

FREEZE-DRYING OF FUNGAL HYPHAE

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Methods of long-term preservation in which metabolism is inhibited during storage are preferable to maintenance on agar slants. The last mentioned method is laborious because regular transfers onto fresh media are required. In addition, repeatedly subcultured strains may undergo mutations.

Current methods for long-term preservation are freeze-drying and preservation at temperatures below -130°C . Cryopreservation methods give high survival rates and are universally applicable. However, some disadvantages are encountered. Methods using nitrogen are rather expensive. Regular supply of liquid nitrogen is not always guaranteed. The dependence of ultra-deep freezers on electricity may be problematic in developing countries. Organisms must be dispatched under frozen conditions or activated before transport.

Freeze-drying is a good alternative. Ampoules can be stored easily in dense packing without any special requirements. Cultures need not be revived on agar slants prior to dispatch. The product is light, inactive and dry, enabling easy distribution by mail.

However, so far only conidia or spores can be freeze-dried successfully. Most attempts to revitalize dehydrated hyphae have failed, except for some successes reported for vesicular arbuscular mycorrhizal fungi (Tommerup, 1988), *Claviceps* (Pertot *et al.*, 1977) and some Basidiomycetes (Bazzigher, 1962).

Previous studies have shown that the survival rate of frozen fungal cells increases considerably when cells are cooled at a rate of $-1^{\circ}\text{C}/\text{min}$ (Mazur *et al.*, 1972; Hwang *et al.*, 1976; Grout and Morris, 1987). Several authors have stressed the

beneficial effect of trehalose (Crowe *et al.*, 1984, 1990; van Laere, 1990; Wiemken, 1990). Addition of peptides to the resuscitation medium is aimed to improve recovery (MacLeod and Calcott, 1976). After optimization of these parameters, hyphae of a test series of Ascomycetes and Basidiomycetes were freeze-dried. Trehalose was added to the growth medium rather than to the protectant to allow absorption by the cell. Absorption would result in protection of cellular membranes as well as membranes of organelles.

Materials and methods.—Cultures tested: *Alternaria bataticola* Ikata *ex* Yamamoto, CBS 532.63; *Alternaria dianthicola* Neergaard, CBS 112.38; *Cercospora rautensis* Massal., CBS 555.71; *Chaetomium globosum* Kunze: Fr., CBS 143.38; *Coprinus psychromorbidus* Redhead & Traquair, CBS 865.85; *Schizophyllum commune* Fr.: Fr., CBS 333.85.

The following media were employed: 400 ml malt extract (TNO, Zeist, The Netherlands: 10% sugar), 600 ml water, 15 g agar (MEA 4%); 200 ml malt extract, 800 ml water, 15 g agar (MEA 2%); 30 g oat flakes (Quaker Oats B.V., Dordrecht, The Netherlands) in 1 L water, 15 g agar (OA); 20 g powdered malt extract (Difco), 1 g peptone (Difco), 20 g glucose, 1 L water (MP); MP and 5% trehalose (MPtreh).

To ensure that the hyphae were vital and undamaged, very young small colonies, 0.5 to 1.5 mm in diam, were freeze-dried. They were obtained according to the following procedure. Organisms were grown in Petri dishes on an uncoated PT cellophane membrane (Bovenkamp-Papier, Veenendaal, The Netherlands) on 15 ml MEA 4%. All strains were incubated at 24°C

except *C. psychromorbidus* (17 C). After one week for *S. commune* and *C. globosum* and up to three weeks for the other organisms, the cultures were lifted from the cellophane and suspended in 10 ml MP in a Waring blender. The suspensions were mixed at low speed for 2×1 sec. Five ml of the suspension were added to 75 ml of either MP or MPtreh in 300 ml Erlenmeyer flasks.

The suspensions were incubated on a rotary shaker (150 rpm) at 24 C, except *C. psychromorbidus* which was incubated 7 days at 24 C and subsequently at 17 C. Colonies of the ascomycetous strains that were produced were harvested 3 days later and colonies of the Basidiomycetes 10 days later.

After harvesting, individual colonies were transferred to 300 μ l 12% skimmed milk (Elk, DMV Campina B.V., Eindhoven, The Netherlands) in a 2 ml constricted ampoule (Wheaton Scientific, Millville, New Jersey).

Colonies were cooled at a rate of -1 C/min to a temperature of -45 C and subsequently to -75 C in 30 sec using a Syllab Icecube 1610. Freeze-drying was performed in a Virtis Unitop 600 SL with Freezemobile 12XL (condensor temperature -80 C; vacuum 5–20 millitor) for 48 h at -45 C followed by 1 or 2 h at 20 C, giving a residual moisture content (RMC) of 4–6 and 2–3% respectively. The ampoules were closed under vacuum.

For revival, pellets were suspended in 10 ml MP and incubated for 16 h at 24 C on a rotary shaker (150 rpm). Afterwards, the mycelia of *A. dianthicola*, *A. bataticola*, *C. rautensis* and *C. globosum* were transferred to 15 ml OA; *S. commune* and *C. psychromorbidus* were transferred to 15 ml MEA 2%. *A. dianthicola*, *A. bataticola* and *C. rautensis* were incubated at 22 C, *C. globosum* at 28 C, *S. commune* at 24 C and *C. psychromorbidus* at 17 C.

