

ISOLATION TECHNIQUES FOR BASIDIOMYCETES

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Basidiomycetes are routinely isolated from fruit body tissue, basidiospore deposits, bulky substrata such as wood, and vegetative mycelial aggregations such as rhizomorphs, mycelial cords, sclerotia and ectomycorrhizal sheaths. Since fruit bodies provide the majority of diagnostic characters used in basidiomycete identification, it follows that those cultures derived from such structures, or which fruit readily, are relatively easy to identify. A useful guide to relevant taxonomic works is provided by Rayner & Boddy (1988) although primarily intended for wood-inhabiting species. Non-fruiting isolates derived from vegetative material require more taxonomic and cultural experience. However, many species in mycologically better-studied parts of the world can still be distinguished using keys based on cultural characters alone, such as wood-inhabiting Aphyllophorales (see Stalpers 1978), *Mycena* (see Treu & Agerer, 1990) and North American ectomycorrhizae (see Hutchinson, 1991).

Basidiospore Collection

Basidiospores from the majority of species naturally fall from fruit bodies under the influence of gravity and so deposits of spores can usually be obtained at room temperature overnight on a clean surface placed beneath portions of fruit bodies. A bowl or beaker is inverted over the fruit bodies to protect the falling spores from draughts. Stipes should be removed where necessary and some fruit bodies may have to be wedged to permit vertical deposition from their basidia. Ideally some spores should be collected on a glass microscope slide because the dimensions and other morphological features of mature spores are useful in identification. It is worthwhile remembering that younger fruit bodies are less likely to be substrata for other fungi and so less likely to produce a misleading spore deposit. To minimise the chances of isolating mycoparasitic fungi, spore morphology should be checked against published

descriptions. Glass slide sandwiches, cellophane sheets and aluminium foil are all useful for keeping spore deposits clean when in transit and before they are used to isolate germling mycelia (see below). When consulting some taxonomic keys, however, it must be borne in mind that spore colour is described as seen against a background of white paper (see Moser, 1978), which may differ slightly but significantly from that recorded for spores on glass.

Isolation Techniques

Single Spore Isolation

Such isolates are preferred to those subcultured from plates of interacting germlings (polyspore isolates) in all studies where it is important to know the origin and particularly the genetic origin of the mycelia used.

A drop of sterile distilled water is added to the edge of a spore print collected on a non-absorbent surface. The resulting spore suspension is then spread across the surface of agar media in Petri dishes. Frequently-used isolation media include 2% MEA, RTMEA and PDA whereas for ectomycorrhizal fungi, Fries Agar and MMN are used (see list of media components). Dilutions of the spore spreads are made by adding further drops of sterile distilled water and removing spores in suspension to a fresh agar plate on a sterile spreader until, using a x 10 microscope objective, approximately one spore is visible per field of view as viewed through the base of an unopened dish.

Some spores will germinate overnight at 15-20 C some may take a week or two; and others are open to experimentation. Germination inducers that have been applied to certain basidiomycetes include, alone or in combination, fungal mycelium, yeasts such as *Rhodotorula glutinis*, chemicals such as activated charcoal (Ali & Jackson 1989; Fries, 1987) and heat treatment (40 C) for dung fungi. However any agent thought to be ecologically relevant is worth trying with persistent non-germinators.

If the spore print proves to be contaminated, then a fresh attempt should be made using a medium containing 0.1g l⁻¹ novobiocin (Sigma) or 2mg l⁻¹ benomyl (Benlate, DuPont) to combat respectively bacterial or *Trichoderma* contaminants. Such measures are equally applicable for any contaminated basidiomycete culture, regardless of origin. When germlings are microscopically visible, but before they have branched sufficiently to cast doubt on their single-spore origin, the Petri dish lid is removed and the agar surface scanned microscopically with a x 10 objective until a single germling is located at the centre of a field of view. The objective is then racked away from the dish and a dummy objective rotated into position. This device (a modified La Rue cutter) consists of a protruding metal tube, which may be periodically

flame-sterilised, mounted in place of the lenses of a normal microscope objective. The diameter of the tube is just less than that of a field of view as observed with a x 10 objective. By lowering the dummy objective until it briefly depresses the agar surface, the previously observed target germling's position will be marked by a circular indentation. After transferring the dish to a stereo dissecting microscope, a small plug of agar bearing the germling is cut from within the marked circle using a mounted tungsten needle that may be repeatedly sharpened in molten sodium nitrite. It is necessary to carry out needle sharpening in a fume hood. The mounted tungsten wire is placed in molten sodium nitrite and the tip is stroked on the base of the shallow metal dish containing the liquid. The dish is constantly heated from beneath a tripod by a gas burner to prevent the sodium nitrite solidifying. The germlings are subsequently cultured in isolation. It should be emphasised that clamp connections can form on single spore, ie unmated, isolates of several basidiomycete species and so do not always indicate that germlings have interacted by hyphal fusion. To ensure that the desired fungus has been isolated by this or the following methods, its cultural characteristics should be checked against published details if possible.

