THE FORMATION OF PROTOPLASTS FROM MUCOR SPECIES

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Methods were developed for the reproducible production of high yields of stable protoplasts from mesophilic and thermophilic strains of Mucor. This is a pre-requisite for the genetic analysis and manipulation of Mucor by cell fusion or transformation and is of special importance in these species since conventional genetic analysis by recombination studies is not feasible. The cell wall lytic enzyme used was the concentrated culture fluid of Streptomyces sp no. 6 grown on chitosan and chitin. Using germlings of M. circinelloides f. lusitanicus the optimum conditions for protoplast formation were determined as: 0.5-1.0 mg · ml⁻¹ streptozyme (1.2 units chitosanase · ml⁻¹) plus 2.0 mg · ml⁻¹ novozym 234 or 1%(v/v) helicase in 0.5 M-sorbitol, 0.01 M-sodium phosphate buffer, pH 6.5 for 3-4 hours at 23 °C. Treatment of 10^7 · ml⁻¹ germlings gave yields of up to 3×10^7 · ml⁻¹ protoplasts with regeneration and fusion frequencies of up to 40% and 1.7%, respectively. With the thermophilic strains growth at low temperature (25 °C) was critical for the subsequent formation of stable protoplasts. Synchronous growth and germination was obtained at this temperature by a short pre-incubation (1-3 hours) of the sporangiospores at 40 °C.

1. INTRODUCTION

The filamentous fungi belonging to genus Mucor are of biochemical and industrial interest. This is primarily because of the production by certain species of a number of extracellular enzymes such as acid proteases (3, 27), lipases (37), amylases (2) and cellulases (36). In addition, they provide a useful system for the study of the biochemical basis of morphogenesis since some species can be induced to undergo a morphogenetic change from hyphal to budding yeast-like growth in response to various environmental stimuli (4). The genetic analysis of Mucor species and any mutants derived therefrom is hampered by the difficulties in utilising the sexual cycle of these organisms. Mating of (+) and (-) strains does not always result in the formation of zygospores, and in some species the numbers produced are low while in others their development remains incomplete (34). Moreover, the successful germination of zygospores under laboratory conditions is rare and is preceded by a considerable period of dormancy (12, 14, 42). Formal genetic analysis by recombination studies is thus not feasible. In this context the study of protoplast formation in Mucor becomes of special interest since the alternative genetic techniques of cell fusion and transformation depend in most cases on the availability of large numbers of viable protoplasts. The efficient transformation of Mucor circinelloides by incubation of protoplasts with plasmid DNA in the presence of polyethylene glycol and $CaCl_2$ is reported in an accompanying paper (18).

The formation of protoplasts requires the removal of the cell wall by digestion with a lytic enzyme(s). The vegetative spores (sporangiospores) of Mucor are not a suitable source of protoplasts due to the extreme resistance of the cell wall to lytic digestion (20). The high content of melanin (10% dry weight) (5) is implicated in this resistance (20) as it is a known inhibitor of cell wall lytic enzymes (11). During germination, a vegetative wall is formed de novo underneath the sporangiospore wall, later emerging to become the germ tube wall (6). The lack of continuity between the spore and vegetative walls is correlated with marked differences in their composition, including the absence of melanin in the latter. Chitin and chitosan become the main components; in M. rouxii for example they constitute 9.4% and 32.7% of the hyphal cell wall, respectively (5). Chitinases isolated from several sources are available commercially but they are unable or only partially able to hydrolyse chitosan. Chitosanases are not yet commercially available but are produced as extracellular enzymes by a variety of microorganisms (17, 24, 26, 28). The culture fluids from some of these microorganisms, or the chitosanases purified therefrom, can digest the hyphal cell walls of Mucor (20, 28, 30) and enable the formation of protoplasts from Mucor (15, 22, 26, 30) and the related genus Phycomyces (9). The present paper reports the successful formation of protoplasts from a number of Mucor species using the concentrated culture fluid of Streptomyces sp. no. 6. The effects of some parameters are examined in order to maximise protoplast yields and the various methods reported for streptozyme preparation are compared.

2. MATERIALS AND METHODS
2.1. Strains
Streptomyces sp. no. 6 was obtained from D. Jones (the Macaulay Institute for Soil Research, Aberdeen, Scotland). Strains of Mucor used were the mesophiles Mucor circinelloides f. lusitanicus CBS 277.49 (= M. racemosus ATCC 1216b (33)), M. racemosus no. 50 (19) and M. rouxii CBS 416.77 and the thermophiles M. miehei CBS 182.67, M. miehei CBS 370.65, and

M. pusillus IMI 96211. The auxotrophic mutants used in protoplast fusion experiments were isolated after mutagenic treatment of M. circinelloides f. lusitanicus CBS 277.49 sporangiospores with UV-irradiation in this laboratory by Dr. M.I.G. Roncero (31).

2.2. Chemicals
Unless stated otherwise, all chemicals were supplied by Sigma. The chitin and chitosan were of practical grade prepared from crab shells. The enzymes used in section 3.2.1 (iv) and their sources were: novozym 234 (prepared from Trichoderma harzianum, mainly containing α-1,3 glucanase activity, received as a gift from Novo Industries A/S, Denmark), helicase (L'Industrie Biologique, France), laminarinase (β-1,3 glucanase ex mollusca, Calbiochem, U.S.A.), β-glucanase isolated from Penicillium emersonii (BDH Biochemicals, England) and chitinase (Sigma, activity = 3.0 units · mg⁻¹).

