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Determination of growth characteristics and lipolytic and proteolytic activities of *Penicillium* strains isolated from Argentinean salami

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Abstract

The growth of *Penicillium* spp. on the surface of meat-based dry fermented sausages provides them with a protective effect against some undesirable microorganisms. *Penicillium* also acts as an antioxidant, minimizes the risk of excessive drying, and it is responsible for flavor development due to the decomposition of proteins, free fatty acids and lactic acid.

With the aim of developing starter cultures, important physiological properties such as growth and proteolytic and lipolytic activities were evaluated on 13 mold strains belonging to the genera *Penicillium.* These strains were isolated from Argentinean dry fermented meat sausages named "salami". The selection was based on color, mycellium appearance and growth characteristics. The most important factors of the drying process of salami, such as temperature (14 and 25 °C), water activity (a_w) (0.90, 0.95 and 1.00) and presence of 2.5% sodium chloride (NaCl), were analyzed.

Although all strains analyzed were able to grow under the different conditions evaluated, they showed different growth velocity (K = mm/day) in response to temperature, a_w and presence of NaCl in the media. All strains showed both proteolytic and lipolytic activities under the studied factors of the drying process. Nevertheless, differences in inter-species and even intra-species were found. The addition of NaCl gave a stimulant effect to the proteolytic activity at 25 °C, but the response at 14 °C was variable. The same variability was observed in the presence of salt, both at 25 and 14 °C, when the lipolytic activity was assayed.

According to our results, detailed assays of the physiological capacities of indigenous strains proposed as starter cultures are required.

Keywords: *Penicillium*; Lipolytic activity; Proteolytic activity; Growth characteristics

1. Introduction

The possible role of *Penicillium* spp. on the drying process of fermented meat sausages, apart from the formation of the typical appearance, is multiple. Some of its functions are antioxidative effect, minimization

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of the risk of excessive drying and development of a characteristic flavor due to the decomposition of proteins, fat and lactic acid (Philipp and Pedersen, 1988). Lipases from molds which grow on the surface of these meat products increase the levels of most free fatty acids and, therefore, play a role in determining the flavor and aroma of the product (Selgas et al., 1999). To the same extent, a relationship between the sensory properties of dry fermented meat sausages and the proteolytic activity of these molds was established (García et al., 2001; Geisen, 1993). Even though there are many studies in the literature about the influence of the mold growth on flavor of sausage (Ockerman et al., 2001a,b; Hwang et al., 1993a,b; Singh and Dincho, 1994; Geisen et al., 1991), there are very few studies about proteolytic and lipolytic activities of the indigenous molds which grow on the surface of dry fermented sausages.

The presence of penicillia on the surface of these varieties of meat sausages can be considered as being desired, since several authors have reported a protective effect of some of these molds against some undesirable microorganisms (Leistner, 1990; Geisen et al., 1992; Singh and Dincho, 1994). This mold coating on raw dry sausages is considered to be an indicator of good quality and complementation of the process of ripening by many manufacturers (Singh and Dincho, 1994).

The traditional source of molds on raw dry sausages is the natural house mycoflora. This often consists of heterogeneous molds composed of representatives of different genera and species (Dinchev, 1981; Berwal and Dinchev, 1991). Many of these molds are undesirable and may lead to serious problems for both the consumer and the producer. Some of these molds are capable of producing mycotoxins (Leistner, 1984; Fink-Gremmels and Leistner, 1990; López Díaz et al., 2001) and they also present quality problems associated with color and mycelium appearance.

The requirements of quality and safety in dry meat sausages is achieved by the use of starter cultures. These molds should be well adapted to the sausage environment. This gives them an advantage in the competition with other molds (Andersen, 1995). The dominating species of the mycoflora are probably the same species that contribute most to the typical appearance, flavor and aroma. In order to develop new starter cultures the first step should be to determine which mold species prevail on fermented sausages. Besides, those cultures should be selected from the environment where they will be used.

With the aim of developing starter cultures, in previous research, we identified 45 strains isolated from the surface of regional sausages and we evaluated their morphological characteristics (Pose et al., 2003). From these strains, 32 were discarded because of their imperfect color and mycelium appearance and growth deficiencies. The remaining 13 strains, all of them corresponding to the genera *Penicillium* (9 *Penicillium nalgiovense*, 1 *Penicillium chrysogenum*, 1 *Penicillium olsonii*, 1 *Penicillium solitum*, 1 *Penicillium griseofolvum*) were subjected to characterization with respect to the suitability as starters. In this regard, technically important physiological properties were determined such as lipolytic and proteolytic activities as well as the growth characteristics.

