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Isolation and Regeneration of Protoplasts from the Cellulolytic Thermophilic Fungus *Sporotrichum thermophile*

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Protoplasts of filamentous fungi have been used for studies on cell wall enzyme localisation (MacLellan and Lampen, 1963; Beteta and Gascon, 1971), on permeability of the cell membrane (Kuo and Lampen, 1972; Scarborough and Schulte, 1974), and on isolation of cell organelles such as vacuoles, nuclei and mitochondria (Villanueva and Garcia-Acha, 1971). The biosynthesis of a new wall by regenerating protoplasts has retained the attention of many workers (de Vries and Wessels, 1975; Ramos and Garcia-Acha, 1975; Benitez *et al.*, 1975) as the sequential synthesis of individual components may help to understand its final functional organisation. In addition the regeneration of the new wall is the direct evidence that such structures have retained the complete capacity to form a normal mycelium.

The present paper reports on the conditions necessary for the formation and regeneration of protoplasts from *Sporotrichum thermophile*. This investigation was undertaken with the future aim of studying the possible implication of some cell wall enzymes in cellulolysis.

Materials and methods

A) Organisms: The strain of *Sporotrichum thermophile* (Var. 2) used in this study was isolated by Barnes (1974) from a paper compost, maintained on Difco Sabouraud-glucose agar slants and stored at 2° C for several months. *Micromonospora chalcea* was obtained from the Spanish Type Culture Collection in Salamanca (CECT 3195) and stored at 2° C on a medium containing: oat meal 6%, pasteurized milk 500 ml, agar 2%. The organism was reinoculated on solid GAE medium (see below) before it was used to produce lytic enzymes.

B) Lytic enzymes for the digestion of *Sporotrichum thermophile* cell walls were obtained by growing *M. chalcea* in a medium containing: (w/v)$NaNO_3$ 0.1% K_2HPO_4 0.2%, $MgSO_4 \cdot 7 H_2O$ 0.1%, NaCl 0.1%, yeast extract (Difco) 0.05%, chitin (Fluka) 0.25% and laminaran (Koch and Light) 0.25%. Cultures were shaken at 28° C for 5 days on a reciprocal shaker. The mycelium was removed by filtration, the filtrate dialysed against distilled water at 4° C for 24 hours and then stored at −20° C. β-1,3-Glucanase and protease are the main enzymatic components of the lytic system obtained in this way, but traces of chitinase and β-1,6-glucanase are normally also present in the enzymatic preparation (Villanueva *et al.*, 1973).

C) Protoplast isolation and purification: S. thermophile was grown at 44° in a liquid medium containing: glucose 1%, asparagine 0.1%, yeast extract (Difco) 0.05%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7 H_2O$ 0.05%, $FeSO_4 \cdot 7 H_2O$ 0.001% (medium GAE). Erlenmeyer-flaks (250 ml) containing 100 ml GAE-medium were inoculated at 44° C on a reciprocal shaker. After 10 hours growth the mycelium was collected by filtration, washed several times with distilled water and resuspended in the lytic system of *M. chalcea* with an osmotic stabilizer [usually with 1.2 M NH_4Cl]. Protoplasts were obtained after incubation at 37° C for several hours, liberation being followed by phase contrast microscopy. They were purified by filtration through a thin layer of cotton wool sandwiched between 4 layers of gauze, in order to eliminate the bulk of the mycelial debris. The protoplasts were recovered by centrifugation (600 x g) and washed several times with 1.2 M NH_4Cl. In the presence of this osmotic stabilizer the protoplasts were stable for many days at 4° C.

