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AN IMPROVED METHOD FOR THE PRODUCTION OF PROTOPLASTS OF *Podospora anserina*.

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INTRODUCTION

We pretend with this work to improve the conditions of protoplast production and regeneration of *P. anserina*, and so make easier the genetic works with this organism. Most experiences with protoplasts of *P. anserina* used natural *Trichoderma* lytic extract to get them (1). However its production and regeneration was low: about 10^7 protoplasts/g and 1-3% regeneration (2) or 10^6 protoplasts/ml and 5-15% regeneration rate (3). Prompted by these results, we have tried to increase the yield of the process, and so provide of better material for the works of fusion and transformation of protoplasts.

MATERIAL AND METHODS

ORGANISMS.

The wild s strain of *Podospora anserina* used in this work was gently provided by K. Esser. For details see (4). The *Trichoderma harzianum* CBS 354-33 strain was used to produce lytic enzymes against *P. anserina*.

MEDIA.

Podospora media. Microconidia production medium (MPM): medium according to Esser (4). Microconidia germination medium (MGM): medium according to Esser (4). Acetate minimal medium (AMM): minimal medium of Prillinger and Esser (5) with sodium acetate (10 g/l) as sole carbon source. Malt extract medium (MEM): contained malt extract 5 g, glucose 5 g, yeast extract 5 g per liter. Corn meal medium (CMM): medium acecording to Esser (4). Rabbit food medium (RFM): rabbit food commercial pellets 25 g, distilled water 1 l. Boil the rabbit food and let steep for ½ hour and filter through cheesecloth. Soluble starch medium (SSM): soluble starch 15 g, yeast extrac 4 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g per liter. Protoplast regeneration media: above media supplemented each with 0.7 M $MgSO_4$. Agar was added at 20 g/l for solid cultures.

Trichoderma medium. Medium according to Sánchez et al. (6).

BUFFERS AND STABILIZERS.

Several buffers were assayed at different pHs: Tris-HCl, Tris-maleate-NaOH, citrate-phosphate, sodium phosphate, potassium phosphate and some experiences were done with no buffer. Furthermore, as stabilizers and at different molarities were tested NaCl, KCl, $MgSO_4$, sorbitol, mannitol and saccharose. Best results were obtained with 50 mM potassium phosphate and 0.7 M $MgSO_4$ pH 6.0, and with this stabilized buffer were realized the rest of the experiences.

GROWTH OF CULTURES.

Liquid cultures were done with 25 ml of medium in 250 ml Erlenmeyer flasks on a rotatory shaker (200 rpm). *P. anserina* mycelium was propagated on solid agar plates of MPM. Microconidia were harvested and inoculated to MGM (Fig. 1), and after 3 days they were transferred to AMM for 2 days, Then the mycelial pellets were grinded with a Potter and hyphal fragments were transferred to MEM flasks for 2 days, if not otherwise specified. All cultures were at 27°C.

PREPARATION OF PROTOPLASTS.

Mycelia from the various shake flask cultures were recovered aseptically by filtration, washed three times with osmotic stabilizer and resuspended in stabilized buffer containing the lytic mixture. Incubations were done at 27°C with gentle shaking, and protoplast number was determined by using a Neubauer counting chamber.

When assayed, pretreatment of mycelium with a thiol compound prior to digestion with lytic enzymes was realized with 0.01 M dithiothreitol or 0.7 % mercaptoethanol in stabilized buffer for 1 hour.

REGENERATION OF PROTOPLASTS.

Protoplasts were purified through a fritted glass filter to remove the hyphal fragments, and were washed twice by centrifugation in osmotic stabilizer. Then they were regenerated in osmotically stabilized (0.7 M $MgSO_4$) solid and liquid media.

ENZYMES AND CHEMICALS.

Novozym 234 was kindly provided by Novo Industri A/S, Denmark. Chitinase and Chitin were purchased from Sigma, USA. Cytohelicase was obtained from L'Industrie Biologique, France. Cellulase CP was from Sturge, UK. Cellulase Onozuka R-10 was purchased from Yakult, Japan.

RESULTS AND DISCUSSION

At first we attempted to choose the best enzymatic system for the production of protoplasts. We tried some lytic mixtures, and results can be seen in Tab. 1 (not all the combinations are presented). *Trichoderma* extract obtained by us provides low number of protoplasts, similar to (2). Furthermore Cytohelicase behaves in a similar way, as spected (3). However, Novozym 234 has been the most effective, although combinated with other enzymes can improve on this result (anyway Novozym 234 alone is cheap and suitable enough for the production of protoplasts). The optimal concentration of Novozym 234 is 10 mg/ml for 5 hours of incubation, or 20 mg/ml for 3 hours as presented in Fig. 3.

Potassium phosphate 50 mM was the most effective buffer; others gave lower numbers of protoplasts. Experiences with no buffer produced more protoplasts, but smaller and not as viables.

