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International Journal of Food Microbiology 52 (1999) 97–103

International Journal of Food Microbiology

www.elsevier.nl/locate/ijfoodmicro

Water activity and temperature effects on growth of *Eurotium amstelodami*, *E. chevalieri* and *E. herbariorum* on a sponge cake analogue

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Received 8 December 1998; received in revised form 7 July 1999; accepted 5 August 1999

Abstract

Eurotium is a widespread storage fungal genus that has been frequently isolated from bakery products. The objective of this study was (i) to obtain a method for studying the growth of xerophilic fungi on bakery products, and (ii) to determine the effects of water activity (a_w), temperature, isolate and their interactions on mycelial growth of *Eurotium* spp. on an analogue medium of sponge cake. Statistical analysis showed that there were intra-isolate differences ($P<0.001$) due to a_w, temperature, isolate, and two- and three-way interactions. Optimum growth of all isolates over $a_w \times$temperature range tested showed optima at 0.90 a_w and 30°C, with an interval of growth rate of 3.8–5.1 mm·d^{-1}. At 0.75 a_w, growth was less than 0.15 mm·d^{-1}, if there was any.

Keywords: *Eurotium* spp.; Bakery products; Fungal growth; Water activity; Temperature

1. Introduction

In the last few years, the bakery products and flour confectionery sector has witnessed particularly intense technological progress which has brought clear and tangible changes, not only in terms of commercial and qualitative characteristics of the products, but also in terms of process innovation. Usually, bakery products are packaged in plastic films after baking and cooling, and they are consumed within 1 or 2 months. Postprocess contamination is unavoidable (Ponte and Tsen, 1987), and a wide range of moulds, such as *Penicillium*, *Aspergillus*, *Cladosporium* and *Eurotium* species, gain access to the product surface prior to packaging (Fustier et al., 1998).

Bakery products are classified as products of intermediate moisture content. On the other hand, the nutritional composition of different bakery products will differ and influence fungal growth. Contamination by xerophilic organisms in these kind of products usually comes from the postbaking cooling period, as the high cooking temperatures is normally

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0168-1605/99/$ – see front matter
PII: S0168-1605(99)00131-2

enough to eliminate previous contamination (Roessler and Ballenger, 1996).

The mycoflora of intermediate and low moisture foods is dominated by relatively few fungal genera. By far the most widespread and probably the most important in biodeterioration are species of *Eurotium*, *Aspergillus*, and *Penicillium*. Species of these fungi are found in foods at all a_w levels but usually they cause spoilage below 0.90 a_w. From the point of view of food spoilage and loss, *Eurotium* species are probably the most destructive of all. They are usually the first colonisers of improperly dried stored commodities, and as they grow, they raise a_w, allowing other species (like potentially toxigenic Aspergilli and Penicillia) to take over. *Eurotium* species do not produce any important mycotoxin, but they do produce a variety of secondary metabolites, and they cause oxidative rancidity problems in grains and nuts (Hocking, 1988).

Several studies about the ecophysiology of *Eurotium* spp. (Ayerst, 1969; Magan and Lacey, 1988; Wheeler and Hocking, 1988) included effects of a_w and temperature. These studies were carried out on synthetic media. Other workers studied the effect of a_w, temperature and atmosphere packaging in the growth or germination of *Eurotium* spp. also in synthetic media (El Halouat and Debevere, 1997; Haasum and Nielsen, 1998).

Nutrition source may influence the minimum a_w for growth and consequently, studies on artificial substrates may not accurately reflect capabilities for growing on the natural substrate (Magan and Lacey, 1984).

This study was carried out to obtain a new method to study the growth of xerophilic fungi on bakery products and to determine the effect of a_w, temperature, isolates and their interactions on mycelial growth profiles of two isolates of each species of *E. amstelodami*, *E. chevalieri* and *E. herbariorum*, on an analogue medium of sponge cake, representative of Spanish bakery products of this kind.

2. Material and methods

2.1. Fungal isolates

The fungal isolates used in this study were two of each species of *E. amstelodami* (A1, A2), *E. chevalieri* (C1, C2) and *E. herbariorum* (H1, H2), isolated from bakery products (Abellana et al., 1997). The references in brackets are the codes of cultures held in the Food Technology Department of the University of Lleida culture collection.