D) Cell wall regeneration: Winge's medium (glucose 2%, yeast extract 0.3%) with an osmotic stabilizer was used to study cell wall regeneration. The process was again followed by phase contrast microscopy. In order to follow new wall synthesis, a fluorescent brightener (Calcofluor White M 2 R.S. New, obtained from Cyanamid Inc.) was added at 0.1% and regeneration followed by fluorescence microscopy (A.M. Paton and S.M. Jones, 1971)

E) Electron microscopy: After centrifugation (600 x g) the mycelium was fixed with glutaraldehyde 4% for 1 hour in the presence of an osmotic stabilizer. After washing several times with the same osmotic stabilizer, the hyphae were fixed with OsO_4 1% during 1 hour and rewashed several times as above, dehydrated with acetone and embedded in the standard medium of Spurr (1969). The ultrathin sections were stained with uranyl acetate and lead citrate and observed in the electronmicroscope.

Materials: Lipase (from hog pancreas) and cellulase (from *Aspergillus niger*) were purchased from Sigma, St. Louis, USA; Helicase from Industrie biologique française, Gennevilliers, France; Glusulase from Endo Laboratories, Garden City N.Y., USA.

Results and Discussion

The main enzymatic activities of the lytic system of *Micromonospora chalcea* are β-1,3-glucanase and protease (Villanueva *et al.*, 1973). This lytic system with an osmotic stabilizer destroyed the cell walls of *Sporotrichum thermophile* releasing protoplasts. With the aim of accelerating protoplast liberation, several other enzymes were added to the lytic system of *M. chalcea.* The addition of lipase (4 mg/ml) doubled the number of protoplasts liberated after about 6 h incubation. Lipase has been reported as an accelerating enzyme for protoplast production by Garcia-Acha *et al.* (1966). Cellulase, glusulase, β-glucuronidase and helicase were also tested but did not improve yields of protoplasts. The action of the lytic system of *M. chalcea* and lipase suggests that the cell wall of *S. thermophile* is probably composed in part,

