

# PRODUCTION OF FUNGAL PROTOPLASTS FROM SELECTED WOOD-DEGRADING FUNGI

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## ABSTRACT

Studies of the effects of various chemicals on fungal growth are difficult to perform on filamentous fungi because of the difficulty of observing the protoplasm through the rigid hyphal wall, and because most activity occurs in the region near the hyphal tip. However, hyphae can be exposed to certain enzymes that degrade the cell walls, producing protoplasts. There are few reports of protoplast production from economically important wood decayers. In this report, protoplasts were produced from hyphae of the common wood-degrading fungi *Phanerochaete chrysosporium*, *Postia placenta*, *Gloeophyllum trabeum*, and *Trametes versicolor* with a commercially prepared mixture of cell-wall degrading enzymes (Novozyme 234). Protoplasts were more readily produced from young hyphae; however, regeneration was better with older hyphae. Magnesium sulfate and mannitol (0.5 M) performed comparably well as osmotic stabilizers.

*Keywords:* Basidiomycetes, protoplasts, regeneration, white rot, brown rot.

## INTRODUCTION

The presence of rigid cell walls, important structurally to fungal hyphae, makes observation of the internal components difficult. Most hyphal activity occurs near the growing tip, where enzymes are secreted and wood-decomposition products are reabsorbed into the hyphae. As a result, studies of the mycelial phase of the fungus require large amounts of hyphal mass for observation of effects on a relatively few cells.

An alternative approach is to remove the cell wall, creating fungal protoplasts (Collings et al. 1988). Protoplasts are increasingly used for inserting desirable genetic characteristics into fungi and plants, but they may also be useful for studying cell morphology under different growth conditions or for monitoring the effects of toxicants (Gadd and White 1985; Gadd et al. 1987; Kihn et al. 1987).

Methods have previously been developed for generating protoplasts from edible basidiomycetes (Kitamoto et al. 1984, 1988; Yanagi et al. 1985; Toyomasu et al. 1986; Sudo and Higaki 1988; Eguchi et al. 1990; Tamai et al. 1990), heartrot fungi (Trojanowski and Hutterman 1984; Trojanowski et al. 1985), and an array of Fungi Imperfecti (Sivan et al. 1990); but there are few protocols available for generating protoplasts from many economically important wood-degrading fungi (de Vries and Wessels 1972; Gold et al. 1983). We therefore undertook to develop protocols for generating protoplasts from three such fungi common to wood products.

## MATERIALS AND METHODS

Protoplast generation was evaluated with *Gloeophyllum trabeum* (Pers.: Fr.) Murr. (Madison 617), *Postia placenta* (Fr.) M. Lars.

and Lomb. (Madison FP94267R), *Phanerochaete chrysosporium* Burds. (BKMF-1767), and *Trametes versicolor* (Fr. : Fr.) Pilát (Madison R105). The isolates were obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wisconsin. *P. chrysosporium*, although not an important wood-degrading fungus in natural systems, was included for comparative purposes, as it has been the subject of intensive investigation for its lignin-degradation capabilities and for protoplast generation (Gold et al. 1983). All fungi were maintained on potato dextrose agar. Small plugs (3-mm diameter) cut from the actively growing edge of each culture were used to inoculate 50 ml of a medium containing 0.1% yeast extract, 1% glucose, and 1% malt extract in distilled water. The flasks were incubated in stationary culture for 2, 4, 6, 8, 10, 12, or 14 days at 28 C; then 0.25 g (fresh wt.) of mycelium was aseptically removed, homogenized in a Waring blender, and filtered by suction. The mycelium was washed twice with sterile, distilled water and twice with 0.5 M mannitol or 0.5 M magnesium sulfate ( $MgSO_4$ ) in maleic sodium hydroxide (NaOH) buffer (pH 5.5). McIlvaine's buffer and acetic acid-NaOH buffer were also tested, but they appeared to inhibit protoplast release and were not used further.