2.3. Media
The complete medium used was YPG (3 g yeast extract, 10 g peptone and 20 g glucose per litre distilled H_2O). The minimal medium was YNB (0.5 g Difco yeast nitrogen base w/o amino acids and ammonium sulphate, 1.5 g ammonium sulphate, 1.5 g glutamic acid per litre distilled H_2O with 1% glucose and 1 µg · ml⁻¹ thiamine and niacin added post sterilization). The media were adjusted to pH 4.5 with 1 M-HCl unless restricted colony growth was required (for viability counts, detection of heterokaryons) in which case the media were adjusted to pH 3.0. 20g · l⁻¹ agar was included for solid media, and in the case of pH 3.0 media, double strength solutions of agar and the other media components were autoclaved separately to avoid acid hydrolysis of the agar.

2.4. Isolation and assay of Streptozyme from Streptomyces sp. no. 6
Streptomyces sp. no. 6 was grown for 7-8 days at room temperature in the medium of Skujins et al. (35) with 0.5% (w/v) chitin and 0.1% (w/v) reprecipitated chitosan (chitosan dissolved in

1% acetic acid, neutralized with NaOH then washed extensively with H_2O) as sole carbon sources. Cells were removed by centrifugation (5000 g, 30 min) and the supernatant brought to 95% saturation with ammonium sulphate at 4 °C and left stirring overnight. The precipitate was collected (5000 g, 60 min), resuspended in a minimal volume of cold 0.02 M-sodium phosphate buffer at pH 6.5, and dialysed extensively against the same buffer at 4 °C. After centrifugation to remove insoluble material, the preparation was lyophilised and stored at -20 °C. Protein was determined according to BRADFORD (10) using a Bio-Rad protein assay kit I with hen egg albumin as standard. Chitosanase activity was determined according to PRICE and STORCK (28). Chitosan was dissolved in 0.05 M-maleic acid to 2 mg · ml^{-1}, diluted 3-fold with H_2O and the pH adjusted to 6.0 with KOH. After preincubation of 375 μl aliquots at 30 °C for 15 min the reaction was started by addition of 125 μl enzyme in 0.01 M-sodium phosphate buffer pH 6.5 giving a final volume of 500 μl containing 0.5 mg · ml^{-1} chitosan. After a further 15 min at 30 °C the reaction was stopped by addition of 100 μl 1 M-KOH and the chitosan precipitated by incubation on ice for 30 min. The supernatant obtained after centrifugation in an Eppendorf minifuge for 10 min was assayed for hexosamine content by the indole method of DISCHE (13). A unit of chitosanase activity was defined as the activity required to produce 1 μmol of hexosamine equivalent (mono- or oligosaccharide) per min.

2.5. Formation of Protoplasts from Mucor

Sporangiospores from cultures grown for 4-6 days at room temperature (mesophiles) or 37 °C (thermophiles) on YPG pH 4.5 agar plates were harvested by gently scraping with a glass rod into distilled H_2O. After one wash in distilled H_2O they were resuspended in YPG pH 4.5 at $10^7 · ml^{-1}$ and germinated at 28 °C (mesophiles) or 25 °C with a 2 hour preincubation at 40 °C (thermophiles) with shaking. Ungerminated spores were removed by filtration through nylon cloth, mesh size 16 μm and the germlings washed twice with 0.5 M-sorbitol. After resuspension in 0.5 M-sorbitol, 0.01 M-sodium phosphate buffer pH 6.5

the germlings were added to an equal volume of streptozyme dissolved in the same buffer to give final concentrations of $10^7 · ml^{-1}$ germlings and 0.5-1.0 mg · ml^{-1} streptozyme (corresponding to approximately 1.2 units · ml^{-1} chitosanase). After incubation at 23 °C for up to 4 hours with gentle intermittent mixing the number of protoplasts produced per ml was calculated by counting a minimum of two samples in a haemocytometer using phase contrast microscopy. When necessary, any undigested hyphae were removed by filtration of the protoplast suspension through nylon cloth, mesh size 10 μm.

2.6. Protoplast Fusion

Protoplasts were prepared as described in section 2.5, and washed once with 0.01 M-sodium phosphate pH 6.5, 0.5 M-sorbitol. After resuspension in the same medium they were subjected to the procedure for protoplast fusion described by OHNUKI et al. (26) with minor modifications. Suspensions of protoplasts from two different auxotrophs were mixed to give a total of 10^7-10^8 protoplast pairs then centrifuged at 400 g for 5 min. The pelleted protoplasts were resuspended in 5 ml of 45% PEG 4000 containing 60 mM-$CaCl_2$ and incubated at room temperature for 30 min. 30 ml of 0.01 M-sodium phosphate buffer pH 6.5, 0.5 M-sorbitol was added and the suspension centrifuged (400 g, 5 min) after which the pellet was resuspended in 10 ml of the same. 1 ml aliquots of suitable dilutions were mixed with 9 ml of 1% (w/v) agar containing 0.5 M-sorbitol (50 °C) then overlayed onto minimal or complete agar media, pH 3.0. Colonies were counted after two days incubation at room temperature.