2. Materials and methods

2.1. Growth characteristics

The effect of temperature (14 ± 1 and 25 ± 1 °C (control)), water activity (a_w) (0.90, 0.95 and 1.00 (control)) and the presence of 2.5% of sodium chloride (NaCl) on the growth of 10 *P. nalgiovense* strains were investigated. Glycerol was used to adjust the a_w of the medium, which was measured by an electric hygrometer (Decagon Aqua Lab CXT-2T).

In all experiments, Malt Extract Agar (MEA) was used as the basal medium, using 10-cm diameter plates. These were inoculated with a needle sterile from colonies developed previously and maintained at 4 °C. The diameter of colonies was measured daily (in duplicate), from the 2nd to the 7th day and was expressed in millimeters. These experiments were performed in duplicate. From the growth curves obtained, the rate of growth (K = mm/day) was calculated by a regression analysis.

2.2. Proteolytic and lipolytic activities

Thirteen strains (9 *P. nalgiovense*, 1 *P. chrysogenum*, 1 *P. olsonii*, 1 *P. solitum*, 1 *griseofolvum*) were used to evaluate their proteolytic and lipolytic activ-

ities. They were named as S1,2; S13,1; S15,3; S15,1; S15,3; S16,2; S17,3; S25,1; S25,3 (*P. nalgiovense*); S3,2 (*P. griseofolvum*); S15,1 (*P. solitum*); S14,4 (*P. chrysogenum*) and S24,1 (*P. olsonii*).

The extracellular proteases were detected on agar plates, using different substrates (Casein agar (CA) and MEA supplemented with 1% of gelatin (MEAG)) at 14 ± 1 and 25 ± 1 °C and in presence or absence of 2.5% of NaCl.

On CA, the enzyme activity was detected as clearer areas surrounding the colony, indicating that hydrolysis of the substrate had occurred since this medium gives an opaque background. Also, it was developed a method to detection proteolytic activity using MEA as basal medium supplemented with 1% of gelatin. In this case, the detection of extracellular proteases was done after staining with Coomassie Blue (0.25% w/v) in methanol–acetic acid–water (5:1:4 v/v/v) for 1 h at room temperature and destining with methanol–acetic acid (Vermelho et al., 1996). Regions of enzyme activity were detected as clear areas surrounding the colony, indicating that hydrolysis of the substrate had occurred. In all experiments, petri plates were inoculated with a needle sterile from colonies previously developed and maintained at 4 ± 1 °C and incubated to day 11. Diameter of halos was measured in millimeters.

Lipolytic activity was determined from the extracellular lipase production in a liquid medium with the following composition (g/l): NaH_2PO_4 12; KH_2PO_4 2; $MgSO_4$ 7; H_2O 0.3; $CaCl_2$ 0.25, and 1% of $(NH_4)_2SO_4$ and 2% of olive oil as a source of nitrogen and carbon, respectively (Coca et al., 2001). The assays were performed at 14 ± 1 and 25 ± 1 °C (control) and in presence or absence of 2.5% NaCl.

Enzymatic activity was evaluated by determination of free fatty acids by spectrophotometric measurements at 715 nm according to Lowry and Tinsley (1976). Conidia of colonies previously developed on MEA and maintained at 4 ± 1 °C were inoculated in Erlenmeyer flasks of 125 ml with 20 ml of sterile medium, to have a final concentration of 10^6 spores/ml in the medium. The cultures were shaken at 100 rpm for 8 days.

All enzymatic activity assays were performed in duplicate.

3. Results and discussion

In previous reports, López Díaz et al. (2002) demonstrated that temperature, water activity and sodium chloride are the most important parameters influencing the growth of *Penicillium* species in fermented dry sausages.

