Revista Iberoamericana de Micología 1992; 9: 31-34

Original

Factors affecting isolation and regeneration of protoplasts from *Trichophyton mentagrophytes*

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Protoplasts from *Trichophyton mentagrophytes* mycelium were produced using the commercially available enzyme Novozym 234. In order to establish optimal conditions, different parameters involved in protoplasts isolation were analyzed and protoplasts production was notably increased. Best results were achieved with 0.7 M KCl as osmotic stabilizer in phosphate buffer 50 mM at pH 5.8. For the purification of protoplasts, techniques based on both centrifugation and filtration were assayed. Also regeneration of protoplasts was studied on different media and osmotic stabilizers. The protoplasts produced with this method lacked a cell wall, and could also be genetically transformed.

Key words: Trichophyton mentagrophytes, protoplasts, isolation, regeneration, dermatophytes.

RESUMEN

Factores que afectan al aislamiento y regeneración de protoplastos de *Trichophyton mentagrophytes*

Se han obtenido protoplastos de Trichophyton *mentagrophytes* mediante el empleo de Novozym 234. Se han analizado diferentes parámetros para establecer las condiciones óptimas y aumentar la producción de protoplastos. Los mejores resultados se obtuvieron con 0.7 M KCl como estabilizante en tampón fosfato 50 mM a pH 5.8. Se han utilizado técnicas de centrifugación y filtración para purificar los protoplastos, y también se ha estudiado su regeneración en distintos medios y estabilizantes osmóticos. Los protoplastos obtenidos de esta forma carecían de pared celular y podían ser transformados genéticamente.

Palabras clave: *Trichophyton mentagrophytes*, protoplastos, aislamiento, regeneración, dermatofitos.

Aceptado para su publicación: 9 de Agosto de 1991

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The growing clinical importance of mycoses in immunodepressed, transplant and cancer patients makes it desirable that researchers working with fungi will have the incentive, the background, and the funding to benefit from new techniques in molecular biology.

One of the most important features in molecular biology studies is the availability of transformation systems. Fungal transformation protocols require the previous step of protoplasts isolation. In our work with transformation systems for the dermatophyte *Trichophyton mentagrophytes*, we found that published procedures using Novozym 234 as lytic system yielded only a few vacuolated protoplasts[1]. In this paper we present a study of factors affecting protoplasts isolation, purification, and regeneration in *T. mentagrophytes*. Under optimal conditions, a great number of purified protoplasts were obtained with a high regeneration rate. The final protocol provides good biological material for use in transformation experiments.

METHODS

Fungus inoculum

The clinical isolate of *T. mentagrophytes* CECT2793 (CBS 570.80) was used throughout this investigation. Microconidia of *T. mentagrophytes* were harvested from growth on solid agar tubes of SDT (40 g dextrose, 10 g mycological peptone, 20 g agar in 1 l of distilled water; pH 6.8) and inoculated into SDT liquid medium. Cultures were incubated on a rotatory shaker at 200 rpm at 28°C for 24 h.

Enzymes and chemicals

Novozym 234 was purchased from Novo Industri A/S (Denmark); 1,4- dithiothreitol (DTT), chitinase, 2 deoxy-D-glucose (2-d-G), and 2-mercaptoethanol (2-ME) were purchased from Sigma (USA), and cellulase CP from Sturge (UK). Tinopal 5BMS was kindly provided by Ciba-Geigy (Spain).

Preparation of protoplasts

Mycelium was recovered by filtration through Nytal mesh (pore size 50 µm), washed with NaCl 0.9% (w/v), pressed and weighed. The resulting wet mycelium was suspended at a concentration of 100 mg/ml in the lytic mixture (50 mM potassium phosphate buffer pH 5.8, 0.7 M KCl, Novozym 234 at 1 mg/ml), unless otherwise specified. Incubations were carried out at 100 rpm and 28°C with orbital shaking. Protoplasts numbers were periodically determined by using a Neubauer haemocytometer counting chamber. The absence of cell wall in protoplasts was checked by staining with Tinopal 5BMS, as already described[3,4]. To test the influence of the pH of the lytic system on the liberation of protoplasts, 50 mM of citrate-phosphate (pH 4.5-6.0) or

phosphate (pH 6.0-8.0) were used.
Pretreatment assays of mycelium were done in stabilized buffer (lytic mixture without Novozym 234) for 1 hour at 100 rpm and 28°C. Pretreatments were done with 10 mM 1,4-dithiothreitol (DTT), 100 mM 2-mercaptoethanol (2-ME), 0.6 mM 2 deoxy-d-Glucose (2-d-G), or preplasmolysis. In addition to these tests, mycelium was also grown in SDT with 100 µm/ml of 2-d-G, or 0.4 M NH_4Cl added. 100 mg/ml of each mycelium were incubated in lytic mixture for 150 minutes with 1 mg/ml of Novozym 234 at 28°C, 100 rpm.