And for this buffer and lytic system, best pH is 6.0, as can be observed in Fig. 4.

As osmotic stabilizers we have employed mineral salts and sugars (Tab. 2). It can be clearly seen that highest yields are obtained with $MgSO_4$, and protoplasts liberated with it are the biggets (Fig. 2). The optimal concentration of the stabilizer is 0.7 M (Fig. 6).

P. anserina becames senescent with age of mycelium, and forms a melaninic pigment which can affect the liberation of protoplasts. So we have tried to work with juvenile mycelium, and we have studied the influence of the growth medium on the production of protoplasts. Conditions are described in 'Material and methods', and results are presented in Fig. 5. Under these circumstances none of the media became the mycelium senescent. Best results were obtained with MEM ($3x10^9$ protoplasts/g and 20% regeneration rate), whereas protoplasts obtained with CMM or RFM were smaller and presented a very low regeneration rate -data according with (2). Mycelia grown on other media provided less protoplasts but in good conditions.

The number of protoplasts/ml can be increased with the treated amount of mycelium (Fig. 7), but the relationship number of protoplasts/g of mycelium remains unchangeable.

Pretreatment of the mycelium with dithiothreitol had no effect on protoplast yields, and mercaptoethanol even reduced its production sometimes.

As a conclusion, best system was Novozym 234 20 mg/ml in potassium phosphate buffer 50 mM, 0.7 M $MgSO_4$, pH 6.0 for 3 hours and with mycelium grown on MEM for 2 days. With these conditions, we have increased the production in 150 to 1500 times with regard to above references.

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Abstract: In this work we describe the conditions to produce good yields of protoplasts of *P. anserina*, obtaining up to $3x10^9$ protoplasts per gram of mycelium, with a regeneration rate of about 20%. Method consists in the use of juvenile mycelium grown on malt extract medium for 2 days, potassium phosphate 50 mM as buffer, 0.7 M $MgSO_4$ as stabilizer, pH 6.0 and Novozym 234 20 mg/ml as lytic system.

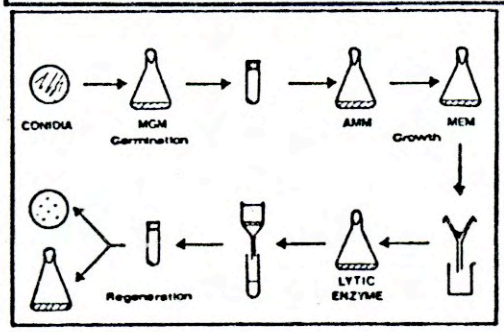

Figure 1. Diagram of the mycelial growth, preparation and regeneration of protoplasts of *P. anserina*. For abbreviations see Fig. 5.

ENZYME SYSTEM	TIME (hours) 1	2	3	4	5
Cytohelicase 20 mg/ml	$1.0x10^5$	$2.5x10^5$	$5.0x10^5$	$7.5x10^5$	$7.5x10^5$
Novozym 20 mg/ml	$3.4x10^7$	$4.7x10^7$	$5.2x10^7$	$4.7x10^7$	$4.7x10^7$
Novozym 10 mg/ml + Cellulase Onozuka 10 mg/ml	$2.5x10^7$	$4.0x10^7$	$6.5x10^7$	$6.7x10^7$	$6.2x10^7$
Novozym 10 mg/ml + Cellulase CP 10 mg/ml	$1.5x10^7$	$2.6x10^7$	$5.1x10^7$	$6.5x10^7$	$5.2x10^7$
Novozym 18 mg/ml + Chitinase 2 mg/ml	$4.5x10^4$	$5.2x10^5$	$6.5x10^5$	$6.6x10^5$	$6.8x10^5$
Trichoderma extract	$5.0x10^6$	$2.0x10^6$	$2.5x10^7$	$3.0x10^7$	$3.0x10^7$
Trichoderma extract + Novozym 10 mg/ml	$5.0x10^6$	$7.0x10^6$	$1.2x10^7$	$1.6x10^7$	$2.0x10^7$

Table 1. Comparison of enzyme systems for the production (in protoplasts/ml) of protoplasts of *P. anserina*.

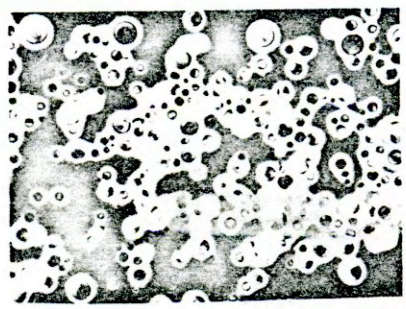

Figure 2. Protoplasts of *P. anserina* obtained under optimal conditions.

