Isolation and regeneration of *Penicillium brevicompactum* protoplasts

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ABSTRACT. The isolation and regeneration of fungal protoplasts is a key step for the establishment of transformation systems, electrophoretic karyotype analysis and fusion between strains, all techniques of broad application on improvement programs of filamentous fungi. To establish conditions for the isolation and regeneration of *Penicillium brevicompactum* protoplasts, that is an excellent pectinase producer, different lytic enzymes and osmotic stabilizers were tested. *P. brevicompactum* protoplasts were obtained at a larger scale when their mycelium was digested with 15mg.mL^{-1} of Glucanex (Novo Nordisk) and 0.8 mol.L^{-1} NaCl as osmotic stabilizer. The best osmotic stabilizer for the regeneration of *P. brevicompactum* protoplasts was 0.8 mol.L^{-1} KCl, with a regeneration frequency of 36.58%. This protocol can be applied in genetic analysis of this *Penicillium* species which, to date has been poorly characterized,.

Key words: *Penicillium brevicompactum*, pectinases; protoplasts.

RESUMO. Isolamento e regeneração de protoplastos de *Penicillium brevicompactum*. O isolamento e regeneração de protoplastos de fungos é um passo fundamental para o estabelecimento de sistemas de transformação, análise do cariótipo molecular e fusão entre linhagens, que são técnicas de ampla aplicação em programas de melhoramento para fungos filamentosos. Neste trabalho foram testados diferentes preparações enzimáticas e estabilizadores osmóticos para estabelecer condições otimizadas de isolamento e regeneração de protoplastos de *Penicillium brevicompactum*, que é um excelente produtor de pectinases. Protoplastos de *P. brevicompactum* foram obtidos em maior quantidade quando o micélio foi digerido com 15mg.mL^{-1} de Glucanex (Novo Nordisk) em NaCl 0,8 mol.L^{-1} como estabilizador osmótico. O melhor estabilizador osmótico para a regeneração dos protoplastos foi KCl 0.8 mol.L^{-1} apresentando uma freqüência de regeneração de 36,58%. Esse protocolo pode ser utilizado em análises genéticas para essa espécie de *Penicillium* cujos estudos têm sido pouco reportados.

Palavras-chave: *Penicillium*, pectinases, protoplastos.

Introduction

Protoplasts are, by definition, all the components of a live cell after experimental removal of the cell wall (Hawksworth *et al.*, 1995). The protoplasts can be obtained in filamentous fungi from the mycelium, germinated conidia or intact conidia, although the protoplastization is more complicated in the last case. For every fungus, enzymatic preparation, osmotic stabilizer and their concentrations must be assayed in order to establish ideal conditions for protoplast preservation, without internal material loss (Azevedo, 1998). They find broad application, based on the concept that they are physiologically normal, preserving all properties of the cell from which they were derived. Protoplasts are important for studies of cell wall synthesis, secretive processes, and biosyntheses of secondary metabolism products. Their direct applications in genetics are protoplast fusion, transformation, and electrophoretic karyotype analysis (Azevedo, 1998).

Protoplast fusion has been used for the improvement of enzyme producing strains, such as cellulases (Furlaneto and Pizzirani-Kleiner, 1992), β-glycosidases (Hoh *et al.*, 1992), pectinases (Solís *et al.*, 1997), amylases (Rubinder *et al.*, 2000) and lipase (Sawicka-Zukowska *et al.*, 2004), penicillin producing strains (Tahoun, 1993), citric acid producers (Kirimura *et al.*, 1988; Martinková *et al.*, 1990), and in fungi of agricultural interest (Viaud *et al.*, 1998; Ogawa *et al.*, 2000).

For transformation protocols, protoplasts are essential in a technique that uses polyethylene glycol (PEG). Although the transformation of fungi can be carried out through techniques like biolistic (Bartsch *et al.*, 2002), or *Agrobacterium*-mediated (Rolland *et al.*, 2003), where protoplast isolation is not necessary,

these techniques require costly equipments. A number of papers reports the fungal transformation using the PEG technique, as described to *Neurospora crassa*, *Aspergillus nidulans*, *Podospora anserina*, and *Coprinus lagopus*, among others (for a review see Fincham, 1989).