Purification and regeneration of protoplasts

Protoplasts were separated from hyphal debris by filtration through a Nytal mesh (pore size 30 µm). To eliminate the lytic mixture, two systems were assayed: direct centrifugation and filtration[3]. The purified protoplasts were regenerated under different conditions in SDT or MMT (10 g dextrose, 1.5 g NH_4NO_3, 1.8 g KH_2PO_4, 0.1 g $MgSO_4$, 20 g agar per liter, pH 6.8) media, supplemented with different osmotic stabilizers.

Protoplast infectivity

To test the protoplast infectivity, the standard method of *in vitro* hair invasion was employed [5]. Protoplasts ($5x10^6$) were inoculated in 2.5 ml of different media (water, 0.2% yeast extract[5] and SDT), each one with human hair pieces with and without 1.2 M sorbitol. Microconidia were employed under the same conditions as infectivity controls[6].
For each test, data are averages of three independent experiments.

RESULTS

Protoplast isolation

Data from previous studies showed the effective liberation of protoplasts from different fungi using the commercial enzyme Novozym 234[4]. For thies reason, we tested protoplast production using different concentrations of Novozym 234. As can be seen in table I, maximum yield was obtained at 5 mg/ml for 150 minutes, but microscopical observations revealed that these protoplasts were highly vacuolated. In contrast, lower lytic enzyme concentrations (i.e. 1mg/ml) liberated non vacuolated protoplasts. Chitinase (0.2 mg/ml) or cellulase CP (1 mg/ml) were also added to the lytic mixture in order to detect any supplementing activity to Novozym. Cellulase did not increase the liberation of protoplasts. Chitinase produced the same effect as an increase in the amounts of Novozym. The addition of bovine serum albumin (BSA) to the lytic mixture had no apparent effect (data not shown).
As osmotic stabilizers, we employed isoosmotic sugars and mineral salts[7]. The highest yields were obtained with 0.7 M KCl (figure 1). When using other osmotic stabilizers, only a portion of this amount could be achieved, namely 60% for NaCl or mannitol, and 30% for sorbitol or sucrose. Protoplast liberation was influenced by the pH of the lytic system, as can be seen in figure 2. The optimum pH value was 5.8.
Mycelium was pretreated with different compounds to assess their effect on the release of protoplasts (table II). Pretreatment with 2-mercaptoethanol had no effect on protoplasts yield, except that they were more vacuolated:

Table I. Effect of Novozym 234 concentration on the production of *T. mentagrophytes* protoplasts. Data expressed in protoplasts x 10^6/ml.

Time (min)	Novozym 234 concentration (mg/ml)				
	0.1	0.5	1.0	2.5	5.0
0	0.0	0.0	0.0	0.0	0.0
30	0.0	2.2	9.4	37.0	50.0
90	0.3	11.0	31.0	34.0	90.0
150	0.6	11.0	35.0	79.0	180.0
210	0.8	13.0	23.0	80.0	100.0

Table II. Effect of different pretreatments on the production of *T. mentagrophytes* protoplasts. Data expressed in protoplasts x 10^6/ml.

Mycelium pretreatment	
Control	26.0
Preplasm.	6.7
DTT	10.0
2-ME	24.0
2-d-G	9.4

SDT additives	
2-d-G	22.0
NH_4Cl	4.5

DTT = 1,4-dithiotheirol
2-ME = 2-mercaptoethanol
2-d-G =2 deoxy-d-Glucose
Preplasm.= preplasmolysis