STABILIZER	Saccharose 0.58 M	$MgSO_4$ 0.7 M	Mannitol 1.1 M	NaCl 0.7 M	Sorbitol 1.1 M	KCl 0.9 M
Protoplasts/ml	$7.9x10^6$	$5.2x10^7$	$4.0x10^4$	$2.9x10^5$	$5.4x10^4$	$5.9x10^6$
Protoplasts/g	$4.5x10^8$	$3.0x10^9$	$2.3x10^6$	$1.6x10^7$	$3.1x10^6$	$3.3x10^8$

Table 2. Effect of different osmotic stabilizers on the formation of protoplasts. Only bests results for each stabilizer are presented here. Conditions were: potassium phosphate buffer 50 mM, $MgSO_4$ 0.7 M, pH 6.0 and 20 mg/ml of Novozym 234 for 3 hours.

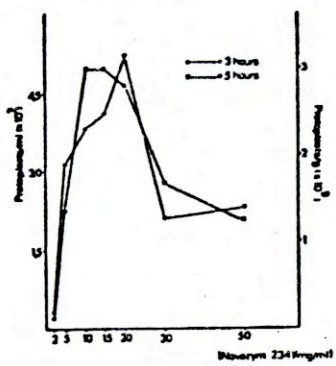

Figure 3. Effect of the concentration of Novozym 234 on the formation of protoplasts, in potassium phosphate buffer 50 mM, 0.7 M $MgSO_4$, pH 6.0.

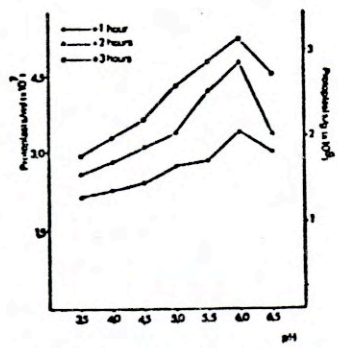

Figure 4. Influence of pH on the production of protoplasts. Solutions contained potassium phosphate buffer 50 mM, 0.7 M $MgSO_4$, Novozym 234 20 mg/ml.

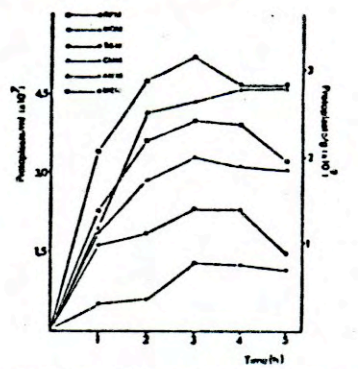

Figure 5. Influence of culture medium on the production of protoplasts, in 50 mM potassium phosphate buffer, 0.7 M $MgSO_4$, Novozym 234 20 mg/ml, pH 6.0. Media are, AMM: acetate minimal medium, CMM: corn meal-medium, MEM: malt extract medium, MGM: microconidia germination medium, RFM. rabbit food medium, SSM: soluble starch medium.

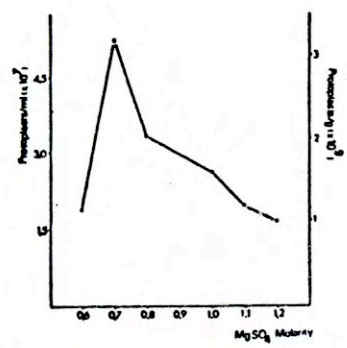

Figure 6. Effect of different concentrations of $MgSO_4$ on the production of protoplasts, using potassium phosphate buffer 50 mM, Novozym 234 20 mg/ml, pH 6.0 and incubation for 3 hours.

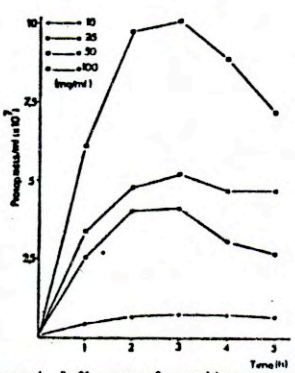

Figure 7. Influence of mycelium concentration on the liberation of protoplasts. Conditions were: potassium phosphate buffer 50 mM, 0.7 M $MgSO_4$, Novozym 234 20 mg/ml, pH 6.0.

CONCLUSIONS

We have optimized the conditions for the liberation and regeneration of protoplasts of *Podospora anserina*. Optimal conditions are:

1. The best enzymatic system is Novozym + Chitinase or Cellulase Onozuka. However, Novozym by itself provides results almost as satisfactory.
2. Optimal amounts of Novozym are 10 mg/ml for treatments of 5 hours, and 20 mg/ml for 3 hours.
3. The best pH for the production of these protoplasts is 6.0.
4. The use of $MgSO_4$ as stabilizer not only provides the highest yield, but protoplasts liberated with it are the biggest.
5. The optimal concentration of $MgSO_4$ is 0.7 M.
6. The influence of the media of growth of mycelium is very important. We have got our bests results with mycelium grown in MEM.
7. Increasing the amount of mycelium/ml, the number of protoplasts/ml sets up, but the relationship protoplasts/g of mycelium remains unchanged.

With these conditions we have enhanced the production of protoplasts in a factor of 150 to 1500 (see reference 2 of Commentary page).