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Methods for isolation and cultivation of the eukaryotic lichen photobionts - looking for a general method. A review.

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ABSTRACT: Methods for isolation and cultivation of the eukaryotic lichen photobionts used in several protocols and personal experience of the authors are summarized. Possibilities of combination of isolation steps are demonstrated.

KEYWORDS: photobionts, lichens, algae, isolation, cultivation, methods

Introduction

Unique symbiotical nature of lichens predetermine them for study in modern biology. However, the whole lichen thallus, composed from cells of the mycobionts and photobionts is very difficult biological material for experiments, due to its problematical cultivation and extremely slow growth.

Main interest in experimental lichenology is predominantly connected with the production of hundreds of unique lichen compounds which dispose of antibiotic, antitumour and newly with anti-HIV attributes (FAHSELT 1994). The possibilities of

lichens to tolerate extreme amplitudes of abiotical ecological factors, e.g. temperature, water, sunlight, etc. are not less interesting biological properties.

Another fact from the controversies in lichen biology is that on one hand lichens are organisms very sensitive to pollution (bioindication), on the other hand they can tolerate high content of heavy metals (metalotollerant populations of lichens, e.g. from old dumps after metals mining).

It is not fairly clear, which biont (mycobiont or photobiont) is responsible for the extreme resistance (or sensitivity) of lichens. We assume that these qualities are determined genetically and „then the genes which code for these traits could be incorporated into suitable vectors that might be used to transform other organisms such as plants or fast-growing fungi" (AHMADJIAN & al. 1987).

The present paper summarize several protocols and experience of authors, which were successfully used in photobionts isolations. Several methods were published, but we want to demonstrate, that all of them include similar sequence of independent steps and possibilities to combine them depend only from a material facilities of different laboratories.

1. Photobionts isolation

1.1 Selection and preparation of lichen material

Photobionts can be probably more or less successfully isolated from all lichen species. In many cases it is not necessary to use fresh material for isolation. The authors of this paper have personal experience with the isolation of *Trebouxia* photobionts from lichens (e. g. *Cladina mitis*, *Cetraria islandica*) kept one year or more in laboratory (in paper bags) without sunlight and water. KELLER & al. (1995) published isolation and cultivation of *Trebouxia* photobionts from several (25-47 (!)) years old herbarium specimens of the lichen *Xanthoria parietina*.

The knowledge of lichen thallus anatomy can give us very valuable information necessary for photobiont isolation. In the case of heteromerous thallus it is often possible to take out layers mechanically without green cells of photobionts, and consequently eliminate contamination with mycobiont cells. Miscellaneous impurity as dead organisms, seeds, mosses and further foreign objects must be removed from thallus. Many micro-organisms and spores of fungi can be washed up by energical washing in distilated or running water about 1 hour (YAMAMOTO & al. 1987).

For the photobiont isolation from macrolichens it is good to cut thallus into small pieces. YAMAMOTO & al. (1987) used approximately 1 cm of the marginal thallus from fruticose lichens or 1cm^2 of foliose lichens. ASCASO (1980) used approximately 2 grams of damp thallus.

1.2 Homogenization

Prepared lichen material must be (except for „thallus fragment method" where washed thallus is directly spread on agarised medium) mechanically disrupted. One of the possibilities is direct homogenisation in sterilized mortar (e.g.

YAMAMOTO & al. 1987). ASCASO (1980) subsequently used Elvehjem-Potter homogenisator. We have a good experience with homogenisation without mortar but only using Elvehjem-Potter homogenisator. The benefit of this preparation is that experimentators can work full time in sterile environment during the isolation which minimalises contamination, thallus can be homogenised directly in sterilized mineral medium and sterilization of homogenisator in the flame is very easy.

By using „micropipette method" (AHMADJIAN 1967a, 1967b, 1973, 1993) the removed pieces of photobiont layer from lichen thallus are homogenized between two microscope slides and single cells are isolated with a micropipette. By this method of homogenisation it is possible to isolate single photobiont cells directly by micropipette.

1.3 Cleaning of homogenates and selection of the photobionts

In all cases the homogenates include a mixture of different cells (cells of the photobiont as well as cells of the mycobiont). In the case of homogenisation in Elvehjem-Potter homogenisator (where disruption of the tissue is not so consistent) we recommend to filtrate crude homogenate through few layers of pleated sterile cotton. There are three main possibilities for the selection of the photobionts from homogenates.

1.3.1 Yamamoto method

This method (YAMAMOTO & al. 1987, 1993) is based on filtration of homogenate through the sterilized filter with 500 µm mesh size. This suspension is filtrated through next filter with 150 µm mesh size and the residue on the filter is washed three times with sterile water (or mineral medium). After the selection of photobiont cells algae are transferred into the liquid or preferably on agarised mineral cultivation medium.

PEREZ & al. (1985) used another filtration method (gel filtration) for isolation photobionts from the lichen Evernia prunastri.

1.3.2 Micropipette method

Full details on this method are given in AHMADJIAN's papers (e. g. 1967a, 1967b, 1973, 1993) and it is recommended by ETTL & GÄRTNER (1995) too. Using the micropipette (ideal size of tip is 50-75 µm in diameter) cells are transferred through drops of sterile water on microscope slides in order to obtain the diluted suspension of the photobiont cells and then single cells are transferred into a medium. The drop of the final suspension of photobionts can be spread onto surface of a mineral agar too. This method is difficult to manual handiness but requisite result is often obtained in first step. The method is useful for isolation of ascospores, conidia, soredia etc., too (AHMADJIAN 1993).

