How to preserve stocks.

David Perkins

Background

Storage of stock cultures in suspenced animation is essential, both for wild type references and for mutant strains. Not to do so compromizes their integrity. Cultures stored at 5°C must be transferred and regrown periodically to maintain viability. Each cycle of storage and regrowth presents an opportunity for mutation and selection. With certain auxotrophs (e.g. methionine, cysteine, adenine requirers), double mutants with additional blocks in the same biosynthetic pathway are at a selective advantage and accumulate as metabolizing cultures are stored and transferred..

The simplest way to avoid trouble is freezing at -20°C (Perkins 1973). This is highly effective for conidiating cultures, which remain viable for several years without growth or transfer. However, nonconidiating strains do not survive freezing and thawing, and viability of conidiating cultures depends on keeping the conidia dry.

Lyophylization is highly effective, assuring long-term servival (Barratt and Tatum 1950. Wilson 1986. McCluskey, 2000). However, the method is laborious and is difficult to use with many nonconidiating strains. Also, each individual tube can be sampled only once.

Preservation on anhydrous silica gel is usually the method of choice, especially for vigorous conidiating cultures (Perkins 1962, 1977. Wilson 1986. Metzenberg 1994. Brockman and de Serres 1962, Catcheside and Catcheside 1979. Kolmark 1979. Smith 1979). It is effective for relatively long periods and is less laborious than lyophylization. Individual tubes can be sampled repeatedly.

Silica gel can be used to preserve at least nonconidiating strains, but only with special effort, using suspensions of mulled mycelia or preparing as described by Metzenberg. (1994) (see below).

As a last resort, difficult strains may be stored at dry-ice or liquid-nitrogen temperatures (Wilson 1986. Jong and Davis 1979. Pounder and Bowman 1999). See, for example, *How to work with slime and other cell wall-deficient strains*.

Silica gel and lyophylization are preferred as standard methods not only because they ensure viability over long periods, but also because (unlike with cryopreservation and methods dependent on refrigeration) they are not vulnerable to power outages and are not dependent on an uninterrupted supply of dry ice or liquid nitrogen. Strains that are aconidiate, poorly viable, or difficult to grow can often be carried in sheltered condition in combination with a "helper" strain that complements the defect and produces a conidiating heterokaryon which can be readily preserved using standard methods (Perkins 1984, 1993. Metzenberg and Sachs 2002). See *How to use helper strains for maintaining and crossing handicapped recessive mutants, for forcing and resolving heterokaryons, and for determining heterokaryon compatibility*.

Procedures

***Silica gel*:**

The following account of current FGSC usage by Kevin McCluskey updates and supercedes the description published by Wilson (1986):

"The FGSC uses 12-28 Mesh, Grade 408 silica gel, without indicator. We dispense about 3.5 g of silica gel into a 13 × 100 mm screw cap tube and plug the opening with cotton. These are sterilized by baking at >150°C overnight. The sterile tubes are maintained in a 75°C oven to keep them dry. To prepare silica gel stocks, a culture of the strain of interest is grown in a 13 × 100 mm tube on appropriately supplemented agar solidified medium for seven to ten days. One to two ml of 7% reconstituted Carnation brand nonfat dry milk is added to the tube and the surface of the slant is scraped with a sterile loop or spatula. Vigorus vortexing can also be used, but tends to fill the air-space of the tube with a cloud of spores making subsequent handling difficult. Silica gel 'blanks' are capped tightly with sterile caps and pre-chilled in ice. 350 to 500 µl of spore suspension is drawn up into a pasteur pipette and dispensed into

the chilled silica gel trying to spread the solution onto as much silica gel as possible. The tube is capped and vortexed vigorously for one to two minutes, breaking up any clumps in the process. The new stock is returned to ice prior to being placed in a box over dessicant at 5°C. After a week or more, the new stock is tested for viability before being moved to the main storage series.

"The sterile milk is prepared by suspending 3.5 g milk powder in 50 ml of deionized water. This is sterilized in a pressure cooker at 15 pounds for 20 minutes. Sterilizing at higher pressure in the autoclave carmelizes the milk.-The prepared milk is kept in the refrigerator."

Procedures used at Stanford (Perkins 1977) differ in minor respects. (1) Culture tubes (13 × 100 mm) without screw caps or a constricted neck are easier to fill. They are plugged with cotton, sterilized by baking as described above, and stored above dessicant in a dessicator, at room temperature. After introduction of the cell suspension, the cotton plug is trimmed flat and each tube is sealed with two precut squares of Parafilm. (2) By growing cultures on slants made up with 4% agar, conidia can be suspended in the original culture tube, where the stiff agar remains intact during vortexing. (3) Nonfat milk is dispensed to small tubes and sterilized in a pressure cooker for 10 minutes at 5 pounds pressure on two successive days.

The following streamlined variations devised by Metzenberg (1994) seem promising, especially for *fluffy* and other nonconidiating or poorly conidiating strains.