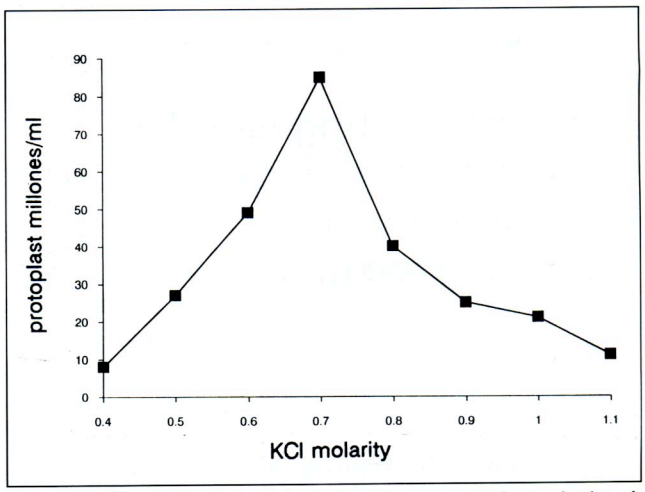

*Figure 1. Effect of KCl concentration in the lytic mixture on the production of protoplasts of **T. mentagrophytes** (100 mg/ml) was incubated in the lytic mixture as described under materials and methods for 2 h 30 min.*

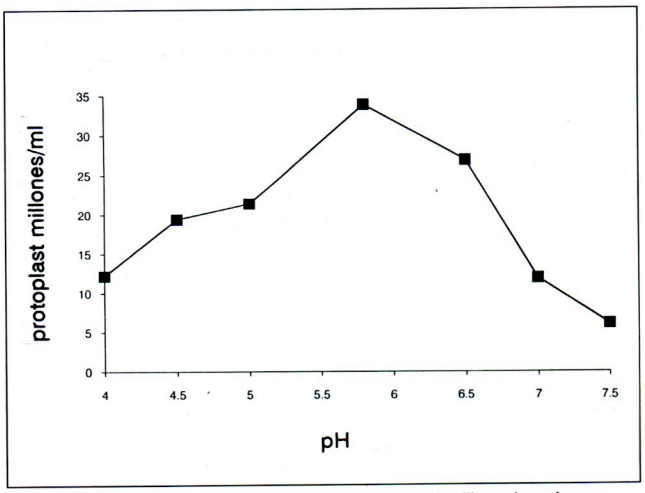

*Figure 2. Influence of the pH of the lytic system on the liberation of **T. mentagrophytes** protoplasts. Mycelium (100 mg/ml) was incubated in the lytic mixture as described under materials and methods for 2 h 30 min.*

1,4-dithiothreitol, 2 deoxy-D- glucose, or preplasmolysis reduced their size and rate of production. We also tested the influence of the addition of 100 µg/ml 2 deoxy-D-glucose or 0.4 M NH_4Cl to the SDT growth medium on the liberation of protoplasts[8]. As can be observed in table II, the mycelium grown with supplemented SDT medium yielded fewer protoplasts than the control.

The influence of the mycelium concentration in the lytic mixture was also investigated. A maximum of protoplast production was obtained at 200 mg/ml of mycelium. Lower concentrations produced fewer protoplasts, although the yield per gram of mycelium was higher at 50 mg/ml. Mycelium concentrations greater than 200 mg/ml decreased the production rates (at 1000 mg/ml only 10% of the number of protoplasts obtained at 200 mg/ml were liberated).

Factors affecting protoplasts purification

Hyphal residues and lytic enzymes must be removed from the protoplasts suspension prior to regeneration. The mycelial debris can be easily separated in *T. mentagrophytes* by a simple filtration through a 30 µm pore size Nytal mesh. To remove the lytic enzymes, two techniques were assayed: filtration and centrifugation. For the first, two types of filter were used, i.e. Sartorius (fibers of cellulose acetate), and Nuclepore (polycarbonate membranes with flat surface), both at two pore sizes (0.22 µm and 0.45 µm). The best filter system was Sartorius 0.45 µm pore size. Resuspension of the filters in the medium to liberate trapped protoplasts increased the final yields 3 to 5 times in all cases. By using this technique, up to 60% of purified protoplasts could be recovered.

The most satisfactory technique to remove the lytic enzymes was by centrifugation. Protoplasts were centrifuged for 5 minutes at different speeds, ranging from 500 to 2500 rpm. The best purification was obtained at 2500 rpm (1075 *g*), allowing about 90% of the protoplasts to be recovered after 3 washes. Centrifugation was chosen as the most efficient procedure to recover protoplasts from *T. mentagrophytes*.

Factors affecting protoplasts regeneration

Data about the regeneration of *T. mentagrophytes* protoplasts have not been previously reported. There are published other protocols for the isolation of protoplasts from this organism1,2, but when we used them the regeneration rates were low, as these protoplasts were vacuolated and evidently degenerated.