2.2. Preparation of the analogue

The medium used in this study was a Spanish sponge cake analogue. It was composed of 273 g of wheat flour, 211 g of vegetable oil, 258 g of sucrose, 258 g of eggs and 4 g of baking powder. The dough sheet after baking had a pH of about 7, and its initial a_w was between 0.60–0.75. The a_w was measured with a Novasina Thermoconstanter TH 200 (Novasina AG, Zurich, Switzerland). The pH was measured with a pH-metre (Crison, micropH 2001, Crison instruments S.A., Alella, Spain) and a penetration electrode for solids (Crison, 52-32, Crison instruments S.A., Alella, Spain). Ingredients were mixed and placed on aluminium plates. Dough was baked in the oven at 160–170°C for 15–20 min. Cooling foil was also put in the oven to sterilise it. After baking, plates were covered with sterile cooking foil to transfer them to the laminar flow bench (Telstar, AH-100, Telstar S.A., Terrassa, Spain). The cakes were exposed to UV light for 10 minutes to eliminate surface contamination. Petri plates (9 cm diameter) with two compartments (Bibby Sterilin Ltd., Stone, Staffs, UK) were used in order to place the analogue slices. The analogue was cut into 3 mm thickness slices with a sterile knife and placed in one compartment of each plate under aseptic conditions. Cakes were covered with a special porous film (Cellophane P400, Cannings Ltd., Bristol, UK) which enabled observation and measurement of mycelial growth to be carried out. This film allows the fungus to obtain nutrients from the substrate. Previous studies have shown that the growth rate is very similar on media 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 grown on malt extract agar containing 20% sucrose (MEA20: 20 g malt extract, 20 g glucose, 1 g peptone, 200 g sucrose, 20 g agar, 1000 ml

distilled water, pH = 5.5; all reagents were supplied by Panreac química S.A., Barcelona, Spain) were used for all experiments. Agar plugs (5 mm diameter) taken from the growing margins of the colonies were aseptically placed in the centre next to the thin wall of each treatment Petri plate, in the edge of the dough sheet. In the other compartment a glycerol–water solution (Dallyn, 1978) was deposited to control equilibrium relative humidity. Plates of the same a_w and temperature were placed in water impermeable plastic containers along with two 100 ml beakers containing a glycerol–water solution which equilibrium relative humidity value was the same as the a_w of the solution of the plates. These solutions were changed every fifteen days. In this way, the equilibration period to reach the target a_w levels was 24 h, maintaining a constant relative humidity in the atmosphere of the Petri plates and at the same time managing to successfully regulate 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 Inc., Buffalo, USA). Measurements were carried out for a maximum of two months. All experiments were carried out with at least three separate replicate Petri plates per treatment.

2.4. Statistical treatment of the results

One-way analysis of covariance was used to analyse colony radius, so effects of single factors (a_w, temperature, isolate), two and three factors could be assessed for statistically significant differences. The SAS System (version 6.12 Institute Inc) statistical package was used for analyses of variance.

3. Results

Fig. 1 shows mycelial growth vs. time at 25°C and at the different levels of water activity tested to each two isolate of the three species of *Eurotium* spp. studied. The behaviour of all isolates was similar, growth was quite rapid at 0.90 a_w and it decreased when the water activity was reduced. *E. chevalieri* was not able to growth at 0.75 a_w. The profiles only show the linear phase of the growth. These data were used to predict the growth rate ($mm \cdot d^{-1}$).

The minimum a_w for growth of the isolates of *E. amstelodami*, *E. chevalieri* and *E. herbariorum* changed with temperature (Table 1). At 0.75 a_w, the lowest level of water stress tested, all of the isolates were able to growth at 30°C, although under this water availability condition the growth was very slow. When the temperature was 25°C only three of six isolates were able to grow.

Temperature influenced the effect of a_w on growth rates. Fig. 2 shows growth rates at four temperatures and water activities of the six isolates of *Eurotium* spp. assayed. Growth rates of all isolates over $a_w \times$ temperature range tested showed maximum growth at 0.90 a_w and 30°C (maximum conditions studied) with an interval of growth rate of 3.8–5.1 $mm \cdot d^{-1}$.

The highest growth rates for all fungi were at 0.90 a_w under the temperature range tested, varying from 0.63 to 5.1 $mm \cdot d^{-1}$. The growth rates decreased as temperature changed from optimal to low marginal conditions. If there was any growth at 0.75 a_w, it was less than 0.15 $mm \cdot d^{-1}$.