The washed mycelium was resuspended in 3 ml of a medium composed of 0.4% Novozyme 234 (NOVO Industria A/S, Bagsvaerd, Denmark), a mixture of cell-wall degrading enzymes, in 50 M maleic-NaOH buffer (pH 5.5) containing 0.5 M mannitol or  $MgSO_4$ . Additional trials were performed with 0.3, 0.5, 0.6, or 0.7 M  $MgSO_4$  as the osmotic stabilizer. The mixture was incubated at 30 C for 2 hours with occasional agitation. The effects of other variables were explored by altering the pH of the enzyme mixture from 4.5 to 6.5 and the exposure time to the enzyme mixture from 60 to 180 minutes. Protoplast formation was initially observed by examining a small sample for evidence of protoplasts. The presence of residual cell wall was monitored by reacting the suspension with fluorescein isothiocyanate

coupled wheat germ agglutinin (FITC-WGA), a lectin specific for the n-acetylglucosamine residues in chitin, the principal polymer of most fungal cell walls. The cells were observed under a Leitz light microscope equipped with filters specific for FITC (Morrell et al. 1985). Cell walls fluoresced strongly, while fungal protoplasts were only weakly fluorescent.

After enzyme treatment, 30 ml of maleic-NaOH buffer containing 0.5 M mannitol was added to dilute the enzyme, and the protoplasts were separated from the mycelial debris by filtration through a coarse sintered-glass filter. The number of protoplasts was estimated with a hemacytometer.

The protoplasts were then diluted 1:1,000 in a medium containing 0.5 M mannitol and 0–3.5% glucose in 50 mM maleic-NaOH buffer (pH 5.5). Glucose was varied to allow observation of the effect of sugar on regeneration. Cell-wall regeneration was evaluated by incubating 2 ml of the protoplast suspension at 28 C for 3–7 days, then spreading the solution on 2% agar containing 0.5% yeast extract, 0.5% malt extract, and 2% dextrose. The protoplasts were incubated for 10–15 days at 28 C and observed for the presence of discrete fungal colonies, which served as the measure of successful regeneration.

Once protoplast regeneration was confirmed, the effects of colony age, glucose concentration, and pH on protoplast production and regeneration were examined for each of the test fungi.

## RESULTS AND DISCUSSION

### *Protoplast production*

Protoplasts were generated from all four fungi tested, although the results differed among species. Protoplast production was greatest with *P. chrysosporium* and *T. versicolor* and lowest with *P. placenta* (Fig. 1A). Previous trials with a different isolate, *P. chrysosporium*, produced significantly higher levels of protoplast release (Gold et al. 1983). These differences may reflect media or isolate variations. Interestingly, regeneration in the current study was approx-