The karyotype of several fungi species has been determined by pulsed-field gel electrophoresis, requiring great protoplast quantities to prepare the agarose inclusions employed by this technique. This allowed the electrophoretic karyotype determination of species such as *Aspergillus nidulans* (Brody and Carbon, 1989), *P. janthinellum* (Kayser and Schulz, 1991), *P. notatum*, *P. chrysogenum* (Fierro *et al.*, 1993), *Agaricus bisporus* (Sonnenberg *et al.*, 1996), *P. marneffei* (Shaoxi *et al.*, 1996), *P. paxilli* (Young *et al.*, 1998), *Pleurotus ostreatus* (Larraya *et al.*, 1999), *P. nalgiovense* (Färber and Geisen, 2000), *A. nidulans* strains with chromosome duplications (Queiroz *et al.*, 2000), and *Crinipellis perniciosa* (Rincones *et al.*, 2003).

Pereira *et al.* (2002) analyzed different species of *Penicillium* for pectinase production and it was detected that *P. brevicompactum* was the best pectin lyase producer. As *P. brevicompactum* have been poorly studied yet, the establishment of a protocol for isolation and regeneration of protoplasts is an important step to introduce this species in a program for pectinase production improvement. For this reason, the aim of this work is to describe a protocol for isolation and regeneration of *P. brevicompactum* protoplasts.

Material and methods

Microorganism and culture medium

The *Penicillium brevicompactum* used in this study was obtained from the Fundação Tropical de Pesquisa e Tecnologia "André Tosello" (Campinas/SP, Brazil) under the registration number CCT 4457. The culture medium used was Potato Dextrose Agar (PDA) with the addition of 2.0g peptone, 1.5g hydrolyzed casein, 2.0g yeast extract, and 1.0mL vitamin solution (0.2mg biotin, 10.0mg ρ-amino benzoic acid, 50.0mg pyridoxine, 50.0mg thiamine, 100.0mg nicotinic acid, 100.0mg riboflavin, and distilled water to complete 100.0mL) in 1000mL of distilled water.

Lytic enzymes and osmotic stabilizers

Lytic enzymes of *Trichoderma harzianum* (LETh) (Sigma Chemicals Co., Toronto, Canada), Glucanex (Novo Nordisk Ferment Ltd., Dittingen, Switzerland), and cellulases were used. Solutions 0.8 mol.L^{-1} KCl, 1.2 mol.L^{-1} $MgSO_4$, 0.8 mol.L^{-1} sucrose, 0.6 mol.L^{-1} manitol, and 0.8 mol.L^{-1} NaCl were tested as osmotic stabilizers. All osmotic stabilizers were prepared in 100m mol.L^{-1} phosphate buffer (pH 5.8). Different combinations between the enzymes and stabilizers were tested, as well as the addition of bovine serum albumin (BSA) (Table 1).

Protoplast isolation

P. brevicompactum conidia were inoculated on Petri dishes containing PDA medium, covered with cellophane paper and incubated up to 24 hours at 25ºC. The mycelium was removed and washed twice in the tested osmotic stabilizer. For the protoplastization, approximately 300mg of fresh mycelium were incubated in 5.0mL tested osmotic stabilizer, containing a combination of lytic enzymes and, in some cases, the addition of bovine serum albumin (BSA) (Table 1) for 3 hours, under agitation (80 rpm), at 30ºC. The protoplasts were separated from the undigested mycelium by filtration and washed twice in the tested osmotic stabilizer by centrifugation at 3000g, during 15 minutes, at 4ºC. After this, the pellet was resuspended and the protoplast number of each treatment determined in a Neubauer chamber.

Protoplast regeneration

The protoplast suspension was diluted and 1 x 10^3 protoplasts were plated in PDA containing different stabilizers for an evaluation of their influence on protoplast regeneration. The stabilizers used in the PDA medium were 0.6 and 1.0 mol.L^{-1} manitol, 0.5 and 1.0 mol.L^{-1} sorbitol, 0.8 and 1.0 mol.L^{-1} sucrose, 0.8 mol.L^{-1} NaCl, and 0.8 mol.L^{-1} KCl. The protoplasts were incubated for 2 to 3 days and the individual colonies were counted in Petri dishes. The PDA medium without osmotic stabilizer was used as control.

