Production of protoplasts from the fungi *Curvularia inaequalis* and *Trichoderma reesei*

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**Summary.** Protoplast formation in *Curvularia inaequalis* was achieved using non-commercial and commercial snail gut enzymes or *Trichoderma harzianum* enzymes. The cells were grown for enzyme treatment on cellophane sheets or in liquid cultures for varying periods of time. The production of *T. harzianum* enzymes is discussed. The highest protoplast yields were $2.6 \times 10^7$ protoplasts/ml enzyme solution. Protoplasts were shown to have zero to four nuclei. Protoplast regeneration was successfully carried out in semisolid agar.

**Introduction**

The technique of protoplast fusion has been shown to be efficient in the development of fungal strains for industrial use (Peberdy 1980). With protoplast fusion it has been possible to cross different fungal strains or even fungal species, thus providing new genetic material for the construction of improved strains. Methods for transformation of fungal protoplasts with recombinant plasmid DNA have also been developed (Case et al. 1979; Ballance et al. 1983).

Fungal protoplasts can be produced by using snail gut juice enzymes (Fawcett et al. 1973), but more generally, microbial exoenzymes are used in cell wall digestion. Enzymes from *Oerskovia xantinolytica* (Broek et al. 1979) and other bacteria belonging to the family *Actinomycetaceae* are cell wall lytic. *Trichoderma harzianum* fungus produces exoenzymes with well-known cell wall lytic activity (Peberdy and Isaac 1976). Some commercially available enzyme preparations have also been reported to be effective in protoplast preparation (Hamlyn et al. 1981).

In this work a method for effective isolation of protoplasts from *Curvularia inaequalis* fungus is presented. *C. inaequalis* produces high amounts of $\beta$-galactosidase with an acid pH optimum suitable in the hydrolysis of lactose in acid whey to glucose and galactose (Mustranta et al. 1981). Conidia of *C. inaequalis* are large (10–30 µm) and carry several nuclei (Müller and Loeffler 1976), which often makes the use of techniques (e.g. mutagenesis) used in strain improvement more difficult. Protoplasts were also prepared from *Trichoderma reesei* QM 9414 (Mandel et al. 1971) and *T. reesei* VTT-D-80133, an efficient cellulase-producing mutant strain (Bailey and Nevalainen 1981).

**Materials and methods**

**Microorganisms and growth conditions.** Protoplasts were prepared from mycelia grown on solid and liquid media. *Curvularia inaequalis* ATCC 34599, *Trichoderma reesei* QM 9414 and *T. reesei* VTT-D-80133 were grown for protoplast formation on cellophane sheets placed on potato dextrose agar plates (PD-agar, Difeo) (Croft and Dales 1979). Sheets were inoculated with heavy conidial suspensions (about $10^7$ conidia/ml) using sterile paint brushes for inoculation and cultures were incubated at 28°C in dark for 15–30 h depending on the fungal strain. For liquid cultures (Isaac and Peberdy 1979), 50 ml medium was placed in 250-ml conical flasks. Media were inoculated with approximately $3 \times 10^3$ spores/ml (*C. inaequalis*) or $2 \times 10^6$ spores/ml (*Trichoderma* strains). Liquid cultures were incubated at 28°C on a rotary shaker (200 RPM) for 15–30 h.

*Trichoderma harzianum* CBS 354.33 was grown for cell wall lytic enzyme production in a bioreactor (Eschweiler AG, FRG) with a working volume of 3 l. The culture medium was the same used in shake flask cultures, except that the carbon source consisted of 3 g/l glucose and 15 g/l dried *Curvularia inaequalis* mycelium. Cultivation was carried out under the following conditions: temperature 30°C, aeration 1 vvm, stirring speed 750 RPM and time 90 h.