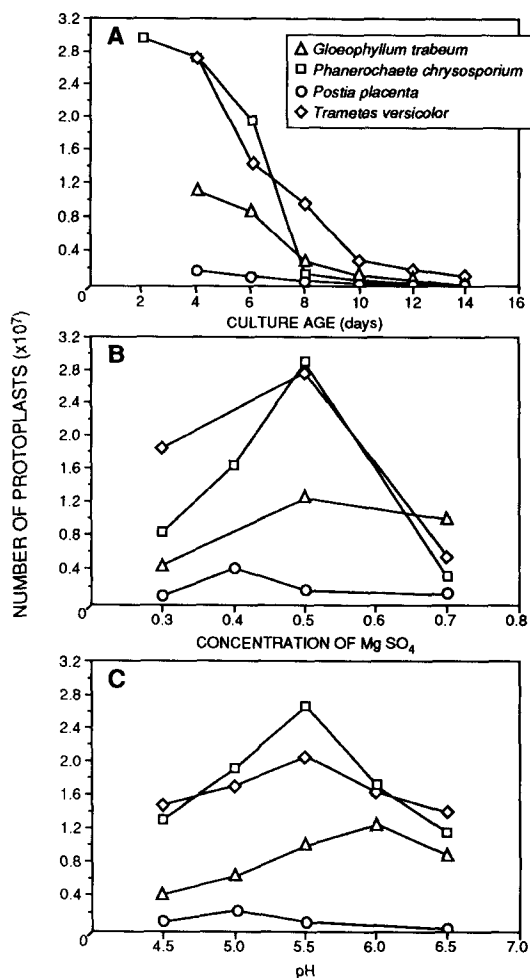


FIG. 1. Effect of (A) hyphal age, (B) magnesium sulfate concentration in the stabilizer, and (C) pH of the extraction medium on the production of fungal protoplasts from cultures of *Gloeophyllum trabeum*, *Postia placenta*, *Trametes versicolor*, and *Phanerochaete chrysosporium*.

imately 5 times that found in the previous study (Gold et al. 1983). Hyphal age played a critical role in protoplast production. In general, protoplast numbers decreased with increasing age of the culture, perhaps reflecting the senescence of large parts of the mycelial mat. Previous studies have employed younger cultures to avoid this problem and enhance protoplast yield (Trojanowski et al. 1985; Eguchi et al. 1990).

Protoplast production was generally greater when 0.5 M MgSO<sub>4</sub> was used as the osmotic

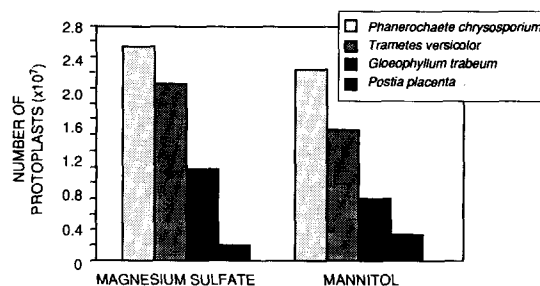


FIG. 2. Effect of 0.5 M magnesium sulfate and mannitol as osmotic stabilizers for releasing fungal protoplasts from liquid cultures of *Gloeophyllum trabeum*, *Postia placenta*, *Trametes versicolor*, and *Phanerochaete chrysosporium*.

stabilizer, although the differences between that stabilizer and 0.5 M mannitol were generally small (Fig. 2). Both stabilizers are used for releasing basidiomycete protoplasts; MgSO<sub>4</sub> perhaps more commonly (Gold et al. 1983; Trojanowski et al. 1985). Increasing the concentration of MgSO<sub>4</sub> from 0.3 M to 0.5 M resulted in increased release of protoplasts with *P. chrysosporium*, *G. trabeum*, and *T. versicolor*, but release declined at 0.6 and 0.7 M (Fig. 1B). *P. placenta* appeared least affected by pH, but only low levels of protoplasts of this species were released at any pH level tested. Protoplast production declined in all test fungi when 0.7 M MgSO<sub>4</sub> was used as the osmotic stabilizer. The pH of the maleic-NaOH buffer also appeared to influence protoplast generation with *G. trabeum*. Optimum numbers of protoplasts released appeared at pH 5.5–6 and declined on either side of that range (Fig. 1C).

#### Protoplast regeneration

Protoplast regeneration provides a relative measure of the effects of enzyme treatment on cell viability. Protoplasts that lack the ability to regenerate presumably either lack nuclei or were damaged at some point during or after the enzyme treatment. In contrast with protoplast release, regeneration improved with increasing age of the cultures of all four species from which they were produced (Fig. 3A). The reasons for this effect are unclear, since older

cells would presumably be less plastic and therefore less capable of responding to the stresses induced during protoplast formation. Interestingly, plating efficiency did not differ markedly among species, suggesting that *P. placenta* retained the same capabilities as the species that produced protoplasts more easily.

The osmotic stabilizer also appeared to influence protoplast regeneration. Magnesium sulfate appeared to inhibit it; therefore 0.5 M mannitol was used as the media in all experiments. The period of exposure to the enzyme preparation also appeared to influence regeneration. *G. trabeum* was sensitive to exposure, while the other three species were unaffected by exposure as long as 3 hours (Fig. 3B).