Results and discussion

Protoplast isolation

To date, no protocol was known to obtain *P. brevicompactum* protoplasts, since the conditions under which protoplasts for other *Penicillium* species were obtained showed no satisfactory results for *P. brevicompactum*. There are important factors that influence the isolation and regeneration of protoplasts like microorganisms, lytic enzyme preparation, osmotic stabilizers, and others (Solís *et al.*, 1996).

This study established conditions for the isolation and regeneration of *P. brevicompactum* protoplasts (Table 1). Glucanex (15mg.mL^{-1}) was the enzyme preparation which allowed the isolation of the highest protoplasts number when 0.8 mol.L^{-1} NaCl was used as osmotic stabilizer, which released 5.75 x 10^7 protoplasts/mL of lytic solution. Glucanex was less efficient when combined with cellulases. Glucanex together with Novozym and 0.6 mol.L^{-1} KCl as osmotic stabilizer was the best combination for the isolation of *Pseudozyma flocculosa* protoplasts

(Cheng and Bélanger, 2000). Hydrolytic enzymes play a key role in the isolation of protoplasts and are currently available in several commercial types. The lytic enzyme of *T. harzanium* is being used for protoplastization of different *Penicillium* species, but was not efficient for the isolation of *P. brevicompactum* protoplasts. These results differ from the ones obtained by Solís *et al.* (1996). These authors reported a maximum release of *Aspergillus* sp. protoplasts when they used the lytic enzyme of *T. harzanium* with KCl or $MgSO_4$ as osmotic stabilizer, both concentrated at 0.7 mol.L^{-1}, and mentioned the need to add BSA to the digestion mixture to neutralize the proteolytic activity in the enzyme preparation. This same enzyme preparation was also most efficient to release protoplasts of *Lentinus lepideus* with 0.6 mol.L^{-1} sucrose as osmotic stabilizer (Kim *et al.*, 2000).

Table 1. Influence of lytic enzymes and osmotic stabilizer in the isolation of *P. brevicompactum* protoplasts.

Culture Medium	Lytic Preparation*	Preparation Concentration (mg.mL^{-1})	Osmotic Stabilizer (mol.L^{-1})	BSA (mg.mL^{-1})	Number of Protoplasts (10^6 x mL^{-1})
PDA	Glucanex	15.0 + 10.0	0.8 KCl	–	0.38
	+		1.2 $MgSO_4$	–	0.00
	cellulase		0.6 Manitol	–	0.00
			0.8 NaCl	–	4.10
			0.8 Sucrose	–	0.94
	Glucanex	15.0	0.8 KCl	–	1.04
			1.2 $MgSO_4$	–	0.73
			0.6 Manitol	–	0.03
			0.8 NaCl	–	57.50
			0.8 Sucrose	–	0.95
	Glucanex	15.0	0.8 NaCl	5.0	58.20
	Glucanex	10.0	0.8 NaCl	5.0	6.65
				–	6.48
	Glucanex	5.0	0.8 NaCl	5.0	3.81
				–	3.43
	LETh	5.0	0.8 NaCl	5.0	3.28
				–	2.53
	LETh	10.0	0.8 NaCl	5.0	5.51
				–	4.48
	LETh	15.0	0.8 NaCl	5.0	5.33
				–	4.71

*The mycelium (300mg) was incubated in 100 mM phosphate buffer (pH 5.8) containing different lytic enzymes and osmotic stabilizers at 30ºC and 80 rpm for 3 hours.

When the lytic preparation Glucanex was used for the release of *P. brevicompactum* protoplasts, the protoplasts were obtained and kept intact without the need of BSA addition to the digestion mixture. BSA addition did not increase significantly the number of protoplasts and, therefore, was not a main requirement (Table 1).

The osmotic stabilizer is an important aspect for an efficient protocol for protoplast isolation. Since the cell wall composition varies according to the species, it is safe to assume that there must be an optimum osmotic concentration for each species (Cheng and Bélanger, 2000), although Solís *et al.* (1996) did not report significant variation at testing KCl, $MgSO_4$, and NH_4Cl (0.7 mol.L^{-1}) for the release of *Aspergillus flavipes* protoplasts.

