Preparation of Protoplasts

1. For *Cochliobolus*, inoculate 100 mL complete medium (CM) in a 500 mL flask with 10^6-10^7 conidia. Alternatively, mycelium from solid of liquid medium may be blended for 5-10 seconds in a sterile blender cup and then used as inoculums. For other fungi, grow as usual on medium known to produce conidia. 150 µg/mL ampicillin may be used to prevent bacterial contamination.

2. Grow 12-18 hours at room temperature (25-28°C) with shaking at 150-250 rpm.

3. Transfer the culture to 40 mL centrifuge tubes and centrifuge at 5000 rpm for 5 minutes at 4°C in a SS34 rotor (Sorvall) or its equivalent. Discard the supernatant and try not to disturb the pellet.

4. Scoop 0.5-0.8 g portions of the pellet and resuspend well in 10 mL of enzyme-osmoticum in a 50 mL Erlenmeyer flask (use 80 mL total of enzyme-osmoticum, 8 x 10 mL).

5. Place flasks at 30-32°C and shake gently (50 rpm) for 1.5-2 hr. 10^8-10^9 protoplasts should be released in this time (count with a hemacytometer).

6. Separate the protoplasts from the intact mycelium and cell wall debris by filtering the protoplast suspension through 4 layers of sterile cheesecloth, then through sterile nylon fabric with a 20-25 µm pore size.

7. Pellet the protoplasts by centrifuging the filtrate from step 6 in 40 mL screw-capped centrifuge tubes at 5000 rpm for 5-6 min in a SS34 rotor at 4°C. Discard the supernatant very carefully trying not to disturb the protoplast pellet. The enzyme-osmoticum can be re-used twice if stored at -20°C.

8. Resuspend the pellets gently in a total of 10 mL 0.7 M NaCl; combine into one tube. Recentrifuge as in step 7. Discard the supernatant.

9. Wash the pellet 3 times with 10 mL STC, pelleting the cells between washes by centrifuging as in step 7. Resuspend protoplasts in 500 µL STC after final wash.

10. Count protoplasts and adjust their concentration to approximately 10^8/mL with STC.