

Laboratory Procedures & Methods: Lichen Biont Isolation

Materials:

- Dissecting microscope with lamp
- Laminar flow hood
- Autoclave
- Sharp steel handled scalpel
- Spear needle (2-3)
- Microscope slides
- Glass pipettes w/ cotton plugs and two pump bulbs
- MEYA and Bold's Basal culture media (see media prep.)
- "Clean" (acid-washed) glassware for stock solutions and media sterilization (see media prep.)
- DI water
- 70% ethanol in spray bottle
- Sterile petri dishes
- Alcohol burner for tool sterilization while working

Mycobiont Isolation:

-Malt Extract Yeast Agar Media Preparation:

Media:

Mix the following ingredients into one 1000mL Duran autoclavable flask:

- 10g agar
- 10g light malt extract
- 1g yeast extract
- 0.5g peptone
- 500mL DI H₂O

Preparation:

- Wrap scalpel, spear needles and place in canning jar or wire basket and place in autoclavable bin with MEYA media flask.
- Autoclave 500mL MEYA media and tools for 50-minute liquid autoclave cycle.
- During autoclave cycle, turn on laminar flow hood and sterilize work surfaces and dissecting microscope stage in the flow hood air stream.
- Position dissecting scope with stage facing air filter and air stream with arm of scope facing **away** from face of filter.
- Once removed carefully from autoclave, allow hot media to cool for no more than 30 minutes in the air stream of laminar flow hood.
- Once media flask is cool enough to handle, pour carefully into sterile Petri dishes at a depth of about 5mm.
- Allow freshly poured plates to cool in stacks of +/-10 until solid within air stream of flow hood (about 30-90 minutes).

-Procedures for Hyphal Isolation Technique:

This method requires use of aseptic conditions and sterile technique.

Freshly collected (within one week) lichen thalli are washed for 5 minutes in running tap water then allowed to soak in DI H₂O for 10 minutes, then brushed lightly with a paintbrush under running DI H₂O.

1. Small sections, about 2cm square are cut from the thallus using a clean razor blade.
2. Under the dissecting microscope in the laminar flow hood, upper cortex and photobiont layer are removed from thallus piece by scraping the lichen using sterile scalpel or sharpened spear needle.
3. Once outer cortex is removed, pieces of the fine thread-like medullary tissue are teased from lichen thallus using a scalpel or sharpened spear needle. These hyphal tissue fragments are then placed on the surface of MEYA containing Petri dishes. Because contamination rates and absence of growth will occur, inoculation rate per Petri dish should be four transfers or more in 10 or more Petri dishes per lichen specimen.
4. Hyphal isolations are allowed to incubate at 15-20 degrees C for 7-14 days or until growth/contamination are evident.
5. When hyphal growth is evident, isolations should be further sectored and transferred to fresh agar media for further isolation and comparison between isolations. Microscopic identification will be necessary to insure the purity and confirmation of lichen species isolation. Side-by-side comparison of different mycobiont isolations for same original lichen thallus will aid in confirming a true axenic mycobiont culture.
6. Once experimenter is confident in purity and identity of mycobiont isolation, the culture should be transferred to MEYA media "slant" test tubes with screw-on lids for storage and future lichen synthesis experiments and mycobiont comparisons.

Mycobiont isolations should be stored at relatively the same temperature range of the free-living lichen or at colder temperatures for long-term storage. Mycobiont cultures can be maintained for several years of experimenting if cultures are periodically re-transferred to fresh MEYA media in sterile test tubes.

-Single Spore Isolation from Discharge by Living Apothecia:

This method and procedure are described in Ahmadjian, The Lichen Symbiosis (ed.1&2) and in Protocols in Lichenology. This technique may be very helpful in not only assuredly obtaining a specific species of mycobiont, but also in contributing to the confirmation of hyphal isolation mycobiont cultures.

This experimenter has not yet completed this portion of the mycobiont isolation procedure, and so has not described it in detail here.

