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Effect of water activity and temperature on growth of three *Penicillium* species and *Aspergillus flavus* on a sponge cake analogue

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Abstract

This study compared the effect of temperature and water activity and their interactions on the rate of mycelial growth of *Penicillium aurantiogriseum*, *P. chrysogenum*, *P. corylophilum* and *Aspergillus flavus* on a sponge cake analogue. As expected, growth rates showed dependence on a_w and temperature. However, no significant differences were observed in the growth rates of different isolates. The minimum a_w values for growth of the *Penicillium* spp. was 0.85–0.90. *A. flavus* was able to grow at 0.90 a_w when the temperature was above 15 °C. This study has shown that fungal growth by these species on a sponge cake analogue, with a composition similar to usual bakery products, is prevented if the a_w is kept at < 0.85.

Keywords: Bakery; Growth; Water activity; Temperature; Moulds

1. Introduction

Mould spoilage of bakery products has been the subject of many studies, and a number of species have been implicated. The most widespread and most important in bakery products are species of *Eurotium*, *Aspergillus* and *Penicillium* (Spicher, 1980; Williams, 1990; Abellana et al., 1997b). Other genera isolated from bakery products have included *Cladosporium*, *Mucor* and *Rhizopus* (Birnbaum, 1981; Spicher, 1984; Pitt and Hocking, 1985; Abellana et al., 1997b), but due to their high a_w requirement for germination and growth, it is unlikely that bakery products will be spoiled by these fungi. *Wallemia sebi* and other xerophilic fungi have also been isolated from these products (Hocking, 1991; Abellana et al., 1997b).

In addition to the economic losses associated with bakery products, another concern is the possibility of mycotoxin production. *Eurotium* species are usually the first fungi to colonize improperly dried, stored commodities, and when they grow, they increase the level of available water allowing other species (e.g. Aspergilli and Penicillia) to thrive. *Eurotium* spp. do not produce any significant mycotoxins (Hocking, 1988), but it is important to know the conditions under which species of *Aspergillus* and *Penicillium* can grow and spoil bakery products, because several

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species produce mycotoxins (Legan, 1993). For example, toxigenic *Aspergillus flavus* has been isolated from 3 of 15 home-stored bakery products (Torrey and Marth, 1977), and toxigenic *Penicillium* spp. have been isolated from wheat flour and bread in the USA (Bullerman and Hartung, 1973). Several species of *Penicillium* produce mycotoxins, including *Penicillium chrysogenum* (El-Banna et al., 1987).

The objectives of this work were to study the effect of water activity (a_w) and temperature, and their interactions on mycelial growth of two isolates each of *P. aurantiogriseum*, *P. corylophilum*, *P. chrysogenum* and *A. flavus* on an analogue of sponge cake used to represent Spanish bakery products.

2. Material and methods

2.1. Fungal isolates

The fungal isolates used in this study were *P. aurantiogriseum* PA1 and PA2, *P. corylophilum* PCO1 and PCO2, *P. chrysogenum* PCH1 and PCH2, and *A. flavus* AF1 and AF2, isolated from bakery products (Abellana et al., 1997b). Codes refer to strains held in the culture collection of the Food Technology Department of the University of Lleida, Lleida, Spain.

2.2. Preparation of the cake analogue

The medium used in this study was a Spanish sponge cake analogue. It was made from 273-g wheat flour, 211-g vegetable oil, 258-g sucrose, 258-g eggs and 4 g of baking powder. The baked analogue had a pH of 7, and its initial a_w was between 0.60 and 0.75. The a_w was measured with a Novasina Humidat ICI Thermoconstanter (Novasina, Zurich, Switzerland). The pH was measured with a pH meter (Crison, micropH 2001, Crison instruments, Alella, Spain) fitted with a penetration electrode for solids (Crison, 52-32). Ingredients were mixed and placed on aluminium plates. Dough was baked in an oven at 160–170 °C for 15–20 min. After baking, plates were surface covered with cooking foil, also sterilised in the oven, during their transfer to a laminar flow bench (Telstar, AH-100, Telstar, Terrassa, Spain). The cakes were then exposed to UV light for 10 min to eliminate surface contamination. Slices of the analogue were placed in Petri dishes (9-cm diameter) with two compartments (Bibby Sterilin, Stone, Staffs, UK). The analogue was cut into 3-mm-thick slices and placed in one compartment of each plate. Cakes were covered with a sterile porous film (Cellophane P400, Cannings, Bristol, UK), which enabled observation and measurement of mycelial growth. This film allowed the fungus to obtain nutrients from the substrate. Previous studies have shown that growth rate is very similar on this medium with and without the cellophane layer (Ramos et al., 1999).