RMC was estimated by the titrimetric procedure of Karl-Fischer (Anonymous, 1990) with a Mitsubishi CA05 Moisture Analyzer coulometric titrator with microprocessor control (Mitsubishi Chemical Industries Ltd., Tokyo, Japan). In this procedure iodine generated coulometrically reacts quantitatively with water in a solvent containing pyridine, sulfur dioxide, and methanol. Ampoules, filled with material, dried for 16 h at 45 C, were weighed. Subsequently, 1 ml Coulomat A (Riedel de Haën A.G., Seelze, Germany) was added to the pellets through the rubber stop with a syringe. The pellets were allowed to

dissolve for 20 min. Afterwards, they were centrifuged (10 min; 5000 rpm). One hundred μ l of supernatant was transferred to the titration vessel. The generator solution cell was filled with 100 ml Coulomat C (Riedel de Haën A.G., Seelze, Germany); the cathode solution cell was filled with 5 ml Coulomat A. One hundred μ l Coulomat A was titrated as a blank. Weights of empty, ethanol-rinsed ampoules were estimated after drying for 1.5 h at 115 C. RMC was calculated according to the following formula:

$$\frac{\text{quantity reagent added}}{\text{quantity reagent titrated}} \times \frac{\text{results analysis} - \text{blank}}{\text{weight pellet} + \text{weight air in ampoule}} \times 100 = \% \text{RMC}$$

Results and discussion.—Strains of *Alternaria dianthicola*, *A. bataticola*, *Cercospora rautensis* and *Coprinus psychromorbidus* had lost the ability to sporulate completely. *Chaetomium globosum* and *Schizophyllum commune* did produce asco- and basidiospores, respectively. In the experiments however, only young colonies consisting of sterile hyphae were freeze-dried. The sporulating strains were included to verify whether the ability to sporulate was retained after freeze-drying.

All strains could be revived (TABLE I). The ascomycetous organisms *Alternaria dianthicola*, *A. bataticola*, *Cercospora rautensis* and *Chaetomium globosum* showed the same characteristics as before freeze-drying, including the production of ascospores by *Chaetomium*.

The survival rates of the two Basidiomycetes studied were found to be lower than those of the Ascomycetes. Revived colonies of *Schizophyllum commune*, incubated in MP medium, were extremely poor and slow-growing; basidia were not produced. When freeze-dried after incubation in MPtreh, a higher growth rate was obtained but colonies remained atypical as no aerial mycelium and no basidiomata were produced. Results with *Coprinus psychromorbidus* were even poorer. Only six out of 25 colonies could be revived.

Survival rates of Ascomycetes as well as Basidiomycetes were significantly higher when the organisms were incubated in a medium containing trehalose. For Ascomycetes 72 out of 98 dried entities grown in MP were viable, while 91 out of 93 colonies incubated in MPtreh could be re-

TABLE I
SURVIVAL OF FREEZE-DRIED COLONIES AFTER PREINCUBATION IN MP MEDIUM WITH AND WITHOUT TREHALOSE

Species	Preincubation medium					
	MP			MPtreh		
	RMC ^a	Positive ^b	Negative ^b	RMC ^a	Positive ^b	Negative ^b
<i>Cercospora rautensis</i>		4	—			
<i>Alternaria bataticola</i>		18	—		10	—
		16	—		12	—
	5	5	—	6	5	—
	2.2	9	—	2.6	9	—
<i>Alternaria dianthicola</i>		—	9		14	—
		9	6		22	—
	5	—	4	6	3	—
	2.0	9	—	2.9	9	—
<i>Chaetomium globosum</i>		2	7		2	2
<i>Coprinus psychromorbidus</i>		—	8		2	7
	3.3	2	2	4.2	2	2
	5	2	2	6	2	2
<i>Schizophyllum commune</i>		1	8		9	—
	2.5	1	8	3.1	9	—

^a RMC: residual moisture content.

^b Number of positive and negative colonies.

vived. The effect was even more marked in the Basidiomycetes studied. Three out of 21 colonies incubated in MP could be revived, while 13 out of 22 colonies grown in MPtreh proved to be viable (TABLE I).

Residual moisture contents of pellets containing hyphae that were incubated in medium supplemented with trehalose were consistently somewhat higher than when trehalose was omitted. Part of the trehalose protection might be explained by its slightly greater water binding capacity. Trehalose is the only disaccharide that has two water molecules in its crystal (Dawson *et al.*, 1968). In addition, the hydroxyl groups of this compound in particular are generally taken to mimic the dipole of water after dehydration (Crowe *et al.*, 1985).

The survival rate of the melanized strain of *Alternaria bataticola* was higher than that of the hyaline mutant *A. dianthicola*. All 84 colonies of *A. bataticola* survived. With regard to *A. dianthicola*, 100% survived when incubated in MPtreh but only 18 of the 37 colonies were viable when incubated in MP (TABLE I). This tendency is in accordance with results published by Breierová (1990), who observed that melanin- or carotene-containing yeasts showed higher survival rates after cryopreservation. The protective value of these compounds against damage caused by freezing and dehydration may be explained by

their ability to scavenge free radicals produced in dehydrated biological materials (Lukiewicz, 1972; Breierová, 1990).

Future research will focus on the stability of the freeze-dried product. Freeze-dried spores or conidia survive storage at 20 C up to periods of 25 years when dried to residual moisture contents below 5%. By extrapolation, the samples are expected to be stable, especially those dried to residual moisture contents ranging between 2 and 3%.

Key Words: freeze-drying, hyphae, trehalose

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