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Formation, Fusion, and Regeneration of Protoplasts from Wild-Type and Auxotrophic Strains of the White Rot Basidiomycete *Phanerochaete chrysosporium*

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A preparation of two commercial enzymes was used to liberate protoplasts from 16-h-old mycelium of *Phanerochaete chrysosporium*. Regeneration frequencies of up to 5% were attained when the protoplasts were plated in a medium containing 10% sorbose and 3% agar. Fusion of protoplasts from different auxotrophic strains in polyethylene glycol-Ca^{2+} produced heterokaryons. Separation of the heterokaryons into their constituent homokaryotic strains could be effected through protoplast release and formation of colonies on regeneration agar.

The basidiomycete *Phanerochaete chrysosporium* and other white rot fungi have potential application in a variety of schemes for the commercial processing of lignocellulose. These organisms have already been used in numerous studies on lignin degradation (3), cellulose degradation (4), and lignocellulose bioprocessing applications (12). Their potential would be enhanced, however, if genetic methods for producing strains with superior capacities were available. In other studies, we described methods for inducing colonial growth and for replica plating with this organism (6); we also elucidated the physiological conditions required for fruit body formation (7). In a subsequent report, we described methods for mutagenesis of *P. chrysosporium* conidia and subsequent isolation and characterization of a variety of auxotrophic marker strains (8). We have also reported on the isolation and characterization of a secondary metabolic mutant of this organism (9).

Recently, considerable interest has been focused on the isolation of fungal protoplasts and their use in fusion and transformation experiments (2, 11, 13, 14). In this report, we describe methods for the preparation of *P. chrysosporium* protoplasts and for the regeneration of mycelia from these protoplasts. In addition, we describe studies on the complementation of auxotrophic mutants through protoplast fusion and the use of protoplasts for recovery of homokaryotic parental strains from heterokaryons.

MATERIALS AND METHODS

Media and reagents. Growth supplements were obtained from Difco Laboratories, Detroit, Mich. Novozyme 234 and Cellulase CP were generously provided by Novo Industries, Bagsvaerd, Denmark, and J. E. Sturge, Ltd., Birmingham, England, respectively. Polyethylene glycol 6000 and sorbose were obtained from Sigma Chemical Co., St. Louis, Mo.; all other chemicals were reagent grade.

Organism. Cultures of *P. chrysosporium* ME446 were maintained on slants of Vogel medium N (18), with thiamin replacing biotin (modified Vogel), (17) supplemented with 3% malt extract and 0.25% yeast extract. Mutant strains were maintained on the above medium supplemented with 0.5% casein hydrolyzate and yeast extract. After 7 to 10 days of growth, conidia were washed from slants, filtered, counted, and diluted as previously described (6).

Cells were grown in Erlenmeyer flasks (250 ml) containing 100 ml of medium on a New Brunswick shaker rotating at a speed of 150 rpm. Flasks were inoculated with 1.0×10^7 conidia; cultures were incubated for approximately 16 h at 28°C. The medium consisted of modified Vogel medium (17) containing 1% glucose, 0.04% yeast extract, and supplements where indicated.

Formation of protoplasts. Mycelia were harvested by suction filtration, with care taken to prevent the mycelium from drying extensively, and washed with 0.6 M $MgSO_4$. Cells (0.1 to 1.0 g) in 1 ml of 0.6 M $MgSO_4$ were treated with Novozyme 234 (0.1 g/g of cells) and Cellulase CP (0.1 g/g of cells) at 38°C with shaking for 3 h. Remaining fragments were removed by filtration (coarse fritted glass funnel), and the protoplasts were sedimented by centrifugation at 4,000 rpm for 10 min and resuspended in 0.6 M $MgSO_4$ or as indicated. The number of protoplasts in suspension was determined with a hemacytometer.

Fusion of protoplasts. Protoplasts from two auxotrophic mutants were separately sedimented by centrifugation, washed with 2 ml of 0.6 M $MgSO_4$, and incubated at room temperature for 1 h. The suspensions were then mixed and sedimented together by centrifugation. The protoplasts were resuspended in 1 ml of 30% polyethylene glycol (prewarmed to 38°C)–10 mM $CaCl_2$–0.05 M glycine (pH 7.5) and incubated for 15 min at 38°C. Magnesium sulfate (0.6 M; 0.5 ml) was then added, and the protoplasts were resedimented by centrifugation and resuspended in 0.6 M $MgSO_4$.