There were significant differences in $a_w \times$ temperature growth rates for the six isolates of *Eurotium* spp. Statistical analysis showed that there were significant intra-isolate differences ($P < 0.001$) due to a_w, temperature, isolate, and two- and three-way interactions (Table 2).

4. Discussion

Mycelial growth of some isolates of *Eurotium* spp. from bakery products was found to be significantly influenced by a_w, temperature, and their interactions. At the highest temperatures tested, the range of a_w conditions for growth was wider than at lower temperatures.

Although minimum a_w for growth has received less attention in the literature than minimum for germination, it is of greater importance because the minimum a_w for growth of the fungus represents the most severe conditions under which it can cause food spoilage (Wheeler and Hocking, 1988). On the other hand, xerophilic fungi exhibit no special temperature

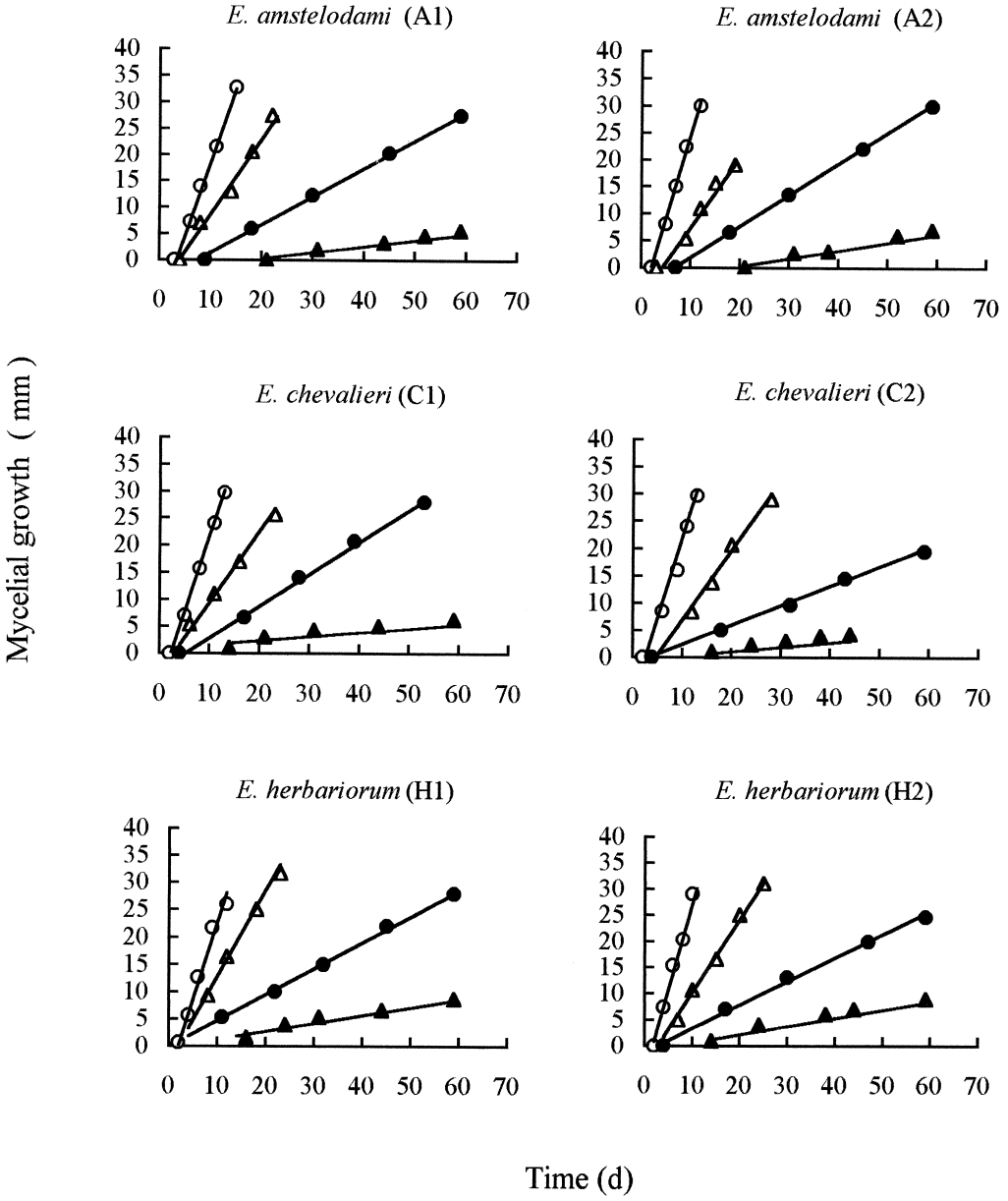

Fig. 1. Effect of water activity on linear phase of mycelial growth of six isolates of *Eurotium* spp. at 25°C on a sponge cake analogue. Water activity levels are 0.90 (○), 0.85 (△), 0.80 (●), 0.75 (▲).

requirements for growth, although most filamentous xerophiles grow best at the 22 to 25°C interval (Beuchat and Hocking, 1990).