3. RESULTS AND DISCUSSION
3.1. Isolation of Streptozyme from Streptomyces sp. no. 6

Earlier reports showed that the cell walls of various members of the genus Mucor could be degraded by the cell free culture fluid of Streptomyces sp. no. 6 (20, 30) or a chitosanase purified therefrom (28). The lytic activity could be induced by growth of the Streptomyces in media containing either Mucor cell walls or chitosan

Table I.
Comparison of crude chitosanase (streptozyme) preparations obtained from Streptomyces sp. no. 6 by three different procedures

Method of preparation	Yield mg protein · l^{-1} culture	Chitosanase Specific Activity units · mg^{-1} protein
As described in section 2.4, with 0.1% (w/v) chitosan and 0.5% (w/v) chitin as sole C-sources in the growth media.	205	2.6
As described in section 2.4 but with 1% (w/v) M. miehei cell walls[a] as sole C-source in the growth media.	47	1.2
Incubation of stationary phase cells from 16 l of culture in 2 l of 0.05 M D-glucosamine hydrochloride, 0.1% yeast extract [b].	91	1.2

[a]) Cell walls were prepared by grinding hyphae in liquid N_2 with a mortar and pestle followed by three passages through a French pressure cell (8000 p.s.i.). They were then repeatedly washed with H_2O in order to remove all contaminating cytoplasmic material.

[b]) According to the method of PRICE and STORCK (28) except that streptozyme was precipitated by addition of $(NH_4)_2SO_4$ to 75% saturation, chromatographed on Sephadex G-50 and then lyophilised.

and chitin (20), or by incubation of stationary phase cells in a medium containing 0.05 M-glucosamine (28). After a comparison of the streptozyme preparations obtained when using each of the three induction methods (Table I), the use of chitosan and chitin as sole C-sources in the growth media was adopted as the routine procedure in this laboratory. It is the simplest procedure in practical terms and gives a streptozyme preparation whose total yield and specific activity is higher than that obtained by the other methods.
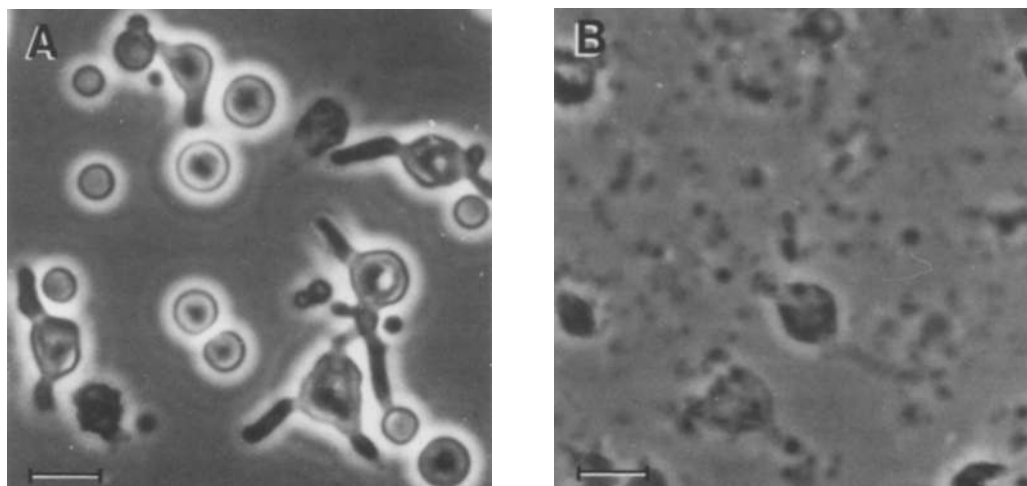

Figure 1. A. The formation of protoplasts from germlings of M. circinelloides f. lusitanicus upon incubation with streptozyme as described in section 2.5. B. The same field after flooding with H_2O showing the osmotic sensitivity of both the protoplasts and degraded hyphae. (Bar = 10 μm).
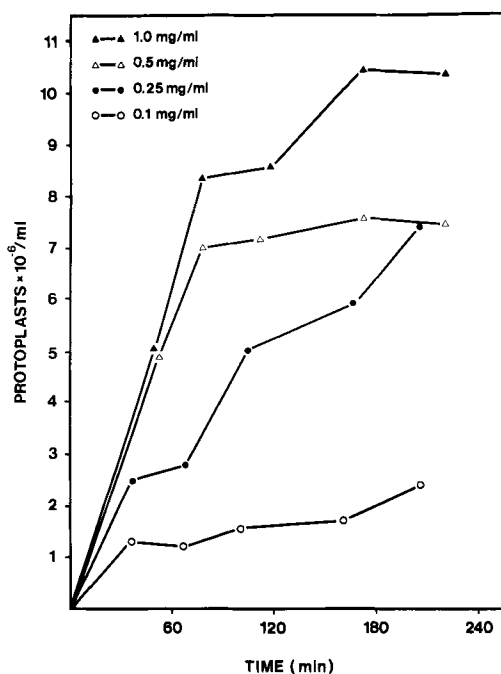
Figure 2. Effect of different concentrations of strepto-zyme on the formation of protoplasts of M. circinel-loides f. lusitanicus. Method is as described in section 2.5 with the final concentration of streptozyme used as indicated. Specific activity of the streptozyme was 1.2 units chitosanase · mg⁻¹.
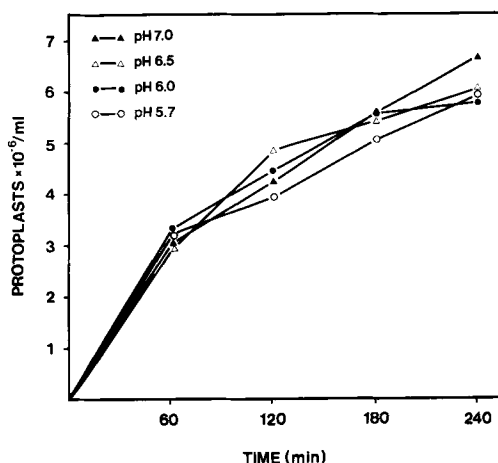

Figure 3. Effect of pH on the formation of protoplasts from M. circinelloides f. lusitanicus. Method is as described in section 2.5 using 0.01 M-sodium phos-phate buffer at the pH values indicated by adjustment of the relative amounts of Na_2HPO_4 and NaH_2PO_4.