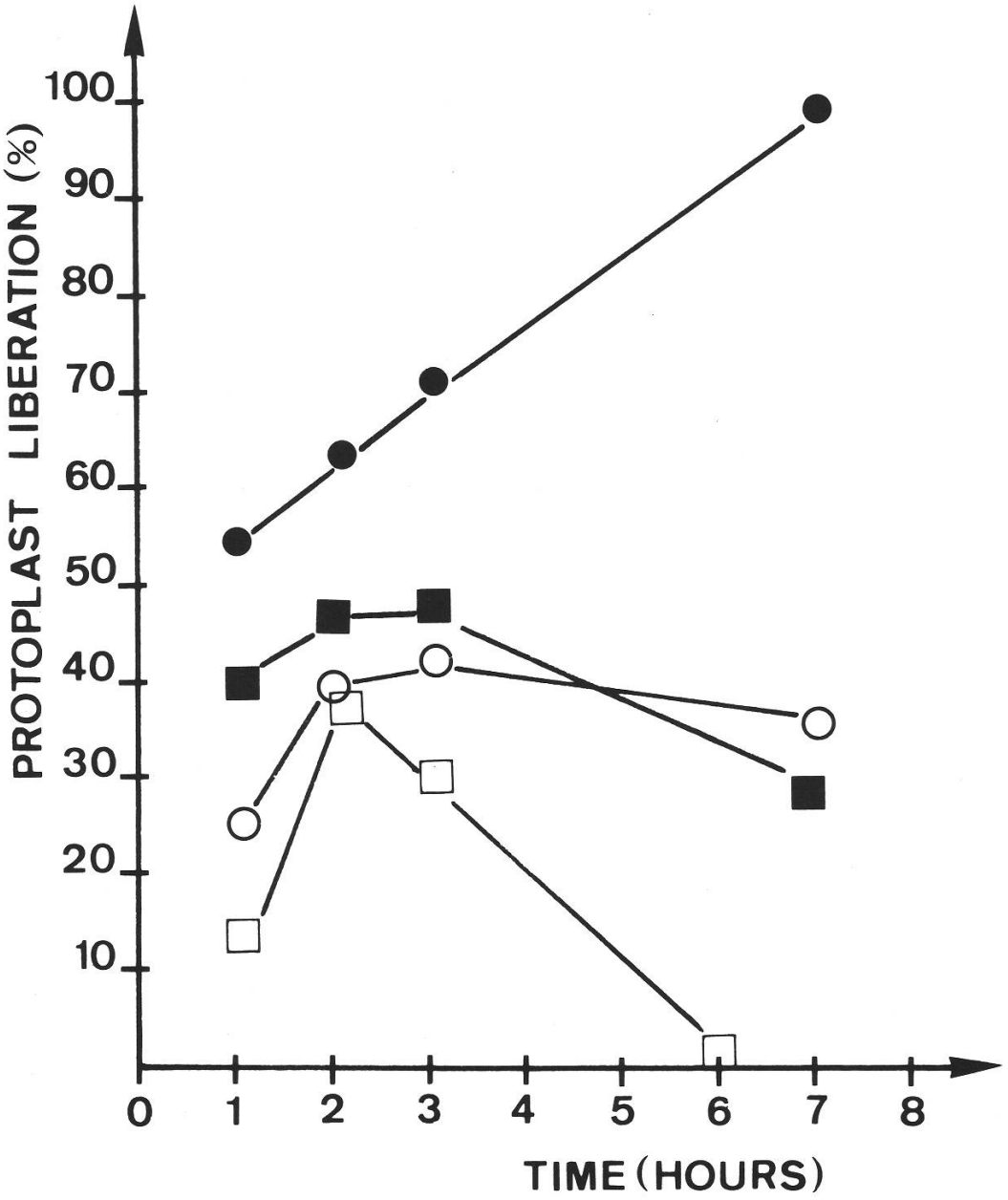


Fig. 1:
Optimal age of *S. thermophile* mycelium to obtain protoplasts. Mycelium was collected and treated as described in „Methods". The osmotic stabilizer was NH_4Cl 1.2 M. The number of protoplasts was calculated as the average of 10 randomly chosen areas of the preparation. The best production was considered as 100% and other values related to this value.

●● Age of mycelium: 10 hours
■■ Age of mycelium: 12 hours
○○ Age of mycelium: 14 hours
□□ Age of mycelium: 16 hours

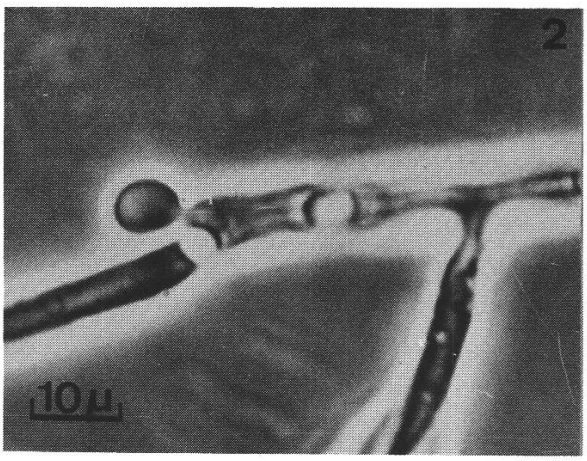
of β-1,3-clucans, proteins and lipids. As pointed out by Bartnicki-Garcia (1968) in his classification of fungi, β-1,3-glucans are important components of the cell wall of many fungi. Lipids in the cell wall have also been reported for numerous species [eg. *Fusarium culmorum* (Nombela and Peberdy, 1971), *Geotrichum lactis* (Ballesta and Villanueva, 1971)].