With respect to the effect of temperature on growth rate (K = mm/day) all strains showed similar patterns in this physiological capability. As shown in Table 1, the highest growth rate was found at 25 °C and at a_w of 0.95. When the temperature was reduced from 25

Table 1
Effect of temperature on the growth rate (K) of 10 strains of *P. nalgiovense* isolated from the surface of Argentinean fermented dry sausages

Strains/ temperature	K (mm/day)					
	a_w = 0.90		a_w = 0.95		a_w = 1.00	
	14 °C	25 °C	14 °C	25 °C	14 °C	25 °C
S1.2	1.64 ± 0.28	2.59 ± 0.13	3.82 ± 0.01	6.11 ± 0.16	2.48 ± 0.05	3.91 ± 0.22
S3.2	2.02 ± 0	2.90 ± 0.30	3.61 ± 0.01	6.65 ± 0.25	2.86 ± 0.22	3.84 ± 0.07
S13.1	1.11 ± 0.13	2.39 ± 0.01	3.81 ± 0.01	7.90 ± 0.05	1.33 ± 0.13	4 ± 0.13
S13.2	1.82 ± 0.01	ND	4.10 ± 0.15	6.07 ± 0.20	2.83 ± 0.07	3.83 ± 0.01
S15.1	1.80 ± 0.20	2.93 ± 0.28	3.64 ± 0.13	8.10 ± 0.01	2.43 ± 0.01	5.98 ± 0.26
S15.3	1.73 ± 0.13	2.39 0	3.51 ± 0.13	6.14 ± 0.02	2.48 ± 0	3.96 ± 0.01
S16.2	1.14 ± 0.22	1.53 ± 0.07	3.64 ± 0.28	7.07 ± 0.36	2.23 ± 0	2.36 ± 0.31
S17.3	1.64 ± 0.01	ND	3.72 ± 0.30	6.81 ± 0.01	2.30 ± 0.13	6.2 ± 0.42
S25.1	1.84 ± 0.07	2.60 ± 0.25	3.18 ± 0.07	5.67 ± 0	2.39 ± 0.13	3.52 ± 0.07
S25.3	1.98 ± 0.5	2.15 ± 0.16	3.81 ± 0.30	6.34 ± 0.12	2.5 ± 0.01	6.5 ± 0.12

Data are presented as mean ± S.D. of growth rates (K (mm/day)).

ND: not detected.

Table 2
Relative reduction (%) of K (mm/day) at 14 °C with respect to 25 °C

Strains[a]	$a_w = 0.90$	$a_w = 0.95$	$a_w = 1.00$
S1.2	37	37	36
S3.2	30	46	25
S13.1	53	52	67
S13.2	ND	32	26
S15.1	38	55	59
S15.3	27	43	37
S16.2	25	48	3
S17.3	ND	45	63
S25.1	29	44	32
S25.3	8	40	61

ND: not detected.
[a] *P. nalgiovense*.

to 14 °C, all the strains showed a decrease in their growth rate, at all a_w evaluated. However, it is important to point out that all the strains analyzed showed an appropriate growth rate at 14 °C and at low a_w, which are the conditions of the drying process. Table 2 shows the percentage of reduction of K. Although all the species showed a decrease in K as temperature was reduced, not all of them showed the same magnitude of decrease. These differences were most important at lower and higher a_w (0.90 and 1.00, respectively) (Tables 1 and 2).

Sodium chloride is added to the initial sausage mixture at concentration in average of 2–3% (Rust, 1994). It influences multiple reactions of the maturation and drying process because it takes part in chemical and enzymatic reactions and it reduces a_w. Besides, NaCl influences the particular flavor of the final product. According to our results, the presence of this level of NaCl has a stimulating effect on the growth of the *P. nalgiovense* strains. Other authors have found a similar stimulating effect (Godinho and Fox, 1981).

P. nalgiovense is the typical species utilized as starter culture for dry sausages in Europe. In our study, we demonstrated that not all strains of *P. nalgiovense* have a similar response against extrinsic factors, and that there is a great variability intra-species in their growth capabilities. For this reason, some strains could be more adequate than others for specific purposes.

With respect to proteolytic activity, all strains showed activity on CA at 25 °C. Seventy percent of the studied strains showed similar patterns although different capabilities were determined. In this manner, 1 strain of *P. nalgiovense* (S25-3) and *P. olsonii* were able to double the proteolityc activity of the other strains. In addition, we were able to determine that one strain of *P. nalgiovense* (S15-1) and *P. chrysogenum* showed a proteolytic activity six and seven times greater than the former species, respectively. At 14 °C all strains showed