3.2. Formation of protoplasts from M. circinelloides f. lusitanicus

When germlings of M. circinelloides were treated with streptozyme as described in section 2.5 digestion of the cell wall occurs, the cell membrane bulges out, is pinched off, and sphe-rical osmotically sensitive protoplasts with an average size of 7 μm are released (Figure 1). The formation of protoplasts preferentially occurs at the hyphal apex which is the major site of cell wall synthesis (7). The total number of proto-plasts formed varied with the conditions used, but it could be three times the total number of germlings treated. Mucor is coenocytic (i.e. has nonseptate hyphae), thus the formation of more than one protoplast per germling indicates an effective resealing of the cell membrane after a protoplast is released.

3.2.1. Factors affecting protoplast formation
(i) Concentration of streptozyme:

An increase in the concentration of strepto-zyme increased the rate of protoplast formation and the final yield of protoplasts obtained (Fi-gure 2). When comparing different batches of streptozyme, their efficacy in producing proto-plasts was directly proportional to the total chitosanase activity present. Thus, 0.5 mg · ml⁻¹ of a batch with a specific chitosanase activity of 2.3 produces equivalent results to 1.0 mg · ml⁻¹ of a batch with specific chitosanase activity of 1.2.
(ii) pH:

Variation of the pH of the phosphate buffer used during incubation within the range of pH 5.7-7.0 had no effect on the activity of the streptozyme (Figure 3). This is consistent with a report (28) that a chitosanase purified from Streptomyces sp. no. 6 culture fluids had op-timal enzyme activity towards deacetylated chi-tosan over the pH range of 4.5-6.5. A decrease in activity was reported to occur at higher pH (28) but the substrate chitosan exists in a colloidal state above pH 6.5, possibly affecting the measu-rement of activity. RAMIREZ-LEON and RUIZ-HERRERA (30) also showed that the activity of their streptozyme preparation on isolated M. rouxii cell walls plateaued between pH 5.0-6.5 with one additional higher peak of activity at pH 7.0-7.5.

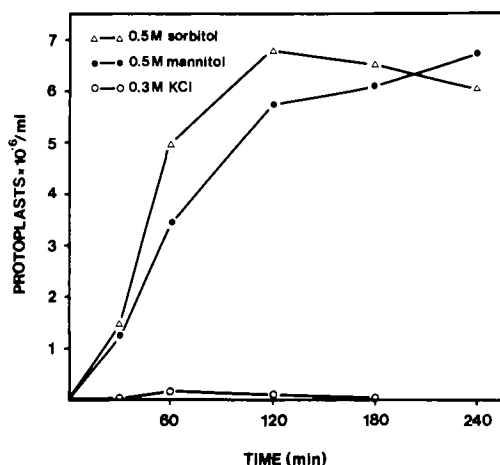Figure 4. Use of different osmotic stabilisers in the formation of protoplasts from M. circinelloides f. lusitanicus. Method is as described in section 2.5.
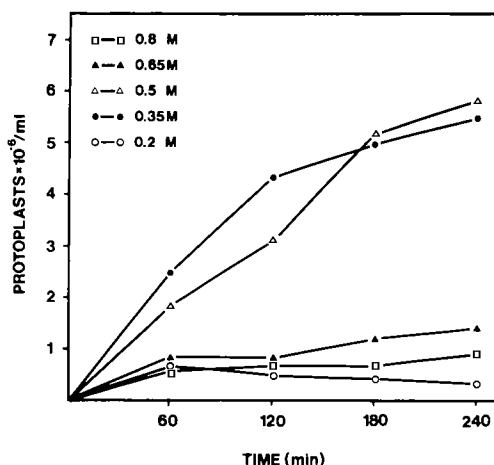
Figure 5. Effect of different concentrations of sorbitol as osmotic stabiliser on the formation of protoplasts from M. circinelloides f. lusitanicus. Methods as described in section 2.5.

(iii) Osmotic stabilisers and their concentration:

Solutions of sugar alcohols such as sorbitol and mannitol proved to be the most effective osmotic stabilisers, while use of salt solutions such as 0.3 M-KCl resulted in no protoplast formation (Figure 4). 0.3 M-solutions of $MgCl_2$, $(NH_4)_2SO_4$ or $MgSO_4$ were as ineffective as KCl and all apparently inhibit the activity of the streptozyme preparation since no cell wall degradation was observed in their presence. The optimal concentration of sorbitol was 0.35 M-0.5 M (Figure 5). At concentrations of 0.65 M or higher no cell wall degradation was observed and at concentrations of 0.3 M or lower, the action of the streptozyme was accompanied by extensive cell lysis rather than protoplast formation.