Most of the studies about ecopysiology of species of *Eurotium* were carried out in a wide values of temperature and a_w, e.g. as by Hill and Lacey (1983) in the case of *Eurotium* species, reporting that they have optimum growth at 0.90–0.95 a_w, but in cereals they represent a real problem at 0.75–0.85 a_w. This underlines of the importance of addressing the real values of a_w and also temperature depending of the type of product being studied. In our case, we worked in the range of a_w relevant to intermediate moisture products.

Table 1
Minimum water activity for growth found at different temperature levels for six isolates of *Eurotium* spp. tested on a sponge cake analogue. Growth is considered to take place when a growth rate higher than 0.1 $mm \cdot d^{-1}$ is achieved

	Temperature (°C)			
	15	20	25	30
E. amstelodami (A1)	0.80	0.80	0.80	0.75
E. amstelodami (A2)	0.80	0.80	0.75	0.75
E. chevalieri (C1)	0.85	0.85	0.80	0.75
E. chevalieri (C2)	0.85	0.85	0.80	0.75
E. herbariorum (H1)	0.80	0.80	0.75	0.75
E. herbariorum (H2)	0.80	0.80	0.75	0.75

Table 2
Analysis of covariance of the effect of water activity (a_w), temperature (T), isolates (I) and their interactions on colony radius of *Eurotium* spp. on a sponge cake analogue

Factor	*df*	MS	*F*
I	5	1768.66	44.10[a]
a_w	3	44389.80	1106.81[a]
T	3	23846.82	594.59[a]
I×a_w	15	181.41	4.52[a]
I×T	15	792.06	19.75[a]
a_w×T	7	3480.36	86.78[a]
I×a_w×T	35	183.45	4.57[a]
Time	1	135203.20	3371.13[a]

[a] Significant $P<0.001$.