TABLE 1. Efficiency of protoplast release and regeneration for strains of *P. chrysosporium*

Strain	Protoplasts (10^8)/g (wet wt) of mycelium[a]	% Regeneration frequency[b]
Wild type	3.0	5.0
Lys1[c]	1.7	1.0
His2	5.0	5.0
Arg1	4.0	0.8
Rib	0.5	2.0
Leu1	1.0	5.0
His2 + Lys1	2.0	1.2
His2 + Arg1	3.8	0.8

[a] Digestion mixtures consisted of 16-h-old cells and Novozyme 234 and Cellulase CP in 0.6 M $MgSO_4$, as described in the text. Digestions were carried out at 38°C, samples were removed, and the number of protoplasts was determined as described in the text.

[b] Regeneration frequency = number of colonies appearing on supplemented regeneration medium/number of protoplasts inoculated × 100.

[c] Complete names and isolation numbers for the auxotrophic mutant strains used in this study have been reported previously (8).

Regeneration of mycelia. Protoplasts in 0.6 M $MgSO_4$ were plated in 15 ml of regeneration agar at 45°C. The regeneration agar consisted of 10% sorbose, 1% yeast nitrogen base, 0.01% amino acid supplements where indicated, and 3% agar adjusted to pH 4.8. Plates were incubated at 28°C for 1 week.

RESULTS

Formation and regeneration of protoplasts. Under the conditions described, the formation of protoplasts from *P. chrysosporium* mycelium was linear for the first 3 h of incubation, after which the rate decreased considerably. Wild-type hypae from cells which had been grown for 16 h yielded approximately 3×10^8 protoplasts per g (wet weight). Approximately the same number of protoplasts was obtained from the mycelia of each of the mutants and heterokaryons used in this study (Table 1). When either Cellulase CP or Novozyme 234 was used alone, the yield of protoplasts was approximately 5 and 50%, respectively, of the yield when the enzymes were used together. Treatment of the wild-type protoplasts with 0.2% sodium dodecyl sulfate in water for 3 min led to rapid lysis as determined by microscopy. Sodium dodecyl sulfate-treated preparations yielded less than 0.001% as many colonies (probably originating from conidial contaminants) on regeneration agar. Treatment with sodium dodecyl sulfate had no effect on control suspensions of conidia.

The use of 0.8 M sorbitol (13) or mannitol instead of $MgSO_4$ led to similar yields of protoplasts, but these alcohols resulted in colony spreading when the protoplasts were subsequently plated in regeneration agar. When the $MgSO_4$ was replaced by NaCl, only approximately 15% as many protoplasts were obtained. Pretreatment of the mycelium for 30 min with 50 mM dithiothreitol in 50 mM phosphate buffer (pH 7.0) had no effect on protoplast yield.

The efficiency of regeneration of various protoplasts is also shown in Table 1. The regeneration frequency (or the number of viable colonies) on regeneration agar in relation to the number of protoplasts varied from 5.0% with the wild-type strain to 0.8% with the Arg1 strain. This variability may reflect differences in supplement requirements for the various mutant strains.

The low level of regeneration may be a result of the use of 10% sorbose as an osmotic stabilizer and colony-inducing agent in the regeneration agar.

Complementation via fusion of protoplasts. Treatment of various strains with 30% polyethylene glycol and 10 mM $CaCl_2$ in 0.05 M glycine (pH 7.5) led to fusion of protoplasts as measured by complementation on minimal plates. The fusion frequency was determined as the number of colonies formed on minimal medium in relation to the number of colonies formed on media supplemented with the nutritional requirements of the parental strains. The fusion frequencies for the heterokaryons His2 + Lys1 and His2 + Arg1 were 0.12 and 0.14, respectively. When either $CaCl_2$ or polyethylene glycol was omitted from the reaction, fusion as measured by complementation did not occur.

Homokaryotization via formation of protoplasts. The results of Table 2 show that monokaryotization occurs via protoplast formation. When protoplasts from the His2 + Lys1 mutant were plated in histidine-supplemented medium, approximately 39% of the resulting colonies were His^-. If the same heterokaryon was plated in lysine-supplemented medium, approximately 69% of the resultant colonies were Lys^-. A

TABLE 2. Homokaryotization via formation of protoplasts[a]

Strain	Histidine		Lysine		Arginine	
	His^-	Het	Lys^-	Het	Arg^-	Het
His2 + Lys1	39	61	69	31		
His2 + Arg1	42	58			31	69

[a] Protoplasts produced from heterokaryons were plated on regeneration medium containing a single supplement (arginine, histidine, or lysine), as described in the text. Colonies were then picked and transferred to slants of minimal medium. Colonies which did not grow on minimal medium were transferred to supplemented slants. Values are percentages of the phenotypes indicated. Het, Heterokaryon (prototrophic).