(iv) Addition of commercially available lytic enzymes:

The preparation of a lytic enzyme mixture such as streptozyme can be time consuming and different batches often vary in lytic activity. In order to circumvent such problems Hamlyn et al. (16) had investigated the possibility of using various commercially available polysaccharases in the formation of protoplasts from a range of fungi. Many preparations were found to be effective even though the active lytic components were often present only as contaminants. The effect of some of these commercially available preparations on germlings of Mucor was

studied (Figure 6). None of the enzymes when used alone had any visible lytic effect, but both novozym 234 and helicase gave increased yields of protoplasts when used in combination with a low concentration of streptozyme. In contrast, the addition of chitinase, laminarinase or Penicillium β-glucanase had no effect on protoplast yields. The lack of effect of chitinase contrasts with the findings of other workers. Price and Storck (28) reported that the decrease in turbidity of suspensions of Mucor cell walls after addition of the chitosanase purified from Streptomyces sp. no. 6 culture fluids was higher when a mixture of chitinase and this chitosanase was used. Similarly, the chitosanase isolated from Myxobacter AL-1 is always used in combination with a commercially available chitinase in the formation of protoplasts from M. racemosus (syn. circinelloides f. lusitanicus, see section 2.1), (15, 22), although the activity of the chitosanase alone is not reported. With the crude chitosanase prepared from Bacillus R-4, Ohnuki et al. (26) found the addition of chitinase and/or sulphatase essential for the production of protoplasts from M. pusillus in yields higher than 5%. The result shown here in Figure 6 could be explained by the presence of chitinase activity in the streptozyme preparation since the
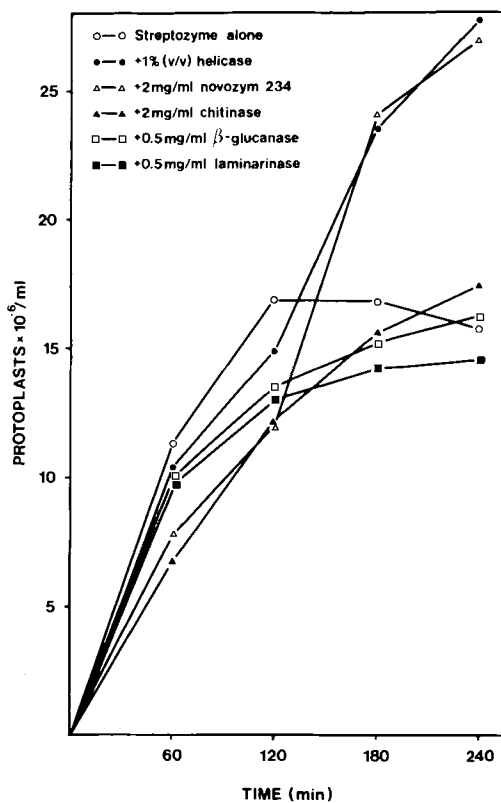
Figure 6. The effect of some commercially available lytic enzymes on the formation of protoplasts from M. circinelloides f. lusitanicus by 0.5 mg · ml⁻¹ streptozyme (1.15 units chitosanase · ml⁻¹). Method is as described in section 2.5. The commercial enzymes were added to the germlings at the same time as the streptozyme and to the final concentrations indicated.
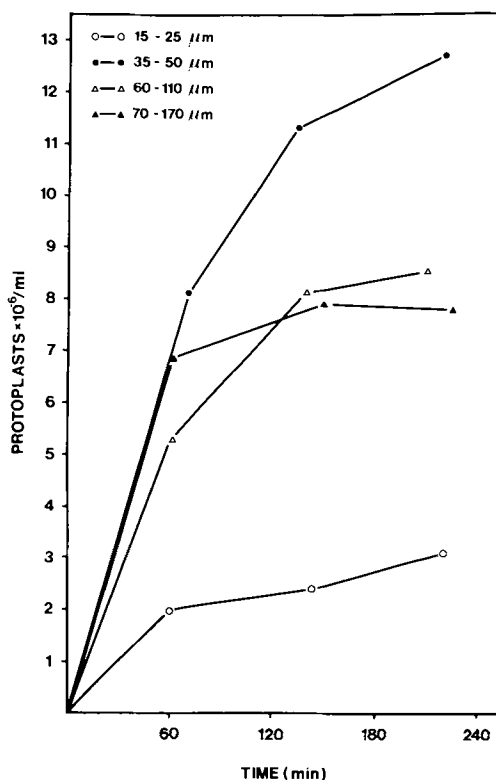
Figure 7. The influence of germ tube length on the formation of protoplasts from M. circinelloides f. lusitanicus. Sporangiospores were germinated until the distance from spore center to hyphal tip was as indicated when they were harvested and treated as described in section 2.5.

growth media for streptozyme production contains both chitin and chitosan.

(v) Extent of germination

Ungerminated sporangiospores were totally resistant to the lytic action of the streptozyme. This resistance was lost upon germination, but was acquired again, though to a lesser extent, as the hyphae aged and grew in length. The effect that these changes in cell wall sensitivity have upon the formation of protoplasts is shown in Figure 7. The yield of protoplasts obtained was highest when germlings had a germ tube length of 35-50 μm. Below this size, digestion of the cell wall at the growing hyphal tip still occurred but the amount of material extruded was decreased,

with a consequent reduction in the number of protoplasts formed. When the germ tube length exceeded approximately 60 μm, the amount of hyphal structure visibly resistant to streptozyme action increased, the protoplast yield decreased, and the homogeneity of the protoplasts obtained also decreased (as evidenced by a greater variation in size, refractility and apparent membrane integrity).