Isolation from Mycelial Aggregations

In general, isolation is more easily achieved from bulky fruit bodies than from smaller structures such as cords, stromata and mycorrhiza. In the case of large fruit bodies, these can be torn open and a small silver of approximately 2mm³ removed from within, taking care to avoid the fertile regions and outer surfaces. The silvers are placed on the surface of media similar in composition to those used for single spore isolation, but must be observed at least every two days for signs of contamination. The morphology and extension rate of common contaminants soon becomes apparent and aids attempts at their early removal. Contamination of mycelia with bacteria or *Trichoderma* can be minimised with media incorporating novobiocin or benomyl and the fruit bodies should be re-sampled after surface sterilisation in 5% domestic bleach or, preferably, in 5-10% H₂O₂. The latter pre-treatment is essential for isolation from all smaller fungal structures. Unicellular contaminants can often be eliminated by cutting out a square of agar containing the contaminated mycelium and replacing it in an inverted position on the agar surface nearby. This technique relies on the ability of mycelia to penetrate the square agar slab so they can be subcultured on reaching the upper surface. Purity checking of mycelia to ensure the absence of algae, bacteria and yeast contaminants is routinely carried out in ISP 1 broth and agar (see list below).

Isolation from Bulky Substrata

If the aim is to isolate those species whose fruit bodies are visible on the

substratal surface, then small slivers should be removed and plated out as for the fruit body samples. It is important to sample as close to the fruit bodies as possible, avoiding any pigmented zones which can form at the boundaries of mycelia, eg : as a result of interspecific interactions. However, if the aim is to study community structure, then samples should be taken within all regions that are bounded by pigmented zones or which have different degrees of staining. Examples of community sampling from wood slices of decayed logs and other sources are provided in Cooke & Rayner (1984).

Hyphal Tip Isolation

To be absolutely sure of the origin of cultured mycelia, the smallest viable unit (ie: a single hyphal compartment, usually an apex) should be isolated. The fungus is grown on an uncoated cellophane membrane (sterilised in distilled water by autoclaving at 121C for 20 minutes) overlying a very weak agar medium, eg: 0.02% MEA. This renders the mycelium sparse enough to permit the severing of an apical compartment using a sharpened tungsten mounted needle below a x 10 microscope objective. Surrounding hyphae are then scraped away with the needle and the dish transferred to the stage of a stereo dissecting microscope. A rectangle of cellophane is cut out with a sterilised scalpel enclosing the desired apex and no other living hyphal fragment. This is then removed using sterilised fine forceps and quickly transferred to a dish of fresh medium at normal nutrient strength before the apex dries out.

List of Media Components

MALT EXTRACT AGAR (2% MEA)	
Malt Extract	20g
Agar	15g
Distilled Water	1000ml
Autoclave at 121 C for 15 minutes	
RAPER & THOM MEA (RTMEA)	
To medium 2% MEA add:	
Glucose	10g
Soy Peptone	5g
POTATO DEXTROSE AGAR (PDA)	
Agar	20g
Dextrose	15g

Add 200g of cleaned, boiled, sieved potato cubes (boiled for 1 hour in 1000ml water). Make up to 1000 ml with distilled water and autoclave at 121 C for 20 minutes. Avoid new potatoes which do not make good media.

FRIES AGAR

Glucose	4g
Ammonium tartrate	1g
KH ₂ PO ₄	0.2g
MgSO ₄ .7H ₂ O	0.1g
NaCl	20mg
CaCl ₂ .2H ₂ O	26mg
ZnSO ₄ .7H ₂	0.88mg
MnSO ₄ .4H ₂ O	0.81mg
FeCl ₃ .6H ₂ O	0.8mg
Malt extract (Difco)	1g
Agar (Difco)	15g
Thiamin	100µg
Pyridoxine	100µg
Riboflavin	100µg
Biotin	25µg
Nicotinamide	100µg
p-amino-benzoic acid	100µg
Pantothenic acid	100µg
Inositol	10,000µg

Make up to 1000ml with distilled water and autoclave at 120 C for 10 minutes.

MODIFIED MELIN-NORKRANS AGAR (MMN)

Glucose	5g
Malt extract	2g
Yeast extract	1g
Potassium phosphate monobasic	0.5g
Ammonium phosphate dibasic	0.25g
Magnesium sulphate	0.15g
Calcium chloride	1% sol'n (5ml aliquot)

Sodium chloride	1% sol'n (2.5ml aliquot)
Ferric chloride	1% sol'n (1.2ml aliquot)
Agar	15g

Make up to 1,000ml with distilled water and autoclave at 121 C for 30 minutes.

TRYPTONE YEAST EXTRACT BROTH (ISP 1)

Bacto-tryptone (Difco)	5g
Bacto-yeast extract (Difco)	3g
Distilled water	1000ml

Adjust pH 7.0 to 7.2 before autoclaving

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