There are several variables that could affect the regeneration rate. First, we decided to choose the best osmotic stabilizer. Protoplasts were therefore inoculated in SDT solid medium supplemented with different osmotic stabilizers. Best results were obtained with the use of sorbitol or sucrose (up to 50% regeneration). Mannitol provided lower frequencies (10%), whereas no regeneration was recorded when either 0.7 M KCl or NaCl were used. To test if this was due to the physiological conditions of the protoplast, or was inherent to the *T. mentagrophytes* strain, we inoculated microconidia onto SDT plates and SDT plates supplemented with either 0.7 M NaCl or KCl. Even with $2x10^4$ viable microconidia per plate, there was no growth on NaCl or KCl plates. Thus we may conclude that this ionic strength must be too high to allow for the growth of *T. mentagrophytes* CECT2793.

Regeneration was also tested in both SDT and MMT media. Excepting the longer incubation times required for MMT, no significant differences were found. However, had an important effect on the regeneration, with a maximum between pHs 4.5 to 6.5. Agar concentration was also important with a regeneration optimum at 1% (w/v). There was still a 20% regeneration without agar, and even 40% of the protoplasts regenerated at 3% (w/v).

Protoplast infectivity

Microconidia or arthroconidia of *T. mentagrophytes* are the hair invasive phases for this organism[6]. It could thus be supposed that protoplasts would not be infective. To test this hypothesis, protoplasts or microconidia were inoculated in water, yeast extract or SDT, each one with sterile hair pieces, with or without 1.2 M sorbitol. Microconidia were always invasive, even in water, and the presence of sorbitol did not alter their infectivity. Protoplasts were not invasive even if they could regenerate, as is indeed the case in yeast extract or SDT media when supplemented with sorbitol.

DISCUSSION

At our laboratory Novozym 234 has demonstrated to be a very efficient lytic mixture for the isolation of fungal protoplasts, and also with *T. mentagrophytes*. We have found that the addition of chitinase or cellulase did not improve the frequencies obtained for this organism, and BSA had no apparent effect on the isolation of protoplasts. As osmotic stabilizer the best system was 0.7 M KCl, and the optimum pH was 5.8. Mycelium pretreatments did not increase yields. The weight of these factors depend on the species, and must be checked for each system. We have tested those factors for *T. mentagrophytes*, and our data improve results obtained by other researchers[1,2]. Scott et al.[2] obtained very few protoplasts, maybe due to the utilization of old mycelium for the preparation of protoplasts. The method of Srikantha and Ramananda Rao[1] liberated more protoplasts, but they were vacuolated. This is because in this case the osmotic stabilizer was $MgSO_4$, which provides protoplasts with big vacuoles.

Protoplasts provided by those systems[1,2] were almost unable to regenerate in our hands. This is a consequence of the degeneration of the protoplasts, which is also evident because of the high vacuolization observed with the microscope. Although there is a good yield of protoplasts with KCl as osmotic stabilizer, we could not observe regeneration with 0.7 M NaCl or KCl in the plates. As microconidia of *T. mentagrophytes* are not able to develop under these conditions, the ionic strength must be also restricting the regeneration of protoplasts. Sugars (i.e. sorbitol or sucrose) provided best results in regeneration, and parameters such as regeneration medium, pH value, and agar concentration in the plates have been optimized.

Besides, the absence of infectivity of these protoplasts has been tested, even if regeneration to mycelium is produced. It is described that microconidia or arthroconidia are the hair invasive phases of *T. mentagrophytes*[6]. So other structures, as regenerated protoplasts, must not be invasive in these conditions.

In summary, the present study shows results that improve the liberation, purification, and regeneration of high yields of protoplasts from *T. mentagrophytes*, which can be used in transformation experiments[9].

ACKNOWLEDGEMENTS

We thank Ciba-Geigy (Spain) for providing us a sample of Tinopal 5BMS.

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NOMENCLATURA DE LAS ENFERMEDADES FUNGICAS (segunda parte)

Informe y recomendaciones del Subcomité de la Sociedad Internacional de Micología Humana y Animal (SIMHA - ISHAM) 1991.
(Odds, Fc et al. J Med Vet Mycol 1992; 30:1-10)

Principios generales de la nomenclatura de las micosis.