A high yield of morphologically homogeneous protoplasts thus requires that the suspension of germlings to be treated with streptozyme is relatively homogeneous with respect to extent of germination (i.e., germ tube length). In the related genus Phycomyces, synchronous germination can be induced by various treatments such as heat shock (48 °C for 3 min) (8).
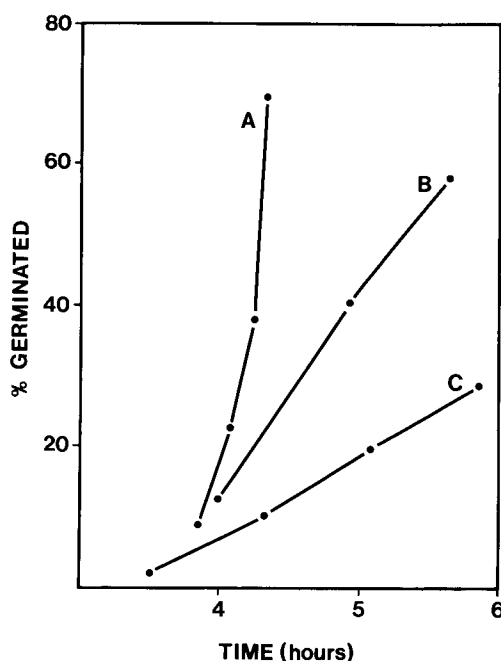
Figure 8. Germination of sporangiospores of M. miehei CBS 370.65 in liquid media at 40 °C. Sporangiospores were harvested from fresh cultures on agar plates (A and B) or obtained from H_2O suspensions stored frozen at -20 °C (C). They were incubated with shaking at a density of $10^7 \cdot ml^{-1}$ in YPG pH 4.5 (A) or YNB pH 4.5 (B and C) with a total of 10 ml media per 250 ml Ehrlenmyer flask. A spore was judged to have germinated when the length of the germ tube was equal to its breadth. When more than 60% of the spores had germinated the accuracy of counting was reduced due to extensive clumping of the germlings.

Unfortunately no specific induction methods are known for Mucor. Germination is generally asynchronous but can be improved by manipulation of the culture conditions. The germination of freshly harvested sporangiospores in a complete media was more synchronous than when using frozen spores or a minimal growth media (Figure 8). A 10-fold reduction in spore density, or a 10-fold increase in the ratio of flask volume to liquid volume gave no further improvement. Use of 0.1 M-phosphate buffer pH 6.5 containing 0.01 M-proline, which is reported to induce synchronous germination in the related genus RHIZOPUS (41), initiated no growth at all. Short incubations (15 min) at temperatures of up to 95 °C had no effect, but generally the closer

the incubation temperature is to the optimal growth temperature the more synchronous is the germination. This can be seen most clearly with the thermophilic species, an example of which is shown in Figure 9. Here the sporangiospores of M. miehei CBS 182.67 can be seen to germinate rapidly (within 5 hours) and relatively synchronously when incubated at 40 °C, but when incubated at the sub-optimal temperature of 25 °C they germinate slowly, extremely asynchronously and many remain ungerminated even after 28 hours incubation. STREETS and INGLE (38) had previously shown that little germination and growth occurred at 25 °C in a strain of M. miehei (NRRL-3420) whose optimal temperature for spore germination was 45 °C. However, substantial growth occurred at 25 °C if a 10 hour preincubation step at 37 °C was introduced, during which time spore germination was completed (38). Figure 9 shows that preincubation at 40 °C for only 2-3 hours significantly increased both the speed and synchrony of subsequent germination at 25 °C. During such short times at 40 °C the spores begin to swell, but the emergence of germ tubes does not occur until some hours later, during incubation at the lower temperature. These regimes are useful, therefore, in studies where sub-optimal growth temperatures are required (see section 3.3).

3.2.2. Regeneration of protoplasts on solid media

The protoplasts of M. circinelloides f. lusitanicus were stable and regenerated at frequencies of up to 40% even after overnight incubation at 4 °C in 0.01 M-sodium phosphate buffer pH 6.5, 0.5 M-sorbitol. A comparison of different plating conditions for the regeneration of these protoplasts on solid media (2% (w/v) agar) showed that a 1% agar overlay consistently gave better results (Table II). Direct spreading of protoplast suspensions onto plates with considerable surface moisture produced equivalently good regeneration but is not preferred because cells clumped and floated on the plate surface, reducing the accuracy of colony counts and hindering the isolation of single clonal colonies. A further disadvantage was the necessity to monitor the amount of moisture on the plates,
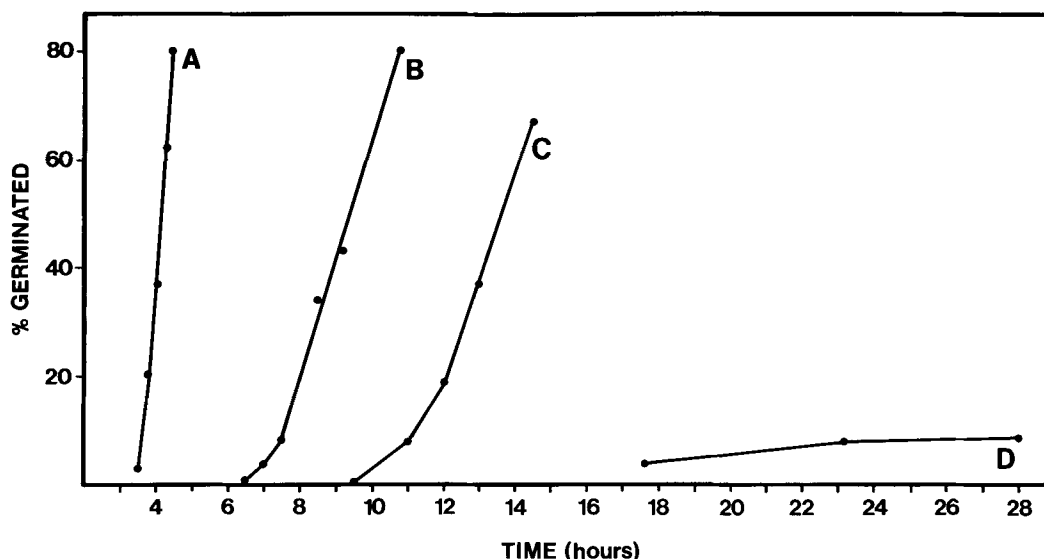
Figure 9. Germination of sporangiospores of M. miehei CBS 182.67 at 25 °C after preincubation at 40 °C for 3 hours (B) or 2 hours (C). Curves (A) and (D) show germination after incubation at 40 °C and 25 °C, respectively.