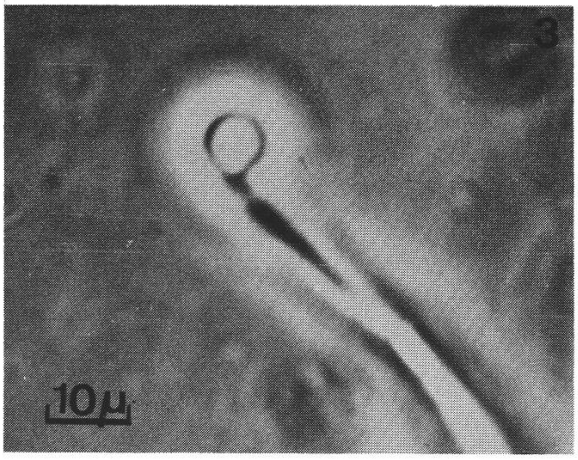
Villanueva (1966) reported that the young mycelium of fungi was the most sensitive to enzymatic lysis. In an attempt to determine the optimal age of mycelum for protoplast isolation from *S. thermophile,* the cells were collected at definite intervals from 10 to 16 hours after inoculation and growth on GAE medium at 44^0C (Fig. 1). The best results were obtained with 10 hours-old mycelium. As the mycelium aged, the number of liberated protoplasts decreased markedly. Osmotic stabilizers are necessary in order to prevent the protoplasts from bursting after their liberation. $(NH_4)_2SO_4$, NH_4Cl, $MgSO_4$, KCl, NaCl, and also mannitol and sucrose were tested as osmotic stabilizers in order to find optimal conditions for osmotic stability of protoplasts. Concentrations from 0.5 to 1.2 M were tested and the best results were obtained with NH_4Cl and $(NH_4)_2SO_4$ at 1.2 M. At higher concentrations the hyphae became plasmolysed and no protoplasts were released. If water was added to a suspension of protoplasts in an osmotic stabilizer at 1.2 M it caused an immediate rupture of the protoplasts, releasing intracellular vacuoles which were resistant to lysis. This phenomenon has also been described by Villanueva and Garcia-Acha (1971) and seems to be a good method to isolate vacuoles.

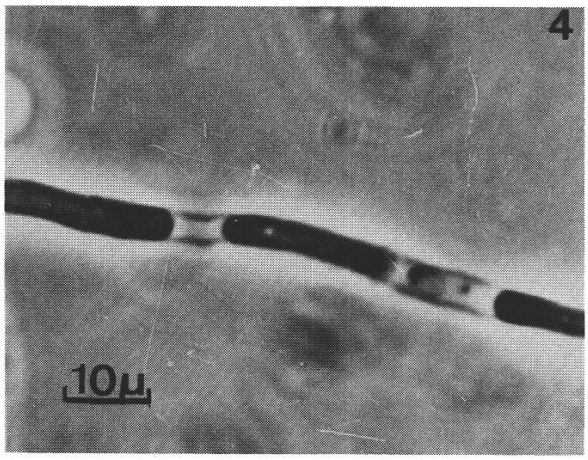
Morphological aspects of protoplast release: protoplasts were liberated from the apical or lateral zones of the hyphae (Fig. 2, 3 and 6) leaving partially empty hyphae (Fig. 4). Protoplasts could then be purified as described in the methods section (Fig. 5).

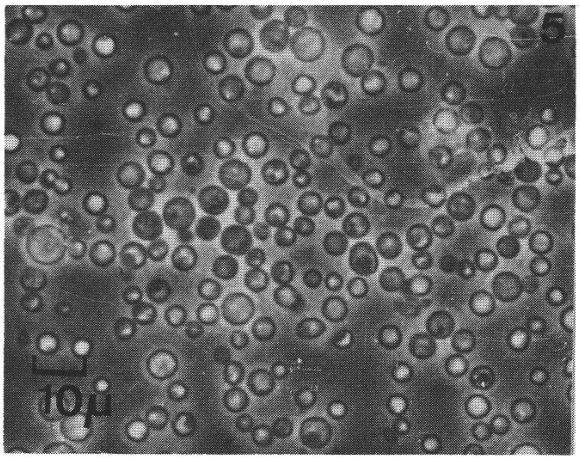
The absence of the cell wall was demonstrated with the fluorescent brightener „Calcofluor White" which is absorbed by the β-linked polysaccharides of the fungus (Maeda and Ishida, 1967). When protoplasts of *S. thermophile* were treated with the fluorescent brightener, no fluorescence was emitted indicating that the cell wall was totally absent. This method has been used to demonstrate the absence of cell wall in protoplasts of *Geotrichum lactis* (Gull *et al.*, 1972) and *Aspergillus nidulans* (Peberdy and Buckley, 1973). The total absence of the cell wall allows the protoplasts of *S. thermophile* to be denoted as true protoplasts rather than spheroplasts.