2.3. Inoculation, incubation and measurement of growth

Actively growing 5- to 7-day-old colonies of the isolates on malt extract agar containing 20% sucrose (20-g malt extract, 20-g glucose, 1-g peptone, 200-g sucrose, 20-g agar, 1000-ml distilled water, pH 5.5) were used for all experiments. Agar plugs (5-mm diameter) cut from the growing margins of the colonies were aseptically placed next to the centre of the dividing wall of each petri dish, touching the cake analogue. In the other compartment, a glycerol water solution with the desired a_w was deposited to control equilibrium relative humidity (Dallyn, 1978). Plates with the same a_w were placed in water-imper-

Table 1
Minimum water activity for growth at 15–30 °C for two isolates of *P. aurantiogriseum*, *P. corylophilum*, *P. chrysogenum* and *A. flavus* tested on a sponge cake analogue

	Temperature (°C)			
	15	20	25	30
P. aurantiogriseum PA1	0.90	0.85	0.85	0.90
P. aurantiogriseum PA2	0.90	0.85	0.85	0.90
P. corylophilum PCO1	0.90	0.85	0.85	0.90
P. corylophilum PCO2	0.90	0.85	0.85	0.90
P. chrysogenum PCH1	0.90	0.85	0.85	0.85
P. chrysogenum PCH2	0.90	0.85	0.85	0.85
A. flavus AF1	N.G.	0.90	0.85	0.90
A. flavus AF2	N.G.	0.90	0.90	0.90

N.G.: no growth. Growth was considered to take place when a growth rate higher than 0.1 mm d^{-1} was achieved.

meable plastic containers together with two 100-ml beakers containing a glycerol water solution with an equilibrium relative humidity value identical to the a_w of the plates. In this way, equilibration to the target a_w levels was achieved within 24 h (unpublished results), maintaining a constant relative humidity inside the petri dishes and also controlling the a_w of the substrate.

The a_w values studied were 0.75, 0.80, 0.85 and 0.90, and the experiments were carried out at 15, 20, 25 and 30 °C. In all cases, observations were carried out daily or as necessary, and the radius of growing colonies measured in three directions. Growth was observed with the aid of a binocular magnifier (Leica, Z45E, Leica, Buffalo, USA). Measurements continued for a maximum of 2 months, and all experiments were carried out with at least three separate replicate petri dishes per treatment.

2.4. Statistical treatment of the results

Radial mycelial extension was measured at different a_w and temperature treatments vs. time. Radial growth rates (mm day^{-1}) at each a_w and tempera-