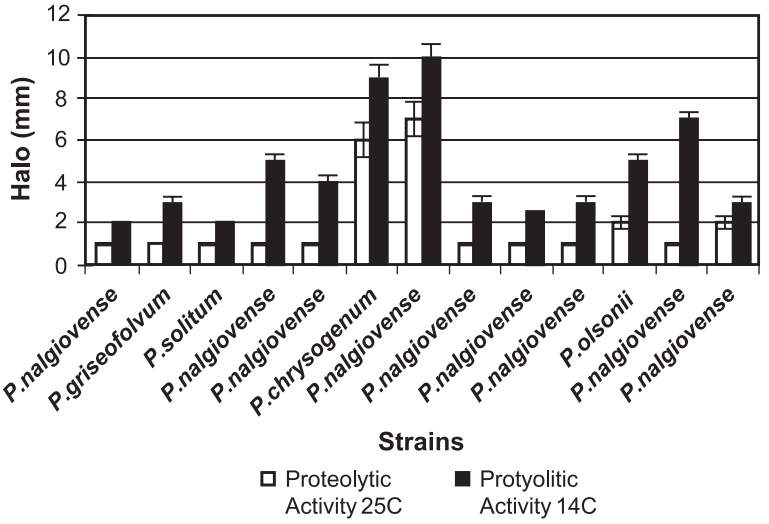

Fig. 1. Profile of proteolytic activity on casein in absence of salt at 25 and at 14 °C for the strains analyzed (mean ± standard deviation of 13 strains, two experiments each strain).

proteolytic activity on CA. This activity was four times greater than at 25 °C, in average (Fig. 1). It is important to point out that temperature had the inverse effect on the proteolytic activity with respect to growth, since growth was lower at 14 °C than at 25 °C.

The addition of NaCl affected all strains analyzed at 25 °C. A stimulating effect was observed on their proteolytic activity. An increase of fourfold was obtained for all strains except for *Penicillium crhysogenum* and *P. nalgiovense* (S15-1), where the proteolytic activity was reduced. Nevertheless, the values of proteolytic activity of these strains are of the same order of magnitude of the rest. At 14 °C, the presence of NaCl did not have the same influence as it did at 25 °C. The physiological responses did not show a general tendency. For some strains, this concentration of NaCl did not affect the enzymatic activity. For other strains, the activity increased or diminished. Again, *P. crhysogenum* and *P. nalgiovense* (S15-1) were the strains that showed higher proteolytic activity at this temperature, double than the others, both in presence and in absence of NaCl.

Similar results were obtained on MEAG at both temperatures. The use of gelatin in the culture medium provided us a qualitative assay to verify, in an initial screening, whether the strains of *Penicillium* spp. secrete extracellular proteases.

Production of extracellular lipases was observed for all strains at 25 and 14 °C. In general terms, the production was higher at 14 °C (Fig. 2). The presence of NaCl either decreased or increased the production depending on the strain considered, at both temperatures analyzed.

Since, in general terms, all strains were capable of growing and producing proteases and lipases under the conditions of the process, the behavior of our isolates from a physiological point of view shows that naturally isolated strains may be used as starter cultures on meat product.

Since our findings show that there are inter-species and even intra-species differences in the physiological capabilities, we believe that exhaustive studies of these capabilities should be performed on indigenous strains aimed to be used as starter cultures, in order to be able to select the best ones for this end.

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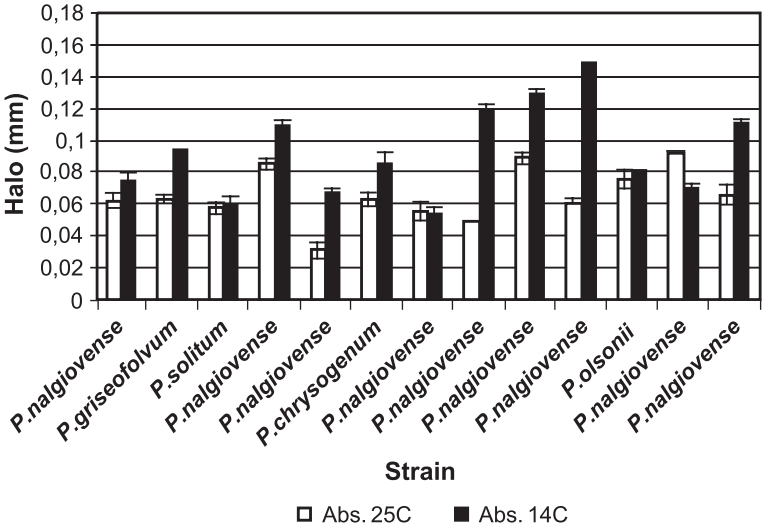

Fig. 2. Profile of lipolytic activity in absence of salt at 25 and at 14 °C (expressed as absorption at 715 nm) for the strains evaluated (mean ± standard deviation of 13 strains, two experiments each strain).

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