Method for obtaining mycelial pads

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Abstract
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Vigfusson, N. and J. Weijer. Improved method for the isolation of sterility mutants. Recent work in this laboratory has involved, in part, the isolation of sterility mutants in Neurospora crassa. The standard method for isolating these mutants is very laborious: conidia after mutagenesis are plated on sorbose medium, individual colonies are isolated after 48-72 hours and subsequently tested for sterility by crossing them with the appropriate mating type. Crosses are made by inoculating dry conidial onto a plate on which the other mating type is already growing.

A more rapid method of isolation involving replica plating has now been introduced. Conidia after mutagenesis are plated on sorbose medium, but at a lower concentration (approximately 20 colonies per plate). After 96 hours there plates are replicated with a velvet pad to a plate containing a lawn of growth of the other (but acnoidal) mating type. Master plate are now held in the freezer to avoid any further growth. After 14-18 days at 25°C, crossing plates can be inspected for reduction of fertility of the replicated colonies. Such colonies are then picked from the master plate for further testing. Colonies showing no reaction whatsoever on the crossing plate are ignored, on the assumption that they were insufficiently replicated by the velvet. (Our observations indicate that mutants showing an extreme degree of sterility still will react with the opposite mating type; however, in such cases the sex reaction does not proceed beyond the formation of very small protoperithecia.)

The above method reduces drastically the number of isolates that must be handled individually as the following figure indicate: 1,103 colonies on 80 plates have been replicated to date. From there, 23 colonies have been isolated as "suspects", four of which have turned out to be mutations to reduced fertility. By comparison, 6,357 colonies have been isolated by the conventional method, yielding 27 mutants. The method has been tested with good results using sterile, semi-sterile, and fertile strains.

Dutta, S. K. The isolation of nuclei from Neurospora crassa conidio. A greater degree of purity of nuclei isolated from conidial cells of N. crassa is obtained by combining the procedures described by Reich and Tsuda (1961 Biochem. Biophys. Acta 53:574) and Munkres et al. (1966 Neurospora News 9: 14) for mycelial cells with some modifications made in our laboratory. The strain used was wild type 74A and all operations are conducted in the cold at 0-4°C. The conidial mass is squeezed dry between Whatman blotting papers and then ground gently with twice its volume of acid-washed sand (prepared by powdering the commercially obtained sand in a Wiley Mill and passing through a 60-mesh screen) until a smooth paste is obtained. About five volumes of sucrose-EDTA (0.5M sucrose, 1mM NaEDTA, 0.01M Tris HCl pH 6.5) is added gradually, stirred into a thick paste, and filtered through four layers of silk cloth. The filtrate is centrifuged at 2000 x g for 25 minutes in a refrigerated centrifuge. This crude nuclear pellet is then suspended in a solution containing 0.5 M sucrose, 2 mM EDTA and 5 mM CaCl₂ at pH 6.5 and centrifuged at low speed (500 x g) for two minutes. Two kind of pellet were noticed. A hard pellet was formed below the loose pellet. The nuclei contained in the loose pellet were dispersed in their own supernatant and centrifuged again at 500 x g for two minutes. This low-speed centrifugation was repeated until no more hard pellet was noticed.

Comparatively pure nuclear pellet was obtained by parsing this final loose pellet through 1.70 M sucrose solution containing 1 mM EDTA. This nuclear pellet was further cleaned from any cytoplasmatic attachments by suspending and stirring for two hours in 10 volumes of saline EDTA (0.08 M NaCl, 0.02 M NaEDTA, pH 6.2), sedimenting at 2000 x g and resuspending in fresh solution of saline EDTA. In our process of chromatin isolation from these nuclease, we use triton-X-100 (0.01 %) along with saline EDTA in order to reduce the surface tension of nuclear membroner. The yield of nuclei was low by this process but a consistent purity (as judged by electron microscopy) was obtained, showing a 5:1 (total protein: DNA) ratio.

Wrathall, C. R. A method for obtaining mycelial pads of Neurospora. Three types of culture conditions are generally used for the cultivation of Neurospora: submerged, shake and agar surface. Of the three, the surface growth is the most difficult to recover for analysis. The use of membroner, such as cellophane and Millipore filters can lead to questionable results, while scraping the agar surface is difficult and time-consuming. In the course of a current investigation of the sexual cycle of Neurospora, a technique for obtaining surface-grown mycelium has been used to great advantage.

A soft agar substrate, made by using Difco agar at a concentration of 0.2%, produces a medium with the consistency of thin "Jello". Growth will take place on the surface of this agar without penetration of the medium. A mycelial pad of sufficient strength to be manipulated is formed in about 60 hours on Synthetic Crossing Medium in a 100 mm diameter petri dish.

Harvesting can be accomplished in one of two ways. 1) The mycelial pad is separated from the rides of the petri dish in which it has been grown and water is run under the agar. The dish is then closed and inverted. The agar surface is then up and it can be loosened and washed away, leaving a circular pad which can be picked up on a piece of circular filter paper of the appropriate size. 2) The pad and agar are poured into a cheesecloth filter, washed with running water and squeezed to remove excess water. This wash is repeated two or three times. The resulting mycelia will be free from agar.
I have found the first of the two methods to be highly satisfactory for obtaining mycelial pads when analysis of portions of each pad is desired.  

