Mycoscience (2010) 51:84–87
DOI 10.1007/s10267-009-0004-4

NOTE

Evaluation of properties of several cheese-ripening fungi for potential biotechnological applications

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Received: 15 April 2009 / Accepted: 28 July 2009 / Published online: 19 December 2009

Abstract Strains of *Penicillium camemberti* and *Penicillium roqueforti* were tested for properties that could be important for future biotechnological applications of these fungi. *Penicillium camemberti* CECT 2267 and *P. roqueforti* NRRL 849 showed the most promising performances in terms of growth, protoplast production, and protoplast regeneration abilities. Transformation of these strains with a plasmid containing gene encoding phleomycin resistance showed that they also have a high transformation frequency. In addition, both strains showed low extracellular proteolytic activity. Thus, these strains have all the characteristics to make them suitable for future genetic improvement, recombinant protein production, and other potential biotechnological applications.

Keywords *Penicillium camemberti* · *Penicillium roqueforti* · Proteolytic activity · Protoplast regeneration · Transformation

For genetics and biotechnological applications of fungi, some specific conditions are necessary, mainly the ability to produce protoplasts, their suitable regeneration in stabilized osmotic media, and their potential for transformation (Peberdy 1979; Fincham 1989; Cheng and Bélanger 2000). Suitable performance in these aspects is important for applications such as electrophoretic karyotyping, protoplast fusion, and genetic improvement using genetic engineering tools. In addition, for applications such as recombinant protein production, a low level of extracellular proteolytic activity is desirable (van den Hombergh et al. 1997). It has been demonstrated that important differences in these properties exist among fungal strains (de Vries et al. 2004), so they must be analyzed case by case to select the most suitable strains.

Penicillium camemberti and *P. roqueforti* are filamentous fungi that are important to the food industry because they are responsible in large measure for flavor in some kinds of cheese, such as Camembert and Brie (*P. camemberti*) and blue-veined cheeses (*P. roqueforti*). However, suitable genetic tools have not been developed for these organisms, thus hindering improvement of their positive features. On the other hand, the mycelia of *P. camemberti* and *P. roqueforti* form part of these cheeses and have thus been eaten by people for centuries without harm to human health. Consequently, these fungi are generally regarded as safe (GRAS) organisms. Since materials produced by GRAS organisms are safe (Hjört 2003), *P. camemberti* and *P. roqueforti* could offer important advantages as potential producers of recombinant enzymes to be used as food additives or for pharmaceutical purposes.

In the work reported herein, we tested the performance of *P. camemberti* and *P. roqueforti* in terms of properties such as growth, protoplast production and regeneration, transformation ability, and extracellular proteolytic activity level. We found two strains with performance that makes them potential candidates for future genetic improvement and biotechnological applications.

The strains used in this work were *P. camemberti* CECT 2267, NRRL 876, and NRRL 877, and *P. roqueforti* NRRL

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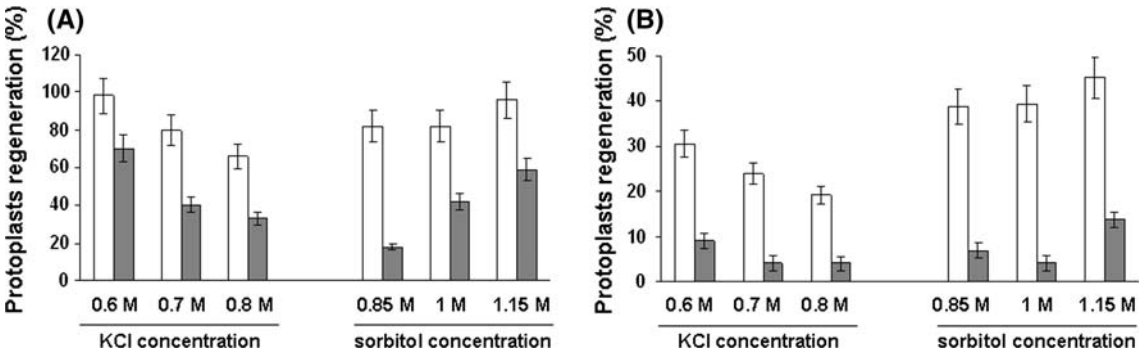