since drying of the surface resulted in a reduced frequency of regeneration (see Table II). Alteration of the pH (pH 3.0-pH 4.5), or use of the defined media YNB instead of YPG produced no significant differences. The figures given in Table II are the results from a single experiment. The absolute values of regeneration can vary somewhat from time to time but the trends remain the same.

3.2.3. Fusion of protoplasts

The fusion of protoplasts prepared from auxotrophic strains was achieved using 45% (w/v) PEG 4000 and 60 mM-CaCl₂ according to the

Table II.
Regeneration of protoplasts of M. circinelloides on solid media

Plating conditions[a]	Regeneration (%)[b]
1% (w/v) agar overlay (50 °C)	39
3% (w/v) agar overlay (50 °C)	11
1% (w/v) low melting point agar overlay (36 °C)	26
Freshly prepared agar plates with a moist surface	31
Agar plates with dried surface	7

[a]) A suspension of protoplasts prepared from M. circinelloides f. lusitanicus CBS 277.49 as described in section 2.5 was diluted in 0.5 M-sorbitol, 0.01 M-sodium phosphate buffer pH 6.5 then plated onto YPG pH 3.0 agar plates by mixing 1 ml with 9 mls of an agar overlay (held molten at the temperature indicated), or by directly spreading 1 ml or 0.5 ml (moist or dry surface plates, respectively) onto the plate surface. All plates and overlay solutions contained 0.5 M-sorbitol as osmotic stabiliser unless otherwise stated.

[b]) Regeneration (%) was calculated by dividing the number of colonies obtained by the total number of protoplasts plated (determined by counting in a haemocytometer). Correction was made for the presence of non-osmotically sensitive cells (undegraded hyphae and sporangiospores) which represented approximately 1.5% of the total count.

Table III.
Fusion of protoplasts of auxotrophs of M. circinelloides[a]

Strains fused	Ile 4A × Met 5A	Cys 1A × Leu 7B
No. of protoplast pairs (A)	6.5×10^7	2.8×10^7
C.f.u.[b] on minimal medium (B)	2.3×10^5	1.3×10^5
C.f.u. on complete medium (C)	1.6×10^7	7.8×10^6
Fusion frequency (B/C)	1.4%	1.7%
No. heterokaryons formed per protoplast pair (B/A)	3.5×10^{-3}	4.6×10^{-3}
Regeneration frequency (C/A)	24.6%	27.8%

[a] Protoplast fusion was carried out as described in section 2.6 using the number of protoplast pairs given above.
[b] C.f.u. = colony forming units after protoplast fusion.

method of OHNUKI et al. (26). The fusion frequencies obtained (Table III) are comparable with the frequencies of 1-6% reported by
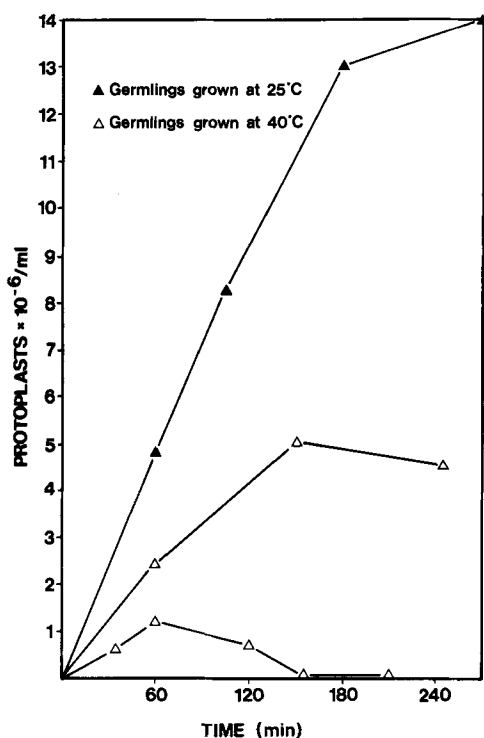

Figure 10. Effect of growth temperature on the formation of protoplasts from M. miehei CBS 182.67. Method is as described in section 2.5 using germlings grown at 40 °C, or 25 °C with a 2 hour preincubation at 40 °C. The results of two seperate experiments using germlings grown at 40 °C are presented to illustrate the variations obtained.