The best stabilizer for protoplasts isolation of *P. brevicompactum* was 0.8 mol.L^{-1} NaCl (Table 1), results that disagree with Dias *et al.* (1997), who established that the best conditions for isolation of *P. expansum* and *P. griseoroseum* protoplasts was when 0.37 mol.L^{-1} KCl was the osmotic stabilize, obtaining larger protoplasts liberation. In another *Penicillium* species for which the electrophoretic karyotype was described, the lytic enzymes, as well as the osmotic stabilizers or their concentrations, also differed. For example: for *P. janthinellum,* the lytic enzyme used was Novozym 234 and the osmotic stabilizer was 0.7 mol.L-1 NaCl (Kayser and Schulz, 1991), for *P. chrysogenum* and *P. notatum* the enzyme was Novozym 234 and 0.7 mol.L^{-1}KCl was the stabilizer (Fierro *et al.*, 1993), for *P. paxilli* it was used Novozym 234 and 1.2 mol. L^{-1} $MgSO_4$ (Itoh *et al.*, 1994), for *P. marneffei* Novozym 234 was the best enzyme (Shaoxi *et al.*, 1996) and for *P. nalgiovense*, lysing enzyme from *T. harzianum* and β-glucuronidase were used with and 0.8 mol.L^{-1} KCl was the best osmotic stabilizer (Färber and Geisen, 2000).

Protoplasts regeneration

The protoplasts regeneration serves as a system model for the study of several cellular processes and in fungal cells it is essential for experiments of genetic manipulation (Kim *et al.*, 2000). The osmotic stabilizer used for the protoplasts regeneration exercises great influence in the regeneration frequency, because it can totally inhibit it. Also, it differs among the species (Solís *et al.*, 1996; Kim *et al.*, 2000).

In this work, several osmotic stabilizers were tested (Figure 1), being verified that the frequency of *P. brevicompactum* protoplasts regeneration was higher (36.58%) when 0.8 mol.L^{-1} KCl was used. That frequency is considered high when compared to the largest frequency obtained by Kim *et al.* (2000) for *Lentinus lepideus*, that observed 3.28% of regeneration using 0.6 mol.L^{-1} sucrose. These same authors verified that, as KCl, NaCl inhibited the protoplasts regeneration. The sugars are normally used as stabilizers for the protoplasts regeneration, being the sucrose used to regenerate protoplasts of *A. niger* (Martinková *et al.*, 1990; Hoh *et al.*, 1992), *P. expansum*, *P. griseoroseum* (Dias *et al.*, 1997), *Beauveria bassiana*, *B. sulfurescens* (Viaud *et al.*, 1998), *Thermomyces lanuginosus* (Rubinder *et al.*, 2000), among others.

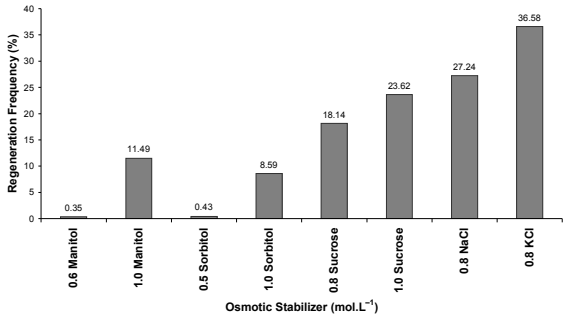

Figure 1. Regeneration frequency of the *P. brevicompactum* protoplats in different osmotic stabilizers. The protoplasts were regenerated in PDA medium at 25ºC.

Conclusion

An efficient protocol for isolation and regeneration of *P. brevicompactum* protoplasts was established, differing from the conditions that usually work for other *Penicillium* species. Approximately 5.75 x 10^7 protoplasts/mL were obtained using 15mg.mL^{-1} of Glucanex as enzymatic preparation and 0.8 mol.mL^{-1} NaCl as osmotic stabilizer, and 36.58% of regeneration frequency was obtained using 0.8 mol.L^{-1} KCl as osmotic stabilizer. This protoplastization protocol is a fundamental step for the genetic manipulation of this species. These conditions can be applied for experiments of protoplast fusion, development of a transformation system and determination of the electrophoretic karyotype of *P. brevicompactum*.

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