ascospore liberation and dispersal in lichens. Lichenologist 5: 33-44

Hale ME (1983) The Biology of Lichens. Edward Arnold, London

Honegger R , Bartnicki-Garcia S (1991) Cell wall structure and composition of cultured mycobionts from the lichen *Cladonia macrophylla, Cladonia caespiticia, and Physcia stellaris* (Lecanorales, Ascomycetes). Mycol Res 95: 905-914

Honegger R, Kutasi V (1990) Anthraquinone production in three aposymbiotically cultured teloschistalean lichen mycobionts: the role of the carbon source. In: Nardon P, Gianinazzi-Pearson V, Grenier MA, Smith DC , Margulis L (eds) Endocytobiology IV. 4th International Symposium on Endocytobiology and Symbiosis, Lyon, July 4-8, 1989175-8. Paris: Institute National de la Recherche Agronomique

Honegger R, Kutasi V, Rufflier HP (1993) Polyol patterns in eleven species of aposymbiotically cultured lichen mycobionts. Mycol Res 97: 359

Kofler L (1970) A method to use spores in quantitative studies on germination. Bryologist 73: 602-606

Kratz WA, Myers J (1955) Nutrition and growth of several blue-green algae. Am J Bot 42: 282

Lallemant R , Bernard T (1977) Obtention de cultures pures des mycosymbiontes du *Lobaria laetevirens* (Lightf.) Zahlbr. et du *Lobaria pulmonaria* (L) Hoffm.: le roles des gonidies. Rev Bry Lichen 43: 303-308

Lawrey JD (1984) Biology of Lichenized fungi. New York, Praeger Publishers

Lilly VG, Barnett HL (1951) In Physiology of Fungi. McGraw-Hill, New York

Mathey A, Holder A (1978) Fluorescence, Iuminescence et pouvoir germinatif des spores dans la famille des Trypethéliacées (lichens pyrénomzcètes). Nova Hedwigia 30:79-110

Nakano T (1987) Isolation and culture methods of lichen photobionts. [In Japanese] Lichen 6: 1-3

Nakano T (1988) Phycobionts of some Japanese species of Graphidaceae. Lichenologist 20: 353-360

Nichols HW (1973) Growth media-fresh water. In: Stein JR (ed) Handbook of Phycological Methods. Culture Methods and Growth Measurements. Cambridge University Press, London, p 7

Oliver E, Crittenden PD, Beckett A, Brown DH (1989) Growth of lichen-forming fungi on membrane filters. Lichenologist 21:387-391

Ostrofsky A, Denison WC (1980) Ascospore discharge and germination in *Xanthoria polycarpa*. Mycologia 72: 1171-1179

Pyatt FB (1968) An investigation into conditions influencing ascospore discharge and germination in lichens. Rev Bryol Lichen 36:323-329

Pyatt FB (1969) Studies of the periodicity of spore discharge and germination in lichens. Bryologist 72: 48-53

Pyatt FB (1973) Lichen propagules. In: Ahmadjian V, Hale ME (eds) The Lichens. Academic Press, New York pp 117-145
Pyatt, FB (1976) Lichen ecology of metal spoil tips: Effects of metal ions on ascospore viability. Bryologist. 79: 172-179
Ramkær K (1978) The influence of salinity on the establishing phase of rocky shore lichens. Bot. Tidsskrift 72: 119-123
Richardson DHS (1971) Lichens. In: Booth C (ed) Methods in microbiology, vol 4. Academic Press, New York, pp 267-293
Roussard M (1969) Contribution a l'étude des ascospores de lichens et de leur germination. Bull Soc Mycol France 85: 355-366
Stanier RY, Kunisawa R, Mandel M,Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (order Chroococcales). Bact Rev 35:171
Starr RC (1980) The culture collection of algae at the University of Texas at Austin. J Phycol 14: 483
Takeshita S, Nakano T, Iwatsuki Z. (1989) Phycobionts of some Japanese species of *Pertusaria* (Pertusariaceae). Pl. syst. Evol. 165: 49-54
Vobis G. 1977. Studies on the germination of lichen conidia. Lichenologist 9:131-136
Watanabe A (1960) List of algal strains in collection at the Institute of Applied Microbiology, University of Tokyo. J. Gen. Appl. Microbiol. 6: 283-292
Whiton JC, Lawrey JD (1982) Inhibition of *Cladonia cristatella* and *Sordaria fimicola* ascospore germination by lichen acids. Bryologist 85: 222-226
Whiton JC, Lawrey JD (1984) Inhibition of crustose lichen spore germination by lichen acids. Bryologist 87: 42-43
Wiedeman VE, Walne PL, Trainor FR (1964) A new technique for obtaining axenic cultures of algae. Can J Bot 42: 958
Yamamoto Y (1987) Tissue cultures of lichens. Proceedings of symposium on Tissue Culture of Lichen and Bryophytes. Osaka, Japan: Nippon Paint company, 14-25
Yamamoto Y, Mizuguchi R, Yamada Y (1985) Tissue cultures of *Usnea rubescens* and *Ramalina yasudae* and production of usnic acid in their cultures. Agric. Biol. Chem. 49: 3347-3348
Yamamoto Y, Kinoshita Y, Takahagi T, Kroken S, Kurokawa T, Yoshimura I (1998) Factors affecting discharge and germination of lichen ascospores. J. Hattori Bot. Lab. 85: 267-278
Yoshimura I, Kurokawa T, Kanda H (1990) Tissue culture of some Antarctic lichens preserved in the refrigerator. Proc. NIPR symp. Polar Biol. 3: 224-228
Yoshimura I, Kurokawa T, Yamamoto Y, Kinoshita Y (1993) Development of lichen thalli *in vitro*. Bryologist 96: 412-421