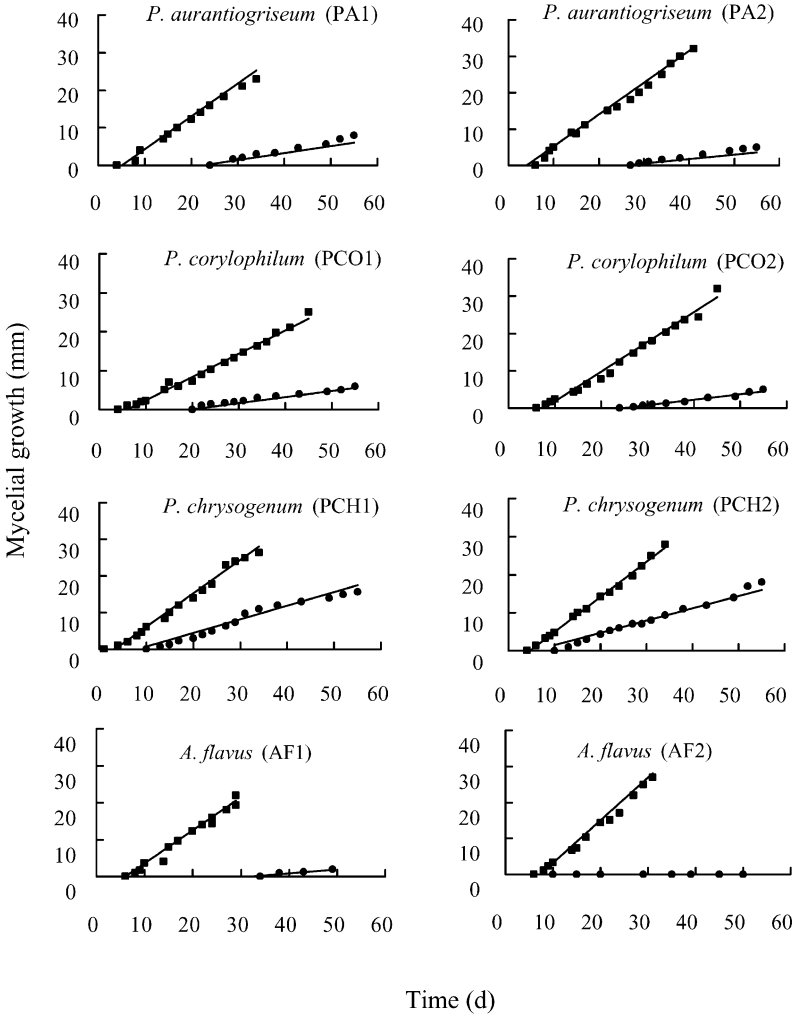

Fig. 1. Effect of water activity on exponential phase of mycelial growth of three species of *Penicillium* and *A. flavus* (two isolates of each) at 25 °C on a sponge cake analogue. Water activity levels are 0.90 (■) and 0.85 (●).

ture treatment were obtained from the slopes of the linear regression of the linear parts of the growth curves (Marín et al., 1995). One-way analysis of covariance was used to analyze colony radius so that effects of single factors (a_w, temperature, species), two and three factors could be assessed separately for statistically significant differences. Analysis of covariance was also used to assess effects of single factors between isolates of each species. The SAS System (version 6.12, SAS Institute, Cary, NC 27513, USA) statistical package was used for analysis of variance.

3. Results

Table 1 shows the minimum a_w for growth of *P. aurantiogriseum*, *P. corylophilum*, *P. chrysogenum* and *A. flavus*. At 0.75 and 0.80 a_w, none of the isolates was able to grow in the range of temperatures studied. The minimum a_w for growth of *Peni-*