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Enzymes. For protoplast production snail gut enzymes from Suc d'helix pomatia (Pharmindustrie IBF), Cytohelicase (Pharmindustrie IBF) Glusulase (Endo Laboratories Inc.), β-glucuronidase (Sigma Co.) and a non-commercial enzyme (from Dr. Farkas, Slovak Academy of Sciences, Bratislava, Czechoslovakia) were used. All snail gut enzyme preparations were ultrafiltered (Diaflow UMZ, Amicon Co., USA) and lyophilized before use.

The cell wall lytic enzyme of T. harzianum was prepared in the following manner: after growth, the T. harzianum cells were removed by centrifugation (10,000 g, 15 min) and the resulting supernatant was ultrafiltered. Filtration was repeated three times with distilled water before lyophilization. The effectiveness of the exoenzymes of T. reesei VTT-D-80133 (Bailey and Nevalainen 1981) against C. inaequalis cell walls was also studied. Enzymes were used at 1% (w/v) concentrations in the osmotic stabilizing solution of 0.65 M KCl in 0.1 M phosphate buffer, pH 5.8. Lytic enzyme preparations were sterilized by filtration.

Protoplast isolation. Cellophane cultures were transferred to clean petri dishes (3/dish) and 20 ml of enzyme solution was added. After 10 min sheets were discarded and the mycelium was left in solution. The mixture of mycelium, lytic enzyme preparation and osmotic support was incubated at 30°C with gentle shaking (30 RPM) for 3-4 h. The release of protoplasts during incubation was followed in a microscope by counting the protoplasts using a counting chamber.

After enzyme treatment protoplasts were separated from mycelial debris by filtration through sintered glass (porosity 1). Protoplasts were pelleted (800 g, 5 min) and washed twice with the stabilizing solution. For regeneration, aliquots of protoplasts were suspended in molten semisolid PD-agar (with 5% KCl) and poured on solid PD-agar plates. Reversion of protoplasts to the mycelial form was followed by examining samples taken at intervals under the microscope. Cell nuclei were stained with acridin orange (Kevei and Peberdy 1977).

Results and discussion

Commercially available Suc d'helix pomatia, Cytohelicase and glusulase snail gut enzymes were not active against Curvularia inaequalis cell walls under the conditions described and no protoplasts were formed. However, with the non-commercial snail gut enzyme preparation $2.6 \times 10^7$ protoplasts/ml of enzyme solution could be obtained (Fig. 1) from cells grown on cellophane sheets for 25 h. Using β-glucuronidase the corresponding yield was $2.0 \times 10^7$ protoplasts/ml. β-Glucuronidase was not used in further experiments.

Enzymatic digestions of cell walls of Trichoderma reesei QM 9414 and T. reesei VTT-D-80133 cellophane cultures (20 h) with the non-commercial snail gut juice resulted in formation of $1.0 \times 10^7$ and $1.1 \times 10^7$ protoplasts/ml respectively (Fig. 2).

From cells grown in shake flasks, harvested at the exponential growth phase, and digested with the non-commercial snail gut enzyme preparation, the protoplast yields per milliliter were as follows: C. inaequalis ATCC 34599, $2.0 \times 10^6$ (Fig. 1); T. reesei QM 9414, $1.2 \times 10^7$ and T. reesei VTT-D-80133, $4.5 \times 10^7$ (Fig. 2).

Protoplast yields of T. reesei obtained in this work compare favourably with the corresponding values given in literature (Benitez et al. 1975; Picataggio et al. 1983).

The marked difference in protoplast yield between T. reesei QM 9414 and T. reesei VTT-D-80133 cells grown in liquid culture may be due to the different morphology of these strains. The mycelium of T. reesei VTT-D-80133 is more branched than that of T. reesei QM 9414. As protoplasts first emerge from hyphal tips presence of more tips in T. reesei VTT-D-80133 could result in more effective protoplasts liberation from this strain.

In Curvularia inaequalis, the number of protoplasts from cultures grown on cellophane sheets was clearly higher (about $2.6 \times 10^7$ protoplasts/ml, Fig. 1) than the protoplast yield of cells from liquid medium ($2.0 \times 10^6$ protoplasts/ml, Fig. 1). However, further