"The method for storing Neurospora strains described by Perkins (1962, 1977) and elaborated in a collection of articles in Neurospora Newsletter 26 has made it possible to keep large collections with much less effort than would be required with the older lyophil method. In Perkins' method, conidia are suspended in sterilized nonfat milk and the suspension is pipetted onto chilled sterile silica gel. For nonconidiating strains, mycelia are mulled or otherwise fragmented in milk to make the suspension. However, even this greatly improved method requires a nontrivial amount of manipulation when large numbers of strains are to be preserved, especially for nonconidiating strains. Preparing each stock consumes a pipet and at least two test tubes: one for growth of the strain and one for preservation. Both tubes need to be labelled, which adds to the effort and to the chance of error. The following method requires no pipetting. The stock is preserved in the tube in which it was grown.

"Perlite, a fluffy white volcanic ash available at any garden center, is put into culture tubes up to no more than 1/5 of their total length. A liquid medium of choice (see below) is added so as just to cover or nearly cover the Perlite and the tubes are autoclaved. The tubes are inoculated and then tapped in a near-horizontal position to form slants which holds their form when the tubes are gently returned to vertical. When the strains have conidiated well, or if nonconidiating, have grown to their limit, baked silica gel, 6-12 mesh, is added to about 4/5 the capacity of the tubes. The tubes are capped, sealed with pre-cut strips of Parafilm, and put at 4ºC. At least 2 weeks later, they are shaken vigorously and stored at 4ºC or in a deepfreeze. The addition of silica gel should be done in a hood for obvious reasons. I have found it convenient to sterilize and dry the gel in an oven in a casserole or open beaker for 2 hours at 450ºF (about 230ºC) and store it in presterilized bottles. Sterile 3 ounce paper dixie cups, upside down and separated by squares of toilet paper, are convenient single-use vessels, and the cups are squeezed to form a pouring lip for adding the gel to the tubes.) A fragment of the dry Perlite or a crystal or two of silica gel will start a new culture of a conidiating strain. For nonconidiating strains, the Perlite is necessary; silica gel crystals usually remain sterile. If the Perlite is difficult to disperse, breaking it up the mass with a sterile bamboo stick can be helpful.

"Recently I have found that "seed beads" are a very convenient alternative to Perlite, especially for non-conidiating cultures. I use very small black or colorless beads from the Czech Republic, available at craft shops or at Discount Beads, POBox 186, The Plains OH 45780; tel. 1-800-793-7592; $25/kilo. I have used 5 g of beads per 18×150 mm tube with 1.5 ml of medium, and form slants by tapping, as with the Perlite. (Presumably, 13×100 mm tubes with about 0.75 ml of medium would work as well.) The holes in the beads trap medium and the mycelium grows into these protected holes. The beads disperse more easily after they are dried with silica gel than does Perlite. The convenience of seed beads often seems worth the greater cost, still only 12.5 cents per tube.

"Finally a caveat. I have not had trouble with cultures dying, but my experience has been very short: about a year with most of the Perlite cultures, and negligible with the seed-bead cultures. Only time will tell how the longevity of these stocks compares with that of stocks made in the traditional ways. Others have found that nonfat milk as a suspending medium enhances survival of lyophil cultures. I have found that Neurospora grows well in otherwise

permissive liquid medium to which nonfat powdered milk has been added to 10% w/v before autoclaving, and I have used this with beads to prepare stocks. However, I do not know whether this improves survival of the stocks. I would appreciate hearing the experience of anyone who decides to try this method."

***Lyophyl*:**

Growth of cultures and suspension of conidia or mycelial fragments is as described above for silica gel. The following account of current FGSC usage by Kevin McCluskey updates and supercedes the description published by Wilson (1986):

"Often the same slant culture is used to prepare silica gel and lyophil stocks. Lyophils are made in 6 × 150 mm tubes that are either prepared from tubing, or custom made (Belco, Inc). One end is sealed and the other is plugged with cotton. The empty tubes are sterilized by baking. Each spore suspension is dispensed in approximately 150 to 200 microliter aliquots into each of six lyophil blanks ~~which~~ and these are resealed with cotton. Each sample is then frozen by holding over liquid nitrogen for two minutes, followed by a brief plunge into the liquid nitrogen. The spores are freeze dried overnight using a Heto LyoLab 3000.

"Prior to the availability of this equipment, freeze drying was carried out over a bed of phosphorus pentaoxide in an evacuated dessicator jar at −20°C. While this technique is useful for labs that do not have access to a lyophilizer, the phosphoric acid produced is dangerous and hazardous.

"After the tubes are dried, they are sealed under vacuum using an oxygen/mixed gas flame."