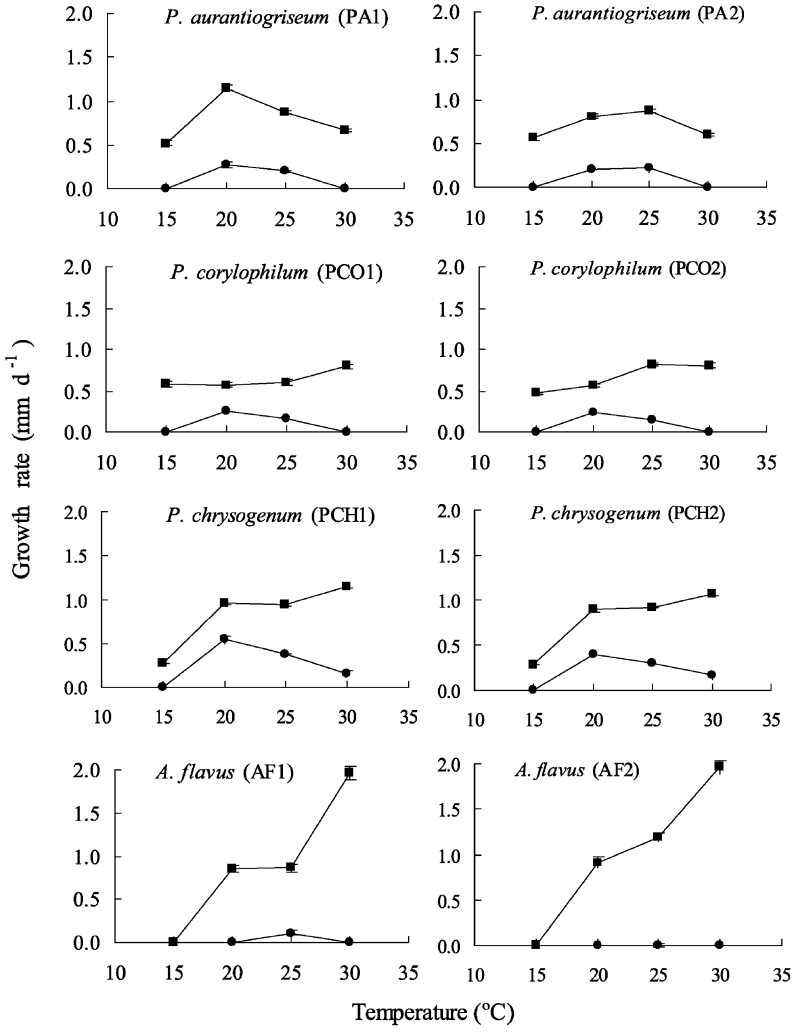

Fig. 2. Effect of water activity and temperature on growth rate of three species of *Penicillium* and *A. flavus* (two isolates of each) on a sponge cake analogue. Water activity levels are 0.90 (■) and 0.85 (●).

cillium isolates was 0.85–0.90 depending on the temperature. The two isolates of *A. flavus* were able to grow at 0.90 a_w when the temperature was above 15 °C.

Fig. 1 shows the effects of a_w on mycelial growth in *Penicillium* species and *A. flavus*. These plots indicate the exponential phase of growth in each case and were used to calculate growth rates (mm day^{-1}). The behaviour of all isolates was similar, growth was rapid at 0.90 a_w and decreased when a_w was reduced. All isolates were able to grow at only 0.90 and 0.85 a_w, though *A. flavus* AF2 grew only at 0.90 a_w. Although there were differences in the growth rate of the three species of *Penicillium* tested, the behaviours of *P. aurantiogriseum* and *P. corylophilum* were similar. The behaviour of the isolates of *P. chrysogenum* at 0.90 a_w was similar to that of the isolates of the other species of *Penicillium*, but growth at 0.85 was faster. Fig. 1 also shows an extension of the lag phase when a_w was decreased from 0.90 to 0.85 a_w.

Changing a_w at different temperatures affected the growth rates of all isolates examined. Fig. 2 compares $a_w \times$ temperature profiles for growth rates of the three species of *Penicillium*. The maximum growth of all isolates, over the a_w range tested, was at 0.90 a_w, but the optimum temperature for growth varied with isolate. Maximum growth rates varied from 0.80 to 1.17 mm day^{-1}. Isolates were not able to grow at 0.80–0.75 a_w; at 0.85 a_w, the growth rate was < 0.5 mm day^{-1}.

Table 2
Analysis of covariance of the effect of water activity (a_w), temperature (T), species (S) and their interactions on colony radius of *Penicillium* spp. and *A. flavus* on a sponge cake analogue

Factor	*df*	MS	*F*
S	3	1384.38	58.26 *
a_w	1	37863.99	1593.34 *
T	3	3754.01	157.97 *
S × a_w	3	758.58	31.92 *
S × T	8	829.67	34.91 *
$a_w \times T$	2	3405.94	143.32 *
S × $a_w \times T$	2	173.89	7.32 *
Time	1	70307.53	2958.58 *

* Significant $P < 0.001$.

Table 3
Analysis of covariance of the effect of water activity (a_w), temperature (T) and isolates (I) for each species of *Penicillium* and *A. flavus* on a sponge cake analogue

Species	Factor	*df*	MS	*F*
P. aurantiogriseum	I	1	2.45	0.13
	a_w	1	13929.60	719.03 *
	T	3	1000.39	51.64 *
	Time	1	16687.80	861.41 *
P. corylophilum	I	1	3.20	0.21
	a_w	1	8360.43	544.53 *
	T	3	603.21	39.29 *
	Time	1	16682.20	1086.54 *
P. chrysogenum	I	1	20.58	0.61
	a_w	1	16691.63	491.27 *
	T	3	3097.49	91.16 *
	Time	1	20149.50	593.04 *
A. flavus	I	1	9.71	0.41
	a_w	1	10473.63	446.86 *
	T	2	2338.14	99.76 *
	Time	1	19417.33	828.45 *

* Significant $P < 0.001$.