Regeneration of the cell walls and hyphae: Regeneration of a new wall occured when protoplasts were incubated at 44^0C in Winge's medium osmotically stabilized with NH_4Cl 1.2 M, and the formation was followed by phase contrast microscopy (Fig. 7 to 9) and, after staining with „Calcofluor White", by fluorescence microscopy (Fig. 10 to 12). The absorption of fluorescent brighteners by regenerating protoplasts has been used by many authors to demonstrate the development of the new wall [Peberdy and Buckley (1972); Gull, Moore and Trinci (1972); Gull and Trinci (1974); Benitez *et al.* (1966)]. Protoplast regeneration of *S. thermophile* occured as follows: protoplasts first increased in size; after about 5 hours of incubation at 44^0C the cell wall began to be synthesised and fluorescence appeared at the periphery of the protoplast. As the time proceeded, a bud was emitted from the protoplast









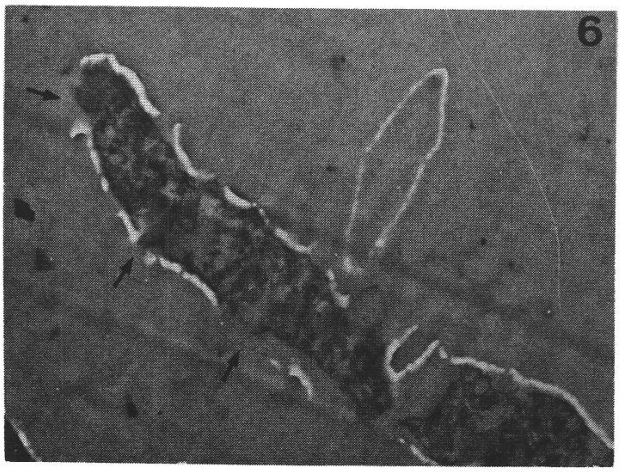
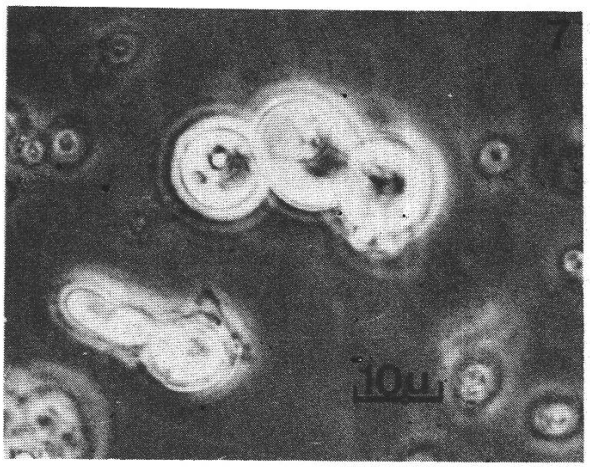
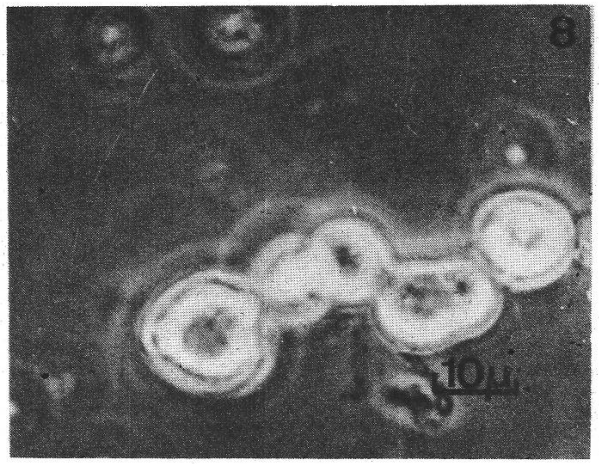