Fig. 1 **a** Percentage regeneration of protoplasts from *P. camemberti* strains CECT 2267 (*white bars*) and NRRL 877 (*gray bars*) using different concentrations of KCl and sorbitol as osmotic stabilizers. **b** The same experiment using *P. roqueforti* strains NRRL 849 (*white bars*) and NRRL 858 (*gray bars*). Protoplasts were isolated as previously described (Chávez et al. 2001), except that Lysing Enzymes (Sigma, 20 mg/ml) were used instead of Novozyme 234. From protoplast suspensions, serial dilutions were conducted and a defined number of protoplasts (100, 500, 1,000, and 1,500 protoplasts) were plated by the overlay method on Czapek agar, supplemented with different concentrations of the osmotic stabilizers KCl and sorbitol. Each protoplast dilution used was plated in triplicate. Plates were incubated at 28°C for 7 days and the number of colonies grown at this time was counted and the percentage regeneration with respect to the number of protoplasts plated was calculated and averaged. As a control, in each experiment protoplasts were plated on Czapek agar without osmotic stabilizers. Standard error is shown by *error bars*

844, NRRL 849, and NRRL 858. The first parameter analyzed was growth on seven different solid culture media: Czapek agar, Power agar (Fierro et al. 1993), trypton soy agar (Difco), potato dextrose agar (Difco), malt extract agar (Difco), Czapek yeast extract agar (CYA; Czapek agar plus yeast extract 5 g/l), and CM medium (Lombraña et al. 2004) plus 1.5% agar. In all cases, we observed that *P. camemberti* CECT 2267 and NRRL 877 grew faster than the strain NRRL 876 (data not shown). In the case of *P. roqueforti*, the strains NRRL 849 and NRRL 858 grew faster than the strain NRRL 844. In addition, spore production of each strain was analyzed on Power agar plates. By counting the average number of conidia from four plates of each strain, we observed that *P. camemberti* NRRL 876 and *P. roqueforti* NRRL 844 exhibited ten times less sporulation than other *Penicillium* strains (data not shown). At this point, strains *P. camemberti* NRRL 876 and *P. roqueforti* NRRL 844 were discarded from further experiments. With the remaining strains, other parameters such as growth in liquid media and protoplast production were measured, with all of them showing good performance; for example, protoplast production was in the range of 10^7–10^8 protoplast/mg fresh mycelia for all these strains, which are suitable values for transformation. However, even though protoplast yields were similar, when they were plated on osmotic-stabilized media for regeneration, significant differences were observed. Figure 1 shows protoplast regeneration on Czapek agar containing different concentrations of KCl or sorbitol as osmotic stabilizers. For *P. camemberti* (Fig. 1a), the strain CECT 2267 showed better regeneration ability than the strain NRRL 877 in all conditions analyzed. In fact, in some conditions (0.6 M KCl and 1.15 M sorbitol), regeneration was around 100% for the strain CECT 2267. In the case of *P. roqueforti* (Fig. 1b), protoplast regeneration was less efficient than for *P. camemberti* strains (see differences in scales in Fig. 1a, b). *P. roqueforti* NRRL 849 showed a strong regeneration ability (about 50%), but the strain NRRL 858, in almost all cases, showed less than 10% regeneration. Frequency of regeneration of fungal protoplasts is highly variable and depends on several factors (Peberdy 1979; Fincham 1989). As a result, and as we observed for *P. camemberti* and *P. roqueforti*, some fungal strains seem to be particularly more amenable to protoplast manipulation and regeneration than others (Peberdy 1979; Cheng and Bélanger 2000). Protoplasts are essential for the transformation techniques used in fungi, and yields of protoplast regeneration obtained in our study compare favorably with other *Penicillium* species for which transformations have been successful (Anné et al. 1974; Geisen et al. 1989). Thus, given that protoplast regeneration is critical for transformation procedures, we selected the strains *P. camemberti* CECT 2267 and *P. roqueforti* NRRL 849 for further experiments.