Freezing:

***Preserving conidiating cultures at −20°C*:** Freezing provides a convenient method that avoids the need for frequent transfer and minimizes chances of accumulating mutational changes, yet takes no more effort than storage in the refrigerator at 5°C (Perkins 1973). Mites and mite eggs are destroyed by freezing. Frozen cultures can be sampled repeatedly without loosing viability, provided that they are returned to the freezer promptly and that conidia remain dry. It is well to let the agar dry down slightly before freezing. Using 75 mm tubes makes for economy of storage space. Dehydration in the freezer can be minimized by storing tubes in sealed plastic boxes or by sealing them with Parafilm. Frozen cultures remain viable for many years. Conidia from a frozen culture can be used directly for fertilization. Eventual loss of viability in aged, dried down cultures is signalled by fading of orange pigment in the conidia.

Survival of nonconidiating cultures is poor after freezing and thawing. Conidia do not survive well if refrozen after becoming wet. Ascospores do not survive freezing. The freezing compartment of refrigerators that incorporate an automatic defrost cycle should be avoided or should be used only if the defrost cycle is inactivated. This can perhaps be done by removing the light bulb that turns on when the refrigerator door is opened.

Freezing at -20°C is used at the FGSC as a means of backing up new or ancillary cultures (for example, stocks from the Perkins wild-type collection that do not have an FGSC number). Slants are sealed with wax film and stored in sealed plastic bags. These cultures are kept frozen on dry ice during sampling.

***Preserving nonconidiating cultures at −80°C*:** Storage of aconidial Neurospora strains by freezing of mycelia as agar plugs and slants at −80°C and −20°C was investigated by Pounder and Bowman (1999). The following is adapted from their account:

"*fluffy A* (FGSC 4960), *fluffy a* (FGSC 4961), and *acon-3* (FGSC 5074) were grown on plates of Vogel's Minimal Medium with 2% sucrose for 3 days at room temperature to a confluent mycelial lawn. Approximately 1 cm square plugs were cut from the agar and placed into sterile 1.5 ml Eppendorf tubes. The tubes were placed in a −80°C freezer with no flash freezing and no glycerol or DMSO added. Agar plugs were retrieved from −80°C after 7 days to 9 months and plated onto the same medium as above. They grew to form a confluent lawn in 3 days. The recovered *fluffy* strains functioned normally in mating type tests. Whole agar slants with mycelia from the aconidial strains were frozen at −80°C. Pieces chipped from the frozen agar grew well on plates following storage of 1 month.

Recovery from agar plugs and slants stored for 1 month (the only time tested) at −20°C was slower than from those stored at −80°C, with 3 to 7 days required for a confluent lawn of mycelia to grow. The recovery of aconidial strains after freezing under these conditions may be due to the larger amount of mycelia stored initially. Sufficient mycelia are present in the 1 cm plugs to allow recovery of the strains, providing a convenient alternative storage technique for aconidial strains."

***Cryopreservation at the FGSC*:** The cryporeservation technique used at the FGSC is described below.This account by McCluskey supercedes that of Wilson (1986).

"While there is an abundant literature on preservation of fungal spores with different cryo-protectants, we have had the best success using the 7% milk as a medium to suspend spores and mycelia prior to mixing 1:1 with 50% glycerol. Slow freezing is preferred to quick freezing, and for strains that we expect to access frequently (such as *fluffy* or *slime*), we make many small aliquots such that we can take one from the freezer without the anticipation of re-freezing it. The freezer stocks are prepared the same whether they will be stored at −80°C or over liquid nitrogen.

"For strains that are highly mycelial, we grow them on agar-solidified medium and cut plugs or blocks of the agar and place that in cryovials which are subsequently flooded with 7% milk and then 50% glycerol in a volume equal to the combined volumes of the agar and milk."

Procedures used by the American Type Culture Collection for preparation, storage, and recovery of cultures are described by Jong and Davis (1979).

***Sheltering of aconidial and poor-growing cultures in heterokaryons*:**

If strains are difficult to preserve because of a recessive defect, a simple solution is to form a conidiating heterokaryon which can readily be silica gelled or lyophylled. See *How to use helper strains for maintaining and crossing handicapped recessive mutants, for forcing and resolving heterokaryons, and for determining heterokaryon compatibility.*

***Storage of ascosporee and perithecia*:**

Ascospores can be stored at 4°C in distilled water, with little loss of viability after at least 18 months (Smith 1973). When cross tubes dry down at room temperature, extruded ascospores collapse and may become sickle-shaped. These remain viable for a long time (never defined), but they must be thoroughly rehydrated before being heatshocked (Strickland and Perkins 1973). When crosses are made in small (10 × 75 mm) tubes, the tube may dry out before all asci have been expelled from the perithecia. Ascospores that remain enclosed in the dried perithecia proceed to ripen fully. These remain viable and show good germination when linear asci ooze out after rehydration of the perithecia (Patricia St. Lawrence, personal communication; Maling 1960). The dehydrated crosses can be stored for at least many months with little loss of ascospore viability.

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