

Two 2.5 liter Fernbach flasks, each containing 500 ml of Vogel's minimal medium, are each inoculated with 4×10^8 wild type conidia 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 usually are evident at the bottom of the flask. The mats are transferred with a glass rod hook to a 500 ml Erlenmeyer flask containing 200 ml of 0.05 M potassium phosphate buffer, pH 6.8, 0.60 M in sucrose. The flask is swirled to effect washing 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 overlaying fine glass wool with 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 pocked 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 when many filtrations are required, as with 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 re-centrifuged as above. Centrifuging in two stages prevents formation of a firm pellet 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 pocking 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 2.5×10^7 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.