Production of Neurospora mycelial protoplasts

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Abstract
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count. Generally, four counts are made on each suspension. By stirring it is possible to get counts that are in very close agreement (less than 1% deviation). The counter gives the actual count in 0.5 ml of suspension; therefore, it is a simple matter to calculate the total number of spores in the remaining suspension. The spores are then pipetted into 80 ml of melted agar, heat shocked, and plated. Bacterial contamination has not been a problem using this procedure.

In order to determine the volume of an ascospore, the instrument was calibrated using paper mulberry pollen of known size obtained from the Coulter company. Figure 1 gives the size distribution of Neurospora ascospores obtained from a cross of two pyr-3 mutants, and what appears to be the size distribution of ascospores of Neurospora macroconidia of a pyr-3 mutant. It can be seen that the curves overlap slightly. Using a 70 \( \mu \) aperture tube, Gillie (1967 Neurospora News 1:1-16) has shown that macroconidia of wild type (74-78B-1a) do not exceed 400 \( \mu \)m in volume. The data presented here were obtained with a 100 \( \mu \) aperture tube and the discrepancy in the size of macroconidia, as measured by the two different aperture tubes, can be attributed to the coincident passage of macroconidia through the 100 \( \mu \) aperture. These data point out the necessity of obtaining ascospore suspension free of massive conidial contamination when using a counting system such as the above. This work was supported by NSF Grant No. GB-5998.

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The procedure given below is currently used in our laboratory to produce protoplast preparations suitable for studying transport and regeneration phenomena. Basically, this method is a further modification (1962 J. Bacteriol. 83:351) of Bachmann and Bonner's method (1959 J. Bacteriol. containing 200 ml of Vogel's minimal medium, one each inoculated with 4x 108 wild type conidio in 10 ml of distilled water. 20,000 units of Penicillin G is added to minimize bacterial contamination. This inoculated culture is allowed to grow without shaking or aeration for 40 hours at 22°C. The resulting mycelial mat floats on the liquid medium. Many non-germinated and slow-germinating conidia are evident at the bottom of the flask. The mats are transferred to 500 ml Fernbach flask containing 200 ml of 0.05 M potassium phosphate buffer, pH 6.8. 0.60 M in sucrose. The flask is swirled and then allowed to stand for five minutes before transfer of the mat to a second wash with fresh sucrose-phosphate buffer. This procedure allows many of the loose, ungerminated conidia to settle to the bottom of the wash flask.

After the second wash, the two mycelial mats (dry weight of each mat approximately 325 mg.) are placed in a wide-mouthed 125 ml Erlenmeyer flask containing 1.0 ml of Helix pomatia extract (source: Industrie Biologique Francaise, Gennevilliers, Seine, France). 10 ml of sucrose-phosphate buffer and 0.1 ml of M/10 glutathione. The flask containing the mats and snail extract preparation is incubated at 30°C with very gentle shaking for six hours. During the first half of the incubation period the flask should be periodically swirled gently by hand to permit release of CO2 which often builds up under the mycelial mat and tends to lift it up out of the enzyme preparation.

At the end of the incubation period, the crude protoplast preparation is poured onto a sterile 10.5 cm Buchner funnel filter prepared by overlining fine glass wool on fine mesh cheesecloth. The flask is rinsed into the funnel twice with 2 ml portions of sucrose-phosphate buffer. This first filtrate is collected in a 125 ml suction flask, but no vacuum is used, resulting in a cleaner filtrate. This first filtrate is then filtered through ten conidial filters packed with fine glass wool and collected in 125 ml suction flasks. Conidial filters are glass tubes about 6 in. long and 1 in. in diameter at the top, tapering to a diameter of 1/2 in. at a point 2 in. above the bottom. The bottom port has a uniform diameter of 1/4 in. and is fitted with a #5 rubber stopper. These filters are used in tandem to reduce the number of transfers required, as with the protoplast preparations. After the final filtration, the glass wool-pocked filters are washed serially with four 3-ml portions of sucrose-phosphate buffer. Here again, no vacuum is used.

The final filtrate obtained is poured into a 50 ml polycarbonate centrifuge tube and spun in a model CL International clinical centrifuge for 12 minutes at 600 rpm at room temperature. Using a Propipet, all but about 0.3 ml of supernatant is drawn off leaving a well-defined layer of protoplasts at the bottom of the tube. This first supernatant is placed into another tube and centrifuged as above. Centrifuging in two stages prevents formation of a firm pellets by the protoplasts and results in at least 75% recovery, based on hemocytometer counts. Centrifuging at speeds higher than 600 rpm or for periods longer than 12 minutes results in poaching of the protoplasts into a firm pellet which does not redisperse.

The protoplasts can be easily redispersed by adding a few drops of sucrose-phosphate buffer (or other suitable osmoticum) and shaking gently. Gradually, more osmoticum can be added with gentle swirling to give a fairly homogeneous dispersion of the desired density. Where required, protoplasts can be washed by re-centrifuging as outlined above. Recoveries during washing are generally quite high, since most of the lighter protoplasts are discarded with the supernatant from the second stage of the first centrifugation. Final yields, after two centrifuge washings, are generally in the range of 2.5 x 107 protoplasts/mycelial mat.

Protoplasts obtained by this method have been observed to give almost quantitative regeneration in sucrose-phosphate buffer supplemented with Vogel's minimal medium. They have been found to respire at a high rate (compared to conidia) even in the absence of a carbon source. They are quite stable for periods of up to 24 hours when mannitol or d(-)-arabinose is used as the osmoticum. (This work was supported in part by a Training Grant in Genetics (T01 GM01316) from the National Institutes of Health to Florida State University). Genetics Laboratories, Department of Biological Sciences, Florida State University, Tallahassee, Florida 32306.