Transformation is a variable phenomenon, and transformation frequency differs from species to species (Daboussi et al. 1989; de Groot et al. 1998). Consequently, the selected strains were then subjected to transformation using p43gdh, a plasmid conferring phleomycin resistance. Table 1 shows the result of several transformation experiments. Both strains had good performance in transformation. However, although *P. camemberti* CECT 2267 produced more viable protoplasts than *P. roqueforti* NRRL 849, the latter strain produced about twice the number of transformants as *P. camemberti*, suggesting that *P. roqueforti* is more amenable to transformation. Following two passages of the transformants on

Table 1 Transformation efficiencies of *P. camemberti* and *P. roqueforti* strains using p43gdh

Strain	Transformants/μg DNA per 10^7 protoplasts
P. camemberti CECT 2267	219 ± 21
P. roqueforti NRRL 849	400 ± 45

Transformation was carried out as described by Cantoral et al. (1987), except that 20 mg/ml Lysing Enzymes (Sigma) were used. Transformants were plated on Czapek agar containing sorbitol at suitable concentration, with phleomycin at 15 μg/mL for *P. camemberti* and 5 μg/mL for *P. roqueforti*. Transformants were analyzed after 7 days of growth. The number of transformants is the average of three independent experiments

Table 2 Proteolytic activity in supernatants of *P. camemberti* CECT 2267, *P. roqueforti* NRRL 849, and control strains INBCC 300 and P20

Strain	Specific activity (U/mg total protein)
P. camemberti CECT 2267	25.3 ± 0.9
P. roqueforti NRRL 849	32.0 ± 1.2
Penicillium sp. INBCC 300	67.5 ± 2.4
P. roqueforti P20	7.1 ± 1.2

P. camemberti and *P. roqueforti* were grown in liquid CAC medium (Moralejo et al. 2002) to induce proteolytic activity. Aliquots (200–400 μl) were taken at 96 h, and proteolytic activity was measured at pH 6.0 using the azocasein method (Larsen et al. 1998). Volumetric activity (U/mL) was defined as by Larsen et al. (1998), and normalized for total protein content measured by the Bradford method (Bradford 1976), thus giving specific activity. Values are the average of three independent experiments

phleomycin-containing media, and three passages on non-selective media, most of *P. camemberti* and *P. roqueforti* transformants were mitotically stable (data not shown).

Finally, proteolytic activity of both strains was assayed. Protease activity was first assayed on plates using several different culture media containing defatted milk, hemoglobin, casein or gelatin, at several pH values, and with or without staining with amido-black to enhance proteolytic activity. In none of these cases was a proteolysis halo observed. However, a halo was observed for *Penicillium* sp. INBCC 300, a natural isolate from cured dry sausage (Laich et al. 1999), used as positive control, suggesting that protease levels in *P. camemberti* CECT 2267 and *P. roqueforti* NRRL 849 are low. This was confirmed by the quantitative determination of proteolytic activity in supernatants of these strains grown in liquid CAC medium (Table 2). At 96 h (peak of protease activity in all the strains), and as expected, *P. camemberti* CECT 2267 and *P. roqueforti* NRRL 849 showed higher levels of proteolytic activity than *P. roqueforti* strain P20, a mutant that produces very low proteolytic activity (Durand-Poussereau and Fèvre 1996), used as a negative control. However, the same strains showed about 2–2.5 times less activity than the strain *Penicillium* sp. INBCC 300. Larsen et al. (1998) described that only high extracellular protease producers, with volumetric values of activity in excess of 70 U/ml at pH 6.0, can be detected by qualitative assays. Our positive control *Penicillium* sp. INBCC 300 shows about 100 U/ml of volumetric activity, sufficient to be detected on plate assays. However, the volumetric proteolytic activity levels of *P. camemberti* CECT 2267 and *P. roqueforti* NRRL 849 were about 50–60 U/ml, low levels that are under the sensitivity of the qualitative method. Thus, these results suggest that both selected strains are mild protease producers.

In summary, *P. camemberti* CECT 2267 and *P. roqueforti* NRRL 849 have strong performance in terms of growth, protoplast regeneration, and transformation, and are mild protease producers. All these characteristics make them suitable for future applications, such as genetic improvement and recombinant protein production.

Acknowledgments This work was supported by FONDECYT Grant No. 11060003 and DICYT-USACH. We wish to thank Dr. Juan F. Martín for supplying the strains and plasmid p43gdh used in this work, and Dr. Jaime Eyzaguirre and Dr. Emilio Cardemil for critical reading of this manuscript.

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