LASKER and BORGIA (22) using their own auxotrophs of the same strain of Mucor, but they are considerably lower than the 5-40% obtained by OHNUKI et al. (26) using auxotrophs of the thermophiles M. pusillus and M. miehei. The total number of heterokaryons formed per protoplast pair, on the other hand, are at least one order of magnitude higher for M. circinelloides (Table III) than for M. miehei and M. pusillus where the frequencies were only $0.35-2.5\times10^{-4}$ (26). Initially it seems somewhat contradictory that the thermophiles give lower total yields of heterokaryons than M. circinelloides in spite of their higher fusion frequencies. This contradiction can be explained however by their extremely low regeneration frequencies (0.017-0.096% when calculated as in Table III using the data provided in Table I of ref. 26). These protoplasts were produced from germlings grown at 37 °C and this low degree of regeneration confirms the observations presented here (section 3.3) that the thermophilic species must be grown at lower temperatures in order to produce more stable protoplasts.

3.3. Formation of protoplasts from other Mucor species: The influence of growth temperature.

Treatment of the mesophiles M. racemosus no. 50 and M. rouxii with streptozyme as described in section 2.5 resulted in the formation of protoplasts similar in yield and properties to those of M. circinelloides f. lusitanicus (section 3.2). When using the thermophilic strains M.
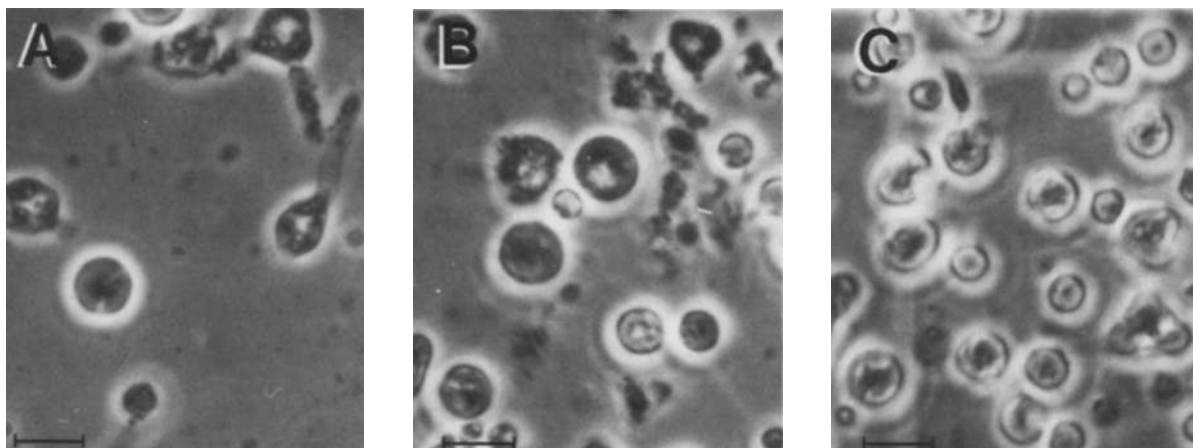
Figure 11. Protoplasts formed from M. miehei CBS 182.67 grown at 40 °C (A and B), or 25 °C with a 2 hour preincubation at 40 °C (C). (Bar = 10 μm). The photographed samples were taken from the protoplast suspensions represented in Figure 10 after 240 min of incubation; in the case of the germlings grown at 40 °C, A and B represent the lower and upper curves respectively. The protoplasts regenerated at frequencies of .003% (A), .17% (B) and 3.5% (C) (determined as described in Table II using 1.0% (w/v) agar overlays).

miehei CBS 182.67, M. miehei CBS 370.65 and M. pusillus IMI 96211 an effect of the growth temperature on the ability to form stable protoplasts was observed. Growth of the germlings at 25 °C prior to streptozyme treatment reproducibly enabled the subsequent isolation of high yields of protoplasts (Figure 10) of homogeneous morphology and with regeneration frequencies of 3-6% (Figure 11-C). When the germlings were grown at 40 °C more variable results were obtained and the final yields of protoplasts were reduced to 10^5-5×10^6 ml^{-1} (Figure 10). The stability of these protoplasts was decreased, as evidenced by the low regeneration frequencies of .003-.3%. Their morphological heterogeneity in terms of size and refractility was increased and the extensive lysis of both hyphae and protoplasts was observed by phase contrast microscopy (Figures 11-A and B). The instability of the protoplasts formed from thermophilic species grown at 37 °C is also indicated in the data of OHNUKI et al. (26) (see section 3.2.3). The deleterious effects described above were aggravated by incubation of the germlings grown at 40 °C for 10-20 min at 4 °C, prior to the incubation at 23 °C with the streptozyme solution. Incubation of cells at low nonfreezing temperatures below growth temperature can

lead to physiological dysfunction, the critical temperature for injury being determined by the gel to liquid-crystalline phase transition temperature of the membrane lipids (21, 23). Incubation at or below the phase transition temperature results in decreased membrane fluidity, altered activities of membrane bound enzymes, increased membrane permeability and eventually in the injury and death of cells and tissues (21, 23). The instability of the protoplasts from Mucor germlings grown at 40 °C might thus reflect injury caused by exposure to the lower temperature(s) of 23 °C (and 4 °C). Growth of these strains at 25 °C might produce physiological changes conferring increased resistance of the cells (and protoplasts) to damage at these temperatures. A decrease in growth temperature is known to be paralleled by a decrease in membrane lipid phase transition temperatures in many different cell types (1, 25, 29, 40) possibly due to the adjustment of the degree of unsaturation of the fatty acid chains of the membrane phospholipids. Decreased growth temperatures have been shown to result in a decrease in the molar ratio of saturated: unsaturated fatty acids in the same cell types (1, 25, 29, 32) and in the thermophilic Mucor species M. miehei and M. pusillus (39).

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