Growth rates of two isolates of *A. flavus* are also shown in Fig. 2. The optimum growth rate was 1.96 mm day^{-1} at 0.90 a_w and 30 °C for both isolates. Although the behaviours of both isolates were similar, *A. flavus* AF1 was able to grow at 0.85 a_w and 25 °C, while the other isolate AF2 grew only at 0.90 a_w. Conditions of $a_w \times$ temperature allowing growth of *A. flavus* were not narrower than the *Penicillium* spp. However, when growth of *A. flavus* occurred, growth rates were higher than those of *Penicillium*.

Significant differences in $a_w \times$ temperature effects on growth rates were observed for all of the species tested. Statistical analysis showed significant differences between species ($P < 0.001$) due to a_w, temperature, species, and two- and three-way interactions (Table 2). No significant differences were observed between isolates of each species tested (Table 3).

4. Discussion

This is the first detailed study of the a_w and temperature relations on growth of three common species of *Penicillium* and *A. flavus* isolated from

bakery products on a sponge cake medium. In this study, mycelial growth of *A. flavus* and three species of *Penicillium* on the sponge cake analogue was found to be significantly influenced by a_w, temperature and their interactions. Furthermore, isolates of the same species have no significant differences in their growth.

The nutritional composition of bakery products differs and influences fungal growth. A recent study carried out with five different Spanish bakery products (Abellana et al., 1997a) showed that the a_w of these intermediate moisture products ranged from 0.71 to 0.79, with pH values between 4.26 and 8.82. The medium used in our study was representative of a bakery product and the results obtained are realistic in relation to what could happen when the bakery products are contaminated with the fungi studied.

The Penicillia most frequently encountered in bakery products have been *P. chrysogenum* and *P. aurantiogriseum* (Hocking, 1988). Fungi present in dough are normally destroyed during baking and contamination occurs during cooling period from spores present in the air. It is possible to minimize fungal growth by implementing hygienic measures for food handling and specific temperatures for transport and storage. Our results show that if the a_w of the bakery products is lower than 0.85, these food cannot be spoiled by the fungal species examined within 2 months.

Our results for minimum a_w for growth differ from those obtained by Ayerst (1969) who used equilibrated malt extract media for a range of species and isolates, including *P. cyclopium* (= *P. aurantiogriseum*) and *A. flavus*. He reported that the minimum for growth of *P. aurantiogriseum* was 0.82 a_w and 0.78 a_w for *A. flavus*. Pitt and Miscamble (1995) studied the colony growth rates for *A. flavus* on malt extract media regulating a_w with glucose and fructose. They found that the minimum a_w for growth of *A. flavus* was 0.82 at 25 °C, 0.81 at 30 °C and 0.80 at 37 °C. Differences observed between these results and those obtained in the present study could be due to the different nutritional status of media, and the method of a_w modification.

Marín et al. (1998) determined growth rates on equilibrated maize meal extract agar modified with glycerol, for a range of species including one isolate of *A. flavus* and *P. aurantiogriseum.* As in our study, they found a significant effect of a_w and temperature on growth of these fungi. The minimum a_w values for growth found by these workers were lower than ours, although growth rates at the same conditions of $a_w \times$ temperature were higher in our work. This may be attributed to the different nutritional effects between the media used by these authors and our analogue.

The importance of carrying out studies in situ instead of in vitro in the bakery products was demonstrated by the work of Reiss (1982) who compared growth of, and aflatoxin production by, *A. parasiticus* and *A. flavus* on malt extract agar, whole wheat bread and a kind of cake. In general, the fungi grew better on cake than on bread. Malt extract agar was a less favourable substrate for aflatoxin synthesis by *A. parasiticus* and *A. flavus* than the two bakery products. In all cases, cake enhanced the production of aflatoxins in comparison with other substrates. The highest yields of aflatoxin B_1 were produced by *A. flavus* on cake. The results from our study showed that if the a_w of bakery products is lower than 0.90, *A. flavus* is not able to grow and consequently, there is no production of aflatoxins.

This study has shown that it is possible to distinguish effectively between tolerances of species to a_w, temperature and their interactions. This study has also provided further information on conditions favoring growth of *Penicillium* spp. and *A. flavus* on bakery products, because the substrate was made with the essential ingredients of this kind of intermediate moisture foods. The method used allows good control of a_w, giving results with higher real application than synthetic media. This information can be used to assess the probability of these fungi being involved in food spoilage problems.

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