1.3.3 Centrifugation methods

There are many protocols which use methods of centrifugations, especially differential centrifugation and gradient centrifugation.

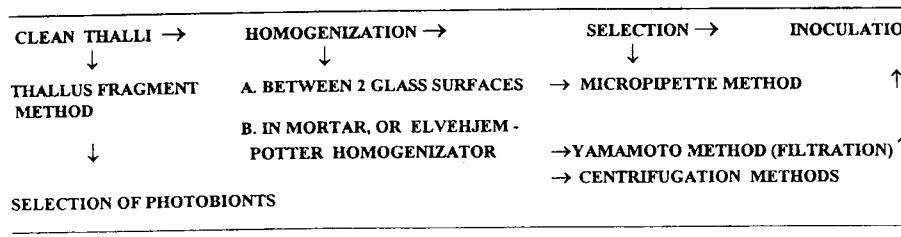

Fig. 1. Horizontal and vertical separation of steps at the lichen photobionts isolation. Arrows indicate the possibilities of their combinations.

Differential centrifugation (DREW & SMITH 1967, RICHARDSON & SMITH 1968, ORUS & ESTÉVEZ 1984) has been used by many photobiont isolators and this method is suitable for isolation of cyanobacterial photobionts too. The small photobionts as *Coccomyxa* sp. were isolated at low-speed centrifugation in clinical centrifuge (not more than 500 rpm) but a lot of cells rested in supernatant. For bigger photobionts (e. g. *Trebouxia*) variable combinations were tested. Lower-speed centrifugation (about 100 x g) was used generally for removing large fragments of thallus (reiterated few times). For the direct photobiont cells collecting from supernatant higher speed centrifugation was used (about 400 x g). Progress of centrifugation was controlled by light microscope and approximately 20-70 % profits are recommended (BUBRICK 1988). Occasionally the homogenates are filtrated (e. g DREW & SMITH 1967) after disruption of the thallus through few teflon filters (with e.g. 30, 20, or 10 μm mesh sizes).

Gradient centrifugation is convenient method for receiving very clear, fresh isolated photobionts. ASCASO (1980) studied the usage of $CsCl_2$ gradients for *Trebouxia* sp. isolation. After thallus disruption the *Trebouxia* cells were resuspended in sucrose (0,25M, 2ml) and carefully layered onto solution of $CsCl_2$ (1.550 $g.cm^{-3}$, 3 ml). Tubes were centrifuged in swinging bucket rotor for 10 minutes at 4 500 rpm. Purified cells were collected from sacharose-$CsCl_2$ interfase and washed. Solution of KI (8g in 10 ml of water) substituted the $CsCl_2$ but it may be harmful after prolonged exposure (ASCASO 1980).

HONEGGER & BRUNNER (1981) used discontinuous sucrose gradients in the study of sporopollenin in the cell walls of *Coccomyxa* and *Myrmecia* photobionts as a procedure for their isolation and purification.

1.4 Inoculation and cultivation of photobionts

For all steps of photobiont isolation routine microbiological procedures are required. Contamined petri dishes (tubes, or Erlenmayer flasks) are removed daily, especially few days after inoculation. Using thallus fragment method, when hand-cut sections of lichen thalli are used directly as inocula, it is necessary to inoculate more cultures - higher in order (large scale of contamination) and to

compare growing algae cultures with photobionts in original thallus (AHMADJIAN 1993). Using micropipette method (Yamamoto method or centrifugation methods) it is possible to obtain clean photobiont cultures in the first inoculation.

1.4.1 Number of cells for inoculation

Inoculum is not given in majority of the papers. Micropipette method requires about 15 photobiont cells as an inoculum. Following centrifugation methods we recommend to dilute photobiont suspension with a medium (or sterile water) in several steps (e.g. 1: 10). In very „slight" suspension no growth is often visible and, on the other hand, dense suspensions cause contamination. Cells are collected by automatic pipette (few µl are pipetted from diluted suspension) and gently layered on an agarised medium (1-2 %). In general, photobionts can be cultivated on many types of media (AHMADJIAN 1967a, 1967b, 1973, 1993, BUBRICK 1988).

1.4.2 Conditions of growth

The colonies of common photobionts (e. g. *Trebouxia*) are visible after two to four weeks, but sometimes after two months. The growing colonies must be inoculated into new medium after 6 weeks.

Growth conditions vary and optimum must be sometimes determined individually. In general, photobionts can be routinely maintained at 15 - 25 ^{0}C (from arctic and alpine lichen species at 10-15 ^{0}C), low intensities of light (500-5000 lux) and pH 6-7 (at lichens from acid substrates less) (e. g. YOSHIMURA & al. 1987, FRIEDL 1989, AHMADJIAN 1993, YAMAMOTO & al. 1993).

Some photobionts are available from culture collections (UTEX, SAG, CCAP, ATCC, ASIB) catalogues of strains can be accessed possible by using „Internet" or references (SCHLÖSSER 1994, TOMPKINS & al. 1995, GÄRTNER 1996).

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