La función del nombre de una enfermedad debería ser la de describir un estado patológico de forma concisa y exacta.
Los nombres de las enfermedades infecciosas deberían indicar tanto la patología como el organismo causal, sin embargo, la mayoría de los nombres usados para describir las infecciones fúngicas proporcionan mejor información al micólogo que al clínico. Una de las razones es la tradición de utilizar una sola palabra (ej. criptococosis, feohifomicosis, esporotricosis) que originalmente indicaba una patología específica, pero que más tarde ha debido calificarse para indicar una forma o condición especial (ej. meningitis criptococcica, feohifomicosis subcutánea, esporotricosis pulmonar, etc.)
La nomenclatura no debería ser motivo de malas interpretaciones, sin embargo en el área de la micología médica parece existir una considerable confusión como resultado, en parte, de la ausencia de nombres para definir patologías específicas y, en parte, por los cambios de nomenclaturas o por el uso de múltiples denominaciones para describir un mismo proceso.
Los nombres de las enfermedades deben ser estables. Nos encontramos en una era en la que las nuevas aproximaciones moleculares y el aumento del interés por la taxonomía producen frecuentes y substanciales cambios en el nombre de los hongos. La tradición de denominar a una micosis por su taxón causal, crea grandes problemas de inestabilidad. por ejemplo las infecciones causadas por especies clasificadas como *Pseudallescheria boydii* han sido referidas de manera variable como "allescheriasis", "petriellidiosis" y "pseudoallescheriasis"; estas diferencias suelen reflejar intentos por mantener el nombre de la micosis de acuerdo con la taxonomía fúngica del momento. El proceso de renombrar puede presentar pocas dificultades para el micólogo conocedor de los problemas taxonómicos pero, para un clínico no familiarizado con con los mismos, puede significar que asuma automáticamente que el nombre de la enfermedad se refiere a diferentes procesos específicos.
Los nombres de una sola palabra reflejan la tradición de las micosis (al igual que de muchas enfermedades microbianas) y favorecen los nombres basados en los taxones fúngicos involucrados, pero es poco claro el beneficio de este método: ¿es mejor decir "alternariosis" en lugar de "infección por *Alternaria*" cuando el nombre no explicita un proceso patológico específico? Si bien el primer modismo es más corto, el segundo recuerda que se trata de dos entidades: la patología y el agente causal.
El Subcomité recomienda, al igual que el informe de la CIOMS (1982), que un nombre aceptable de enfermedad puede ser formado nombrando la entidad patológica y añadiendo las palabras "debido a" o "causada por" el nombre del hongo causal; ello se esquematiza como "patología A debido al hongo X".
El Subcomité considera que es más útil para el clínico y el microbiólogo discutir un diagnóstico individual en términos específicos aunque sean menos concisos.
La inclusión de detalles para la patología y las especies causales, cuando se conocen, tiene una implicación sobre la terapia y el pronóstico que trascienden la necesidad de conformar una nomenclatura más general y sistemática. Una expresión como "quistes subcutáneos causados por *Aureobasidium pullulans*", por ej. es de mayor signoficación para el paciente y en una publicación que "feohifomicosis subcutánea". Pero este último término es un nombre más apropiado para cubrir todo tipo de infecciones subcutáneas causadas por un gran número de hongos dematiáceos.
La forma "genérica" del nombre debe reservarse para ser utilizada solamente en referencia a un rango más amplio de condiciones con un tema clínico común o micológico. El Subcomité opina que es mejor un nombre "genérico" de micosis para mantenr el espíritu de flexibilidad de la nomenclatura específica, siempre que sea posible. Se considera, por ejemplo, que "infección diseminada por *Fusarium*" es superior a "fusariosis diseminada" ya que evita controversias con los que consideran que "fusariomicosis" es más correcto y evita la vulnerabilidad a futuros cambios taxonómicos que afecten el agente causal.
El Subcomité recomienda que los nombres de los hongos sean usados como adjetivos en referencia a enfermedades específicas. Expresiones como "infección por *Microsporum canis*" se ajustan al uso como adjetivo del nombre del hongo y representan un excelente método de construir nuevos nombres de micosis de tipo más general sin provocar controversias ligüísticas.
Inevitablemente se ha topado con nombres genéricos de micosis. Un número escaso de los nombres existentes de micosis han sido publicados previamente sin que aparecieran detractores. El Subcomité está de acuerdo con la lista de nombres que aparecen más adelante, junto con las deficiones y comentarios que se adjuntan. La filosofía prevalente en este listado ha sido mantener los mejor conocidos, ampliamente utilizados y rechazar muchos de los más recientes neologismos micológicos. Por ejemplo, se ha rechazado el término "entoroftoromicosis" ya que puede ser cubierto en un sentido más general por "zigomiocosis" y más específicamente por "infección subcutánea por entomoftorales" o "infección nasal y paranasal por *Conidiobolus coronatus*".