Hedman, S. C. Determination of total phosphorus in Neurospora extracts.

Often one desires to ascertain the total phosphorus content of various Neurospora extracts. The following method has been found applicable for a wide variety of such extracts. This method incorporates various features of previously published procedures as well as some new modifications. There are two parts to the procedure: acid hydrolysis and phosphate determination.

**Acid hydrolysis**: 0.2 ml of extract (containing 1-10 µg of phosphorus) is placed in an acid-cleaned 15 x 150 mm Kimax test tube. 0.3 ml of 5 NH₂SO₄ and 0.9 ml of H₂O are added. The contents are slowly heated over a Bunsen burner until dense white fumes of SO₃ are given off. At this point, the contents of the test tube may be dark-brown to black in color. After cooling the mixture, 0.1 ml of 2 N HNO₃ is added and heated until the contents of the test tube are colorless. The volume is then brought to 1.5 ml by the addition of H₂O and the tube is heated in a 100°C water bath for 5 minutes to hydrolyze pyrophosphates. An acid-cleaned glass marble is placed over the top of the test tube to prevent excessive evaporation.

**Phosphate determination**: To 1.5 ml of hydrolyzed extract are added 1.2 ml of phosphate reagent. The phosphate reagent is made as follows: (a) Stock solution: 50 g of ammonium molybdate.4H₂O are dissolved in 400 ml of 10 N H₂SO₄ with constant stirring. After all is in solution, the volume is brought to 500 ml with additional 10 N H₂SO₄. This stock solution can be stored for several months at room temperature. (b) Preparation of reagent: The phosphate reagent must be made up fresh for each series of assays. To make 20 ml of such reagent, 2.0 ml of stock solution are added to 14 ml of H₂O containing 1.0 mg of FeSO₄.7H₂O. After the ferrous sulfate is in solution, the volume is brought to 20 ml with distilled water.

After five to ten minutes, the absorbance of each tube is read at 710 µm in 1 ml cuvettes of 1.0 cm path length. A reagent blank is used as a reference. Under these conditions, linearity is observed between absorbance and phosphorus content over the range of 10-100 pg. 10 pg of phosphorus routinely gives an optical density of 0.464 ± 0.010.

The phosphate determination by itself can also be utilized to determine the total orthophosphate content as, for example, when assaying for phosphatase activity. The following compounds do not appear to interfere with this method: tris buffer (0.2 M), trichloracetic acid (20% w/v), bovine serum albumin (400 µg/1.5 ml), C₂H₅OH, Cleland's reagent (10⁻⁴ M), or sucrose (0.3 M).


During experiments in which the rate of uptake of different sugars by conidia of a number of different strains of Neurospora crassa was to be estimated and referred to their protein content, a quick and reliable method for measuring the protein content of ungerminated conidio was needed. For technical reasons, we first tried to measure their protein content directly by applying a modification of the Biuret method as described by Beisenherz et al. (1953 Z Naturforsch 8b: 555) to whole conidia.

Individual steps of this modification are as follows: (1) Prepare conidial suspensions of 5 x 10⁷ conidia/ml. (2) Precipitate the protein in 1 ml of the conidial suspension with 0.2 ml 50% TCA, shake well and spin down. (3) Wash two times with a mixture of 3 parts ethyl alcohol and one part ethyl ether and a third time with ethyl ether to remove carotenoid color and other lipids. (4) Let the ether evaporate from the pellet and resuspend in 1 ml Biuret reagent. (5) Incubate for 30 min. at room temperature on a shaker. Centrifuge and transfer the supernatant into 1 cm cuvette. (6) Read the absorption at 546 µm. Add a small amount of KCN powder to the cuvette, stir and wait 1-2 min. until the remaining absorption is constant, and read again. The difference in absorption is due to the protein in the solution (Aₚ). (7) Measure the absorption of the Biuret reagent alone before and after addition of KCN. The difference in the blank Ab to a calibration curve, obtained for bovine serum albumin to obtain mg protein/ml.

Applying this procedure and increasing the incubation time at room temperature from 30 min. to 120 min., a marked increase of the resulting absorption was observed (Fig. 1, curve a). This finding was thought to indicate that not all of the protein in a whole conidium was available freely to the Biuret-reagent. This was confirmed by increasing the temperature of incubation up to 50°C, when a further increase in absorption was observed (Fig. 1, curve b). Neither elongation of incubation-time up to 120 min. nor increase of incubation-temperature up to 50°C had a significant effect on absorption of calibration samples containing bovine serum albumin.

In additional experiments, conidia were therefore disrupted either by treating them 1 to 3 times with the X-press (AB OX, Box 235, Nacka 2, Sweden) with 25 ml volume operated at -25 to -35°C at ca. 2000 kg/sq cm, or by smashing them with glass beads (d = 0.45-0.50 mm) for 5 to 10 minutes in a homogenizer.

![Fig. 1](image_url) Values for whole or disrupted conidia (5 x 10⁷/ml) after incubation with the reagent for different periods of time.