Photobiont Isolation:

-Bold's Basal Medium Preparation:

For procedure of stock solutions preparation, please refer to Ahmadjian (1993) pp.171-172. This procedure details the preparation of Part I stock solutions in 400ml flasks and Part II stock solutions in 1liter flasks; for my experiment these values were made at one-

half the volumes of those outlined by Ahmadjian. In other words: My Part I solutions are all 200ml in volume and all contain half the amount of chemical ingredient per solution; my Part II solutions are only 500ml in volume and again the chemical ingredients listed are also half. In addition to this alteration, I also only made 500ml of photobiont culture media per run of Petri dishes.

Preparation of Photobiont Culture Media and Tools

- Prepare 500ml BBM in 1000ml Duran autoclavable flask
- For green algae lichens add 10g glucose and 5g peptone per 500ml BBM.
- Wrap glass microscope slides in aluminum foil in pairs (X10) and place in glass canning jar or other autoclavable container.
- Place 10 or more glass pipettes w/ cotton plugs in 1qt canning jar.
- Wrap scalpel and spear needles in aluminum foil for autoclaving.
- Fill 2 autoclavable containers with 200ml DI H₂O each.
- Run media, slides, pipettes, tools and H₂O for 50 minute, liquid autoclave cycle.
- While autoclave is running, turn on laminar flow hood and sterilize work surfaces, other tools, and dissecting microscope stage with 70% ethanol.
- Position dissecting scope with stage facing air filter and air stream with arm of scope facing **away** from face of filter.
- Once removed carefully from autoclave, allow hot media to cool for no more than 30-40 minutes in the air stream of laminar flow hood.
- Once media flask is cool enough to handle, pour carefully into sterile Petri dishes at a depth of about 5mm.
- Allow freshly poured plates to cool in stacks of +/-10 until solid within air stream of flow hood (about 30-90 minutes).

-Procedures for Photobiont Isolation by Thallus Homogenate:

This method requires use of aseptic conditions and sterile technique and all procedures are carried out in the air stream of a laminar flow hood.

Freshly collected (within one week) lichen thalli are washed for 5 minutes in running tap water then allowed to soak in DI H₂O for 10 minutes, then brushed lightly with a clean paintbrush under running DI H₂O.

1. Small sections, about 2cm square, are cut from the thallus using a clean razor blade.
2. Under the dissecting microscope, the upper cortex and photobiont layer are removed from thallus piece by scraping the lichen using sterile scalpel or sharpened spear needle on the surface of a sterile microscope slide. The portion not containing photobiont layer is discarded.
3. Once the photobiont layer has been removed, it is transferred to a sterile microscope slide and 2 drops of sterile water from a sterile pipette are added to the extracted photobiont layer.
4. Using another sterile microscope slide on top of the water/photobiont mix gently rub the two slides together to further separate photobionts from cortex and mycobiont cells, then separate the two slides and place them wet-side-up.
5. Individual experimenters may wish to add a drop or two more of sterile water at this point to the two slides of photobiont homogenate. Either way: using a fresh

sterile pipette and another pump bulb, draw up the homogenate from one slide and squeeze 2-3 drops of liquid homogenate onto the surface of solid photobiont media. Repeat this procedure using the same pipette and drop the homogenate from the other slide onto more Petri dishes containing photobiont media.

Photobiont isolations should be kept at a temperature comparable to temperature range of the free-living lichen source. Light intensity should be present but not direct, on a cycle of 12 hours light/12 hours dark.

Once germination of algae is apparent, colonies must be examined for remaining fungal cells inter-mingled with algal cells, this will confirm that true photobiont cultures have been obtained and these should be further isolated onto fresh BBM glucose agar plates. This examination should also be coupled with identification by algae taxonomy and side-by-side microscopic comparisons between different photobiont isolations from the same free-living lichen source.

Once experimenter is confident in the isolate and ID of their lichen photobiont, test tube "slants" of BBM glucose agar media should be prepared and isolates transferred to these tubes for long-term storage and future lichen synthesis experiments.