Fig. 2 and 3: Protoplast formation. Protoplasts emerging through a hole in the wall (phase contrast).
Fig. 4: Partly emptied hyphae after protoplast liberation (phase contrast).
Fig. 5: Purified protoplasts of *S. thermophile* resuspended in NH_4Cl 1.2 M (phase contrast).
Fig. 6: Hypha of *S. thermophile* irregularly attacked (arrow) by the lytic system of *M. chalcea* supplemented with lipase: 4 mg/ml (electron microscope, 9.350 x).

and remained attached to it. This bud was at first non-fluorescent, but later the fluorescence was again observed at its periphery. New buds were formed and finally a new hypha emerged from this chain. At first it was not fluorescent, but later the septae and the tip became strongly fluorescent suggesting a more intensive cell wall formation in these parts.

Not all of the protoplasts regenerated walls and hyphae at the same rate, and completely regenerated forms were only observed after 19 hours of incubation. The majority however, had regenerated a new wall after about 36 hours of incubation an 44°C. Some protoplasts did not regenerate a new wall at all and these were probably unnucleated forms incapable of forming such a wall. A similar regeneration





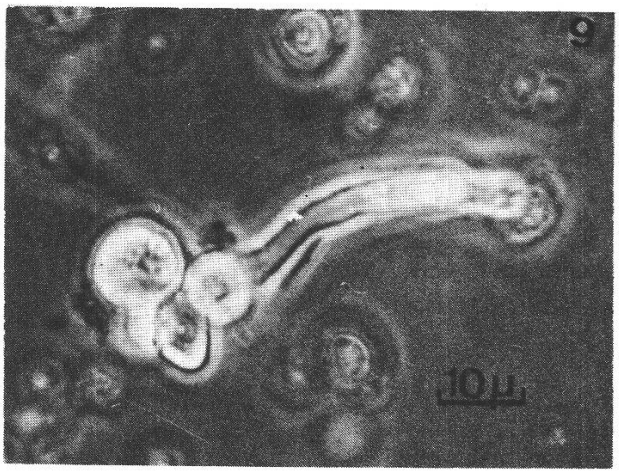
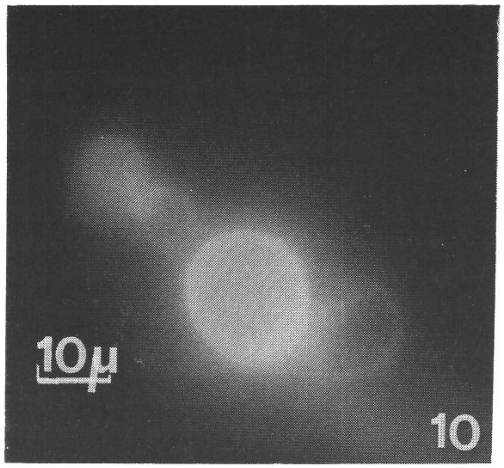
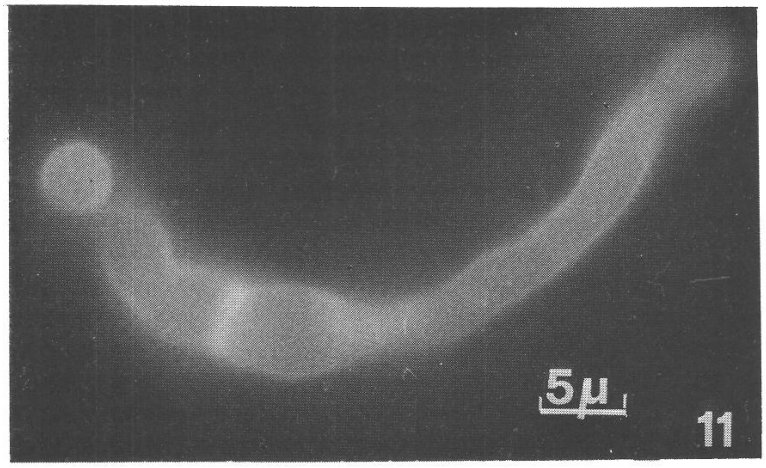


Fig. 7: Regenerating protoplasts after 20 h of incubation (phase contrast).
Fig. 8: Chain of buds emitted from a regenerating protoplast after 24 h of incubation (phase contrast).
Fig. 9: New hypha emerging from the regeneration chain after 36 h of incubation (phase contrast).





