Hendrick, D. and A. G. DeBusk. Production of Neurospora mycelial protoplosts.

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. 78: 550). Two 2.5 liter Fernbach flasks, each containing 500 ml of Vogel's minimal medium, ore each inoculated with 4x 10^8 wild type conidio in IO 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 ot 22°C. The resulting mycelial mot floats on the liquid medium. Many non-germinated and slow-germinating conidio usually ore evident at the bottom of the flask. The mats ore transferred with a gloss 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 stood for five minutes before transfer of the mot 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 myceliol mats (dry weight of each mot approximately 325 mg.) ore placed in a wide-mouthed 125 ml Erlenmeyer flask containing 1 .O ml of Helix pomatia extract (source: Industrie Biologique Francaise, Gennevilliers, (Seine), France), IO 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 b-e periodically swirled gently by hand to permit release of CO2 which often builds up under the myceliol 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 gloss wool with fine mesh cheesecloth. The flask is rinsed into the funnel twice with 2 ml portions of sucrose-phosphate buffer. This first filtmte is collected in a 125 ml suction flask, but no vacuum is used, resulting in a cleaner filtrate. This first filtmte is then filtered through ten conidiol filters pocked with fine gloss wool and collected in 125 ml suction flasks. Conidiol filters ore gloss 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 ore used in tandem to reduce the number of transfers when many filtrations ore required, as with protoplast preparations. After the final filtration, the gloss wool-pocked filters ore 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 drown off leaving a well-defined layer of protoplosts 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 protoplosts con be easily redispersed by adding a few drops of sucrose-phosphate buffer (or other suitable osmoticum) ond shaking gently. Gradually, more osmoticum con be added with gentle swirling to give a fairly homogeneous dispersion of the desired density. Where required, protoplosts can be washed by re-centrifuging as outlined above. Recoveries during washing ore generally quite high, since most of the lighter protoplasts are discorded with the supernatant from the second stage of the first centrifugation. Final yields, after two centrifuge washings, ore generally in the range of 2.5 x 10^7 protoplasts/ mycelial mat.

Protoplasts obtained by this method hove 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 rote (compared to conidia) even in the absence of a carbon source. They ore 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 Tmining Grant in Genetics (T01 GM01316) from the Notional Institutes of Health to Florida State University). ▪ ▪ ▪ Genetics Loboratories, Deportment of Biological Sciences, Florida State University, Tallahassee, Florida 32306.