The effect of pH on regeneration differed from the effect on protoplast production (Fig. 3C). Increasing pH increased regeneration of *P. chrysosporium* slightly, and first improved, then decreased regeneration for the other three fungi tested. Plating efficiency was highest at pH 6.0 for *P. chrysosporium* and at pH 5.0–5.5 for the remaining fungi. Media pH should have substantial effects on protoplasts, since cell membranes are in direct contact with the media and are, therefore, more likely to be affected by subtle environmental changes.

Glucose would be expected to be important in both respiration and in synthesis of the cell-wall material necessary for protoplast regeneration. Increasing glucose concentration generally improved plating efficiency, but the effect was slow and, in some cases, efficiency declined at higher glucose levels. *G. trabeum* regenerated most efficiently in 1% glucose, *P. placenta*, *T. versicolor*, and *P. chrysosporium* most efficiently in 1.5–2.0% glucose (Fig. 3D). Regeneration was minimal when no glucose was present. The differences in effects among species may reflect differential rates of glucose uptake and utilization.

These preliminary results indicate that viable protoplasts can be regenerated from all three common wood-degrading fungi at levels slightly lower than those found with *P. chrysosporium*. Because protoplasts can be used to study the production of various cell-wall de-

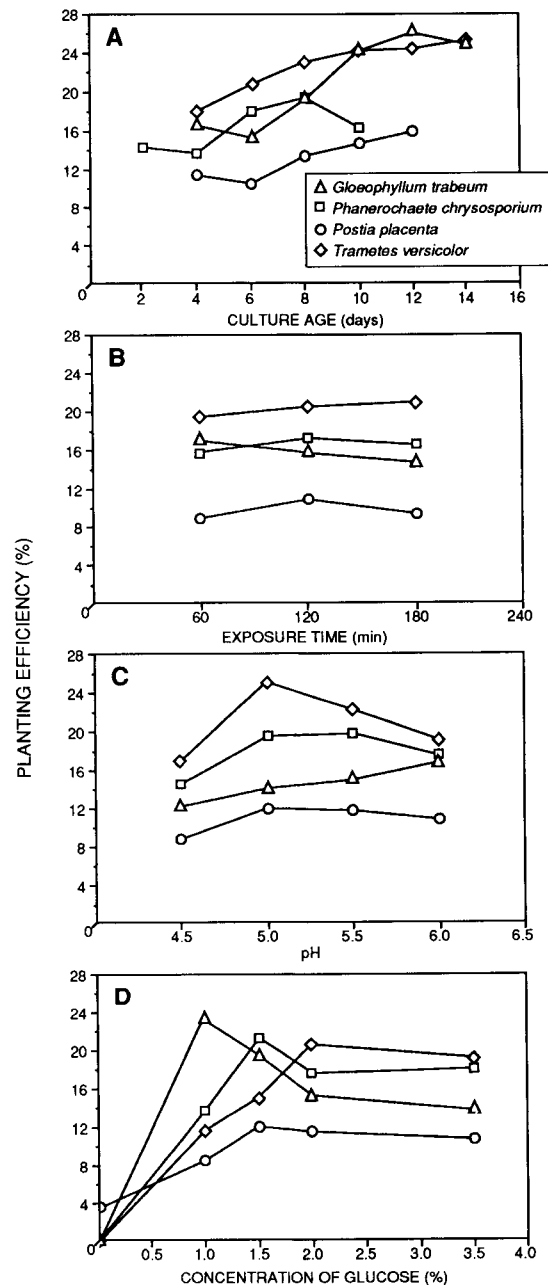


FIG. 3. Effect of (A) hyphal age, (B) enzyme exposure time, and (C) pH of the extraction medium on the efficiency of protoplast regeneration of *Gloeophyllum trabeum*, *Postia placenta*, *Trametes versicolor*, and *Phanerochaete chrysosporium*.

grading enzymes or the effects of selected toxicants on fungal physiology, they are a powerful tool for extending our knowledge of the decay process. Further studies to characterize the physiology of protoplasts from the selected test fungi are planned.

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