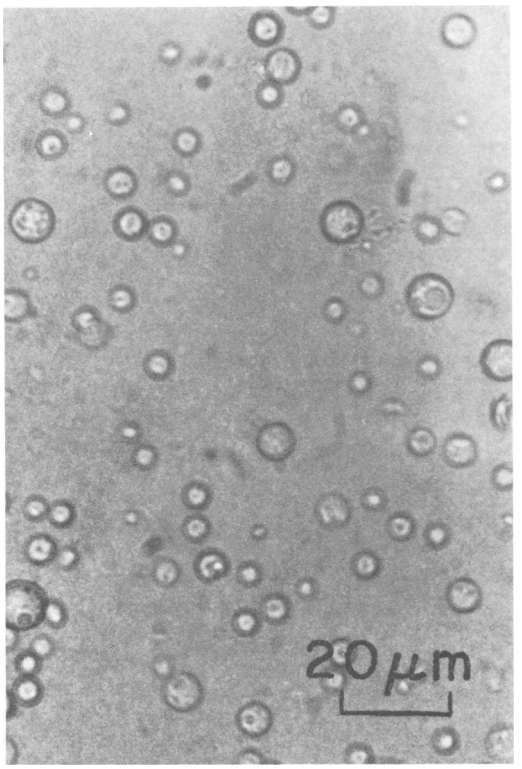

FIG. 1. *P. chrysosporium* protoplasts (2.7×10^7/ml) in 0.6 M $MgSO_4$ were isolated as described in the text. Protoplasts were photographed at 400× magnification with a Zeiss RA phase-contrast microscope.

similarly high yield of homokaryotic protoplasts was obtained from the His2 + Arg1 heterokaryon.

Figure 1 is a photomicrograph of protoplasts produced from the wild-type strain of *P. chrysosporium*. The average diameter of the spheres varied from approximately 3 to 10 μm.

DISCUSSION

In a previous report (8), we described the isolation and complementation of auxotrophic mutants of the lignin-degrading fungus *P. chrysosporium*. Here we have described studies on the formation, fusion, and regeneration of *P. chrysosporium* protoplasts. The commercially prepared enzymes Novozyme 234 and Cellulase CP have been used previously to effect protoplast release from a variety of fungal species (10). Our results extend the use of these enzymes to experiments with *P. chrysosporium*. Although pretreatment of *Saccharomyces cerevisiae* with thiols was required for efficient protoplast release (1, 15), no such pretreatment of *P. chrysosporium* was necessary. Log phase cells (16-h cultures) released protoplasts most effectively. If the cells were allowed to grow for 48 h, approximately 10% as many protoplasts per gram of mycelium were released. Substitution of $MgSO_4$ with sorbitol or mannitol led to an equal yield of protoplasts, but these alcohols prevented tight colony formation in the regeneration agar. Rapid lysis of the protoplasts with concentrations of sodium dodecyl sulfate, which had no apparent effect on conidia, suggests that most if not all of the wall was removed by digestion with Novozyme 234 and Cellulase CP.

The regeneration frequency of mycelia from protoplasts of the different strains varied from approximately 1 to 5% under the conditions described. If the regeneration medium contained only 1% agar, significantly less regeneration was observed. Plating the protoplasts on the surface of the agar reduced the regeneration frequency. The relatively high regeneration frequency with the auxotrophic mutants indicates that this step should not be an impediment to successful experiments with complementation via DNA transformation (2, 11) with this organism.

The results reported here indicate that protoplast fusion is a viable alternative to mycelial fusion (8) for the formation of *P. chrysosporium* heterokaryons. The use of nutritional markers facilitates heterokaryon formation and complementation with mycelium from *P. chrysosporium* (8) and other basidiomycetes (5, 16). However, when other markers such as developmental, secondary metabolic, or pigment mutants are involved, selection pressure for heterokaryon formation would not be present. In such cases, heterokaryon formation via protoplast fusion may be the method of choice.

The results in Table 2 indicate that homokaryotization through protoplast formation is readily attainable. These results also indicate that the original mycelium was heterokaryotic rather than diploid (8). Under the conditions used, more than 30% of the protoplasts formed from heterokaryons were monokaryotic. When protoplasts from the His2 + Lys1 heterokaryon were plated on regeneration medium containing histidine, approximately 39% of the resulting colonies were His^-. Protoplasts from the same heterokaryon gave 69% Lys^- colonies when plated on medium containing lysine. This difference may reflect either the percentage of His^- and Lys^- nuclei in the original heterokaryon or a difference in the stability of protoplasts of different phenotypes. Homokaryons can also be obtained through the use of conidial preparations, as previously reported (8).

Experiments using *P. chrysosporium* protoplasts for genetic transformation, the isolation of fungal DNA, and the possible isolation of active membrane fractions or organelles from lignino-

lytic cells are either under way or planned in this laboratory.

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