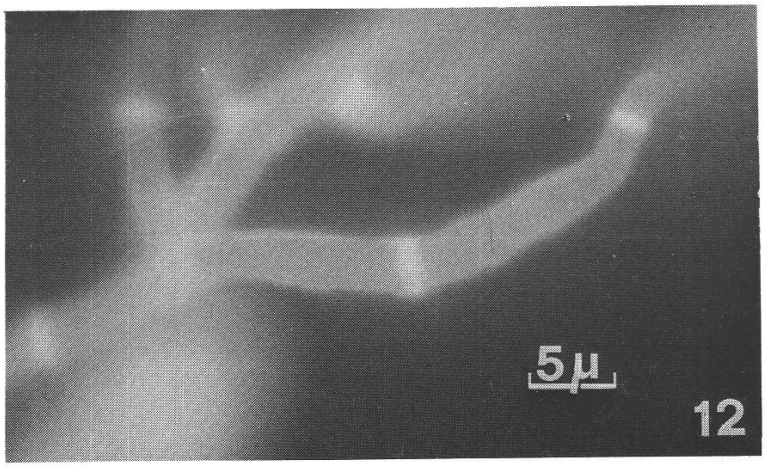


Fig. 10: Regenerating forms after 24 h in Winge's medium (fluorescent microscope, Calcofluor White).
Fig. 11: New hypha emerging from the regenerating chain after 36 h (fluorescent microscope, Calcofluor White).
Fig. 12: New hypha with fluorescent septae after 36 h of regeneration (fluorescent microscope, Calcofluor White).

pattern has been described for several fungi (*Trichoderma viride,* Benitez *et al.* 1975; *Pullularia pullulans,* Ramos and Garcia-Acha, 1975; *Fusarium culmorum,* Garcia-Acha *et al.* 1966) and according to Villanueva and Garcia-Acha (1972) seems to be caracteristic for all true protoplasts, although de Vries and Wessels (1975) reported that in regenerating protoplasts of *Schizophyllum commune* a new hyphae directly emerges from a thick wall structure without budding.

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Summary

Protoplasts from *Sporotrichum thermophile* were obtained by treating 10 hours-old mycelium with the lytic system excreted by *Micromonospora chalcea* which was osmotically stabilized with NH_4Cl or $(NH_4)_2SO_4$ at 1.2 M. The addition of lipase considerably enhanced proplast production. Regeneration of the cell wall lasts about 36 hours at 44°C. The process was followed by phase contrast microscopy and by staining with a fluorescent brightener.

Résumé

Les protoplastes de *Sporotrichum thermophile* ont été obtenus en traitant le mycélium âgé de 10 heures avec le système lytique excrété par *Micromonospora chalcea.* Le stabilisateur osmotique était soit le NH_4Cl, soit le $(NH_4)_2SO_4$ à une concentration de 1.2 M. L'adjonction de lipase stimule considérablement la production de protoplastes. La régénération dure environ 36 heures à 44°C, le processus a été suivi par microscopie à contraste de phase et après traitement au Calcofluor.

Zusammenfassung

Protoplasten von *Sporotrichum thermophile* wurden durch Behandlung eines 10 Stunden alten Myceliums mit einem von *Micromonospora chalcea* ausgeschiedenen, lytischen System erhalten. $(NH_4)Cl$ oder $(NH_4)_2SO_4$ (1.2 M) wurden zur osmotischen Stabilisierung verwendet. Mit Hilfe von Lipasen war es möglich, die Protoplastenbildung erheblich zu steigern. Die Regeneration der Zellwand, welche mit Phasenkontrastmikroskopie und nach Calcofluorbehandlung verfolgt wurde, nimmt bei 44°C etwa 36 Stunden in Anspruch.

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