A METHOD FOR GENERATING PROTOPLASTS FROM *CLITOPILUS PINSITUS*

KENNETH R. STEWART

Squibb Institute for Medical Research,
New Brunswick, New Jersey 08903, U.S.A.

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The basidiomycete *Clitopilus pinsitus* produces a diterpene antibiotic called pleuromutilin[1]. Since its discovery in 1951, the fermentation, structure, and biosynthesis of pleuromutilin have been documented[2]. Pleuromutilin has a limited spectrum of activity, inhibiting primarily the growth of Gram-positive bacteria and of *Mycoplasma*. Pleuromutilin is a fermentation product used for the chemical synthesis of tiamulin hydrogen fumarate, an antibiotic effective for the treatment of dysentery and pneumonia in swine.

Our strain improvement efforts for *C. pinsitus* initially focused on obtaining single cells for conventional mutagenesis. Since this basidiomycete was asporogenous and appeared nonseptate, a method was needed for generating protoplasts. Presented in this report is a rapid and reliable method for isolating and regenerating *C. pinsitus* protoplasts.

For cultivation of *C. pinsitus*, a frozen vial was thawed and inoculated into a 250-ml flask containing 50 ml of a general purpose growth medium. The medium contained glucose 50 g, KH_2PO_4 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $Ca(NO_3)_2 \cdot 4H_2O$ 0.7 g, NaCl 0.1 g, $FeSO_4 \cdot 7H_2O$ 0.05 g, Amberex yeast 1003 (Amber Labs) 12.5 g and soybean oil 4 ml in 1 liter of tap water. The flask was shaken for 3 days at 25°C on a Gyrotory shaker set at 250 rpm with a 6-cm circular orbit. Following incubation, the filamentous cells were removed by centrifugation at $1,000 \times g$ for 10 minutes and were washed 2 times using sterile 0.7 M NaCl. The cells were then suspended in sterile 0.7 M NaCl up to the original volume and were aseptically transferred to a sterile 250-ml flask. A 5.0 mg/ml solution of Novozyme 234 (Novo Laboratories, U.S.A.) was freshly prepared in sterile 0.7 M NaCl and added to the cell suspension at the rate of 1.0 ml per 100 mg (wet weight) of mycelium. A replicate flask was used for the mycelial wet weight determination. It has been reported that Novozyme 234 contains high levels of β-D-glucanase and chitinase activities, important enzymes for hydrolyzing fungal cell wall components[3]. The cell-enzyme mixture was gently shaken at 50 rpm at 25°C for 2 hours.

Following the enzyme treatment, the protoplasts were recovered by filtering the cell suspension through two layers of sterile Miracloth (Calbiochem-Behring Corporation, U.S.A.). The Miracloth retained the filamentous cell debris but permitted the passage of the protoplasts in the filtrate. The resultant protoplast suspension was microscopically examined to confirm that it was free of filamentous cells. The protoplasts were removed by centrifugation at $1,000 \times g$ for 10 minutes and were washed twice using sterile 0.7 M NaCl. Protoplasts were then counted by using an improved Neubauer haemocytometer. Counts usually ranged between 1.0×10^6 and 3.0×10^7 protoplasts per ml. At this point, the desired mutagenic treatment was employed and survivors were regenerated.

Regeneration medium contained sucrose 170 g, sodium succinate 10 g, yeast extract 3.0 g, $MgCl_2 \cdot 6H_2O$ 8.0 g, K_2SO_4 0.3 g, glucose 10 g, Casamino acids 0.5 g, trace element solution 2.0 ml, $CaCl_2 \cdot 2H_2O$ 0.3 g and KH_2PO_4 0.05 g in 1 liter of distilled water. The trace element solution contained $ZnCl_2$ 40 mg, $FeCl_3 \cdot H_2O$ 200 mg, $MnCl_2 \cdot 4H_2O$ 10 mg, $NaB_4O_7 \cdot 10H_2O$ 10 mg and $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$ 10 mg in 1 liter of distilled water. The $CaCl_2 \cdot 2H_2O$ and KH_2PO_4 were sterilized separately and added aseptically when the medium had cooled to 50°C. Solid regeneration medium was made by adding 0.8% agar (Bacto-Agar, Difco Laboratories, Detroit, Michigan, U.S.A.) to the liquid medium.

For regeneration in liquid medium, 0.2 ml of a diluted protoplast suspension was inoculated into 50 ml of liquid regeneration medium in a 250-ml flask. All flasks were incubated at 25°C. It was found that the growth morphology of the regenerants could be directed either to exclusively filamentous growth or exclusively pellet growth depending upon the speed at which the flasks were shaken. Filamentous growth resulted when flasks were shaken at speeds greater than 50 rpm (100 rpm was commonly used). Filaments were detected several days after inoculation by phase contrast microscopy. Pellets were completely absent from these flasks. Once filamentous growth was established, efforts to induce pellet formation by reducing the agitation speed of the

flasks were unsuccessful.

Regenerants grew exclusively as large (2~4 mm diameter) white pellets when flasks were shaken at a low speed (40 rpm) for 7 days. A correlation between the number of protoplasts used as inoculum and the number of regenerant pellets could not be defined. Examination of the pellets revealed that they consisted of a loose network of hyphae arranged around a solid, compact center. While the regulation of pellet formation is not fully understood, it is generally recognized that factors such as inoculum size, growth medium components, and the physical environment within the culture vessel influence pellet growth[4]. In this situation, it appeared that the mild agitation environment was the most important factor in directing regenerant morphology toward pellet formation and growth. The fact that mild agitation promoted pellet formation supports the hypothesis of FOSTER, who proposed that pellet formation most likely occurred when nutrient conditions were unfavorable for rapid and abundant growth and when agitation conditions were very mild as, for example, in shake flasks with deep volumes of liquid in very slow motion[5].

Protoplasts regenerated on the solid regeneration medium after a 7-day incubation period at 25°C. The protoplast regeneration rate was monitored frequently and was found to be between 70 and 80%. High humidity conditions produced by incubating the plates inside a sealed bag with a moist towel and an agar concentration of 0.8% were essential for successful regeneration. Protoplasts failed to regenerate when the agar concentration exceeded 0.8%. One other commercially available agar was tried. Agar (granulated form) from Baltimore Biological Laboratory (Becton Dickinson & Co., Cockeysville Maryland, U.S.A.) was tried and yielded the same results as the Difco product.

For our strain improvement program, it was essential that pure clones of the regenerated culture were isolated. To ensure culture purity, only clones derived from protoplasts regenerated on solid medium were used for future work. Protoplasts that regenerated in liquid medium were not used since these cells most likely experienced cell to cell interactions (such as colliding and sticking to one another) that jeopardized clone purity.

The method described in this report has permitted us to routinely obtain a sufficient number of protoplasts for a mutagenesis and selection program. Using conventional mutagenesis techniques, we have obtained pleuromutilin overproducers which have been recycled into our selection program. To the best of my knowledge, this is the first report of the isolation of protoplasts from this basidiomycete. This method is relatively simple and reliable and may be suitable for obtaining protoplasts from other basidiomycetes for fusion and genetic studies.

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