

## Protoplast Preparation from Mycelium Vaillancourt Lab Method (Venard)

This method is good for strains that don't produce a lot of oval spores. There is some idea that the transformation efficiency will not be as high since some of the protoplasts do not have nuclei. The main thing is NOT to overgrow the cultures since you get a lot of "empty" mycelium that won't protoplast and just contributes to debris. The only parts of the mycelium that protoplast efficiently are the hyphal tips.

1. Isolate spores from 2-3-wk-old PDA cultures of M1.001 grown at 23°C under continuous white light. To isolate the spores, add 10 mls of sterile water to each plate, scrape GENTLY with a rubber policeman or a spatula, filter through several layers of cheesecloth, and rinse the spores twice in 50 ml sterile water to remove the mucilage. Dilute to  $1 \times 10^6$  spores per ml.
2. Add the spore suspension to 1 L Fries Complete Medium (500 mls in each of two 1L flasks) to a final concentration of  $1 \times 10^5$  spores/ml, and incubate at 23-25°C for 36-48 hours on a gyratory shaker at 250 rpm. The culture is ready to use when there are few or no more falcate spores, and when there are many small mycelial "balls" that are no more than a couple mm in diameter. Don't let the culture get too old.
3. Filter the cultures through several layers of sterile cheesecloth to collect mycelium.
4. Prepare a solution of lysing enzymes as follows: Add Lysing Enzymes (100 mg), Driselase (500 mg) and Chitinase (1 mg) (all from Sigma) to 20 mls 1.2 M KCl, mix thoroughly (will be cloudy), and filter sterilize.
5. Add mycelium to the lysing solution in a Petri dish, and incubate with very slow agitation (100 rpm) for 60-90 minutes at 30C. Don't add too much mycelium, the consistency should be "pea-soupy". It will get thinner as protoplasting proceeds, so you may need to reduce the shaking speed to avoid "slopping". Check the suspension frequently for production of protoplasts. MOST OF THE MYCELIUM WILL REMAIN. Don't overdigest, as the enzymes will damage the protoplasts eventually.
6. Pipette the solution **very gently** up and down in a sterile glass pipette to help break up the mycelium and release all the protoplasts.
7. Filter the suspension through a sterile Nynetex membrane.
8. Pellet the protoplasts at 3000 x g at 4°C and resuspend in 15 mls of ice-cold 1.2 M KCl. From this point, keep the protoplasts cold.
9. Pellet again and resuspend in 5 mls of ice-cold STC (1.2 M Sorbitol, 10 mM Tris-Cl, pH 7.5, and 10 mM CaCl<sub>2</sub>) and count them on a hemacytometer.
10. Resuspend at a concentration of  $1 \times 10^8$  per ml.
11. If you have enough, aliquot 300 microliter portions and freeze them at -80. They will lose their efficiency (about half) after freezing, so use fresh if possible. You can store the protoplasts overnight in the refrigerator, but don't store them for longer than that as they will begin to regenerate cell walls.