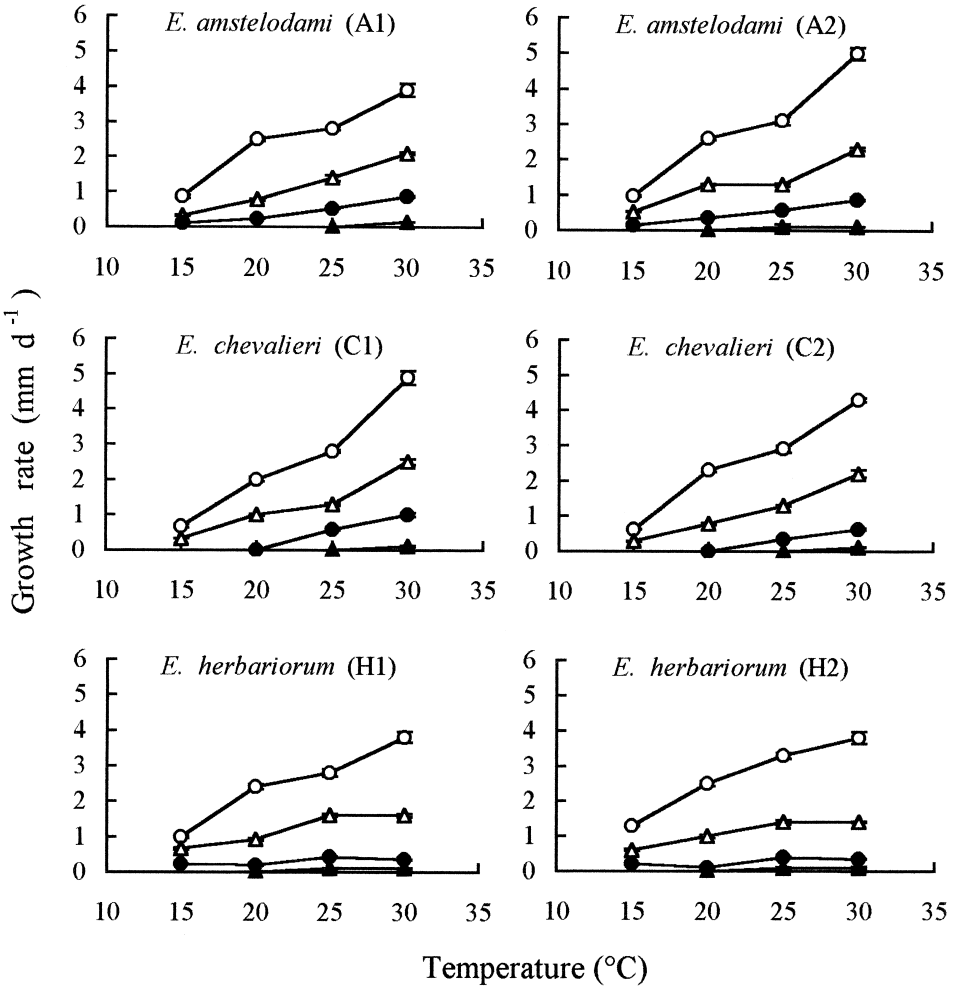

Fig. 2. Effect of water activity and temperature on growth rate of six isolates of *Eurotium* spp. on a sponge cake analogue. Water activity levels are 0.90 (○), 0.85 (△), 0.80 (●), 0.75 (▲). Error bars show standard error of estimated parameters.

Ayerst (1969) found optimum growth of *E. chevalieri* at 0.93 a_w and 33°C, and minimum at 0.71 a_w, and 42°C. The medium used in this study was malt extract agar and the water activity was regulated with potassium hydroxide. Considering that the range of a_w and temperatures studied by this author were wider than ours, the behaviour of *E. herbariorum* in our conditions was quite similar.

Roessler and Ballenger (1996) determined the minimum a_w required for growth of one strain of *E. chevalieri* isolated from cookies. They compared qualitative growth of this isolate on (i) MY70GH agar (Pitt and Hocking, 1997) (ii) on commercially manufactured cookies, in humidity chambers adjusted to various moisture levels, and (iii) on cookies adjusted to various a_w levels in sealed Petri dishes. Temperatures tested were 25 and 30°C. The minimum a_w for growth of the isolate in the humidity chambers was between 0.60 and 0.65 on MY70GH agar and, as low as 0.65, on commercially manufactured cookies. The minimum a_w tested which supported growth of the isolate on cookies with artificially adjusted a_w was found to be between 0.67 and 0.69. These results are slightly different to ours.

The minimum a_w for germination and growth is markedly influenced by temperature, nutrition and the solute used in culture media to control a_w (Magan and Lacey, 1988; Marín et al., 1995). Normally, regulation of a_w has been made by addition of different humectants into the medium as glycerol, salts and sugars. A study carried out by Wheeler and Hocking (1988) with four xerotolerant fungi, including *E. amstelodami*, showed that absolute growth rates depended on the type of solute used to regulate the water activity of the medium. In our work, a_w was regulated with an external solution, which maintained a constant relative humidity in the atmosphere of the Petri plates allowing at the same time a successful regulation of a_w of the substrate. Abellana et al. (1997) detected lower fungal contamination inside the products that on the surface. So, the most important point in our study was to control a_w on the cake surfaces. The most commonly fungi found by these workers were *Eurotium*, *Cladosporium* and the most xerotolerant species of genera *Penicillium* and *Aspergillus*.

The need to assure the microbiological safety and quality of increasingly complex food products has stimulated the interest in the use of mathematical modelling to quantify and predict microbial behaviour. The growth of microorganisms in food systems depends on the effect of multiple variables. Factors that influence microbial growth kinetics include temperature, pH, water activity, oxygen availability, carbon dioxide levels, presence of antimicrobials and nutrient content availability (Buchanan, 1993). Most of the work dealing with predictive modelling has been done with bacteria. The present work is an example of how different environmental conditions can affect the activity of spoilage fungi in bakery products. In addition, this study has shown that it is possible to effectively distinguish between interspecific and intraspecific tolerances of species of *Eurotium* spp. to a_w, temperature and their interactions in our substrate, which is valid to study growth of xerophilic fungi.

In several foods, the use of preservatives has been reduced due to consumer demands. This has resulted in increasing incidents in product spoilage. An aid in preventing fungal spoilage of mildly processed foods would be the application of the hurdle concept or the combined preservation method (Gould, 1995). If this concept is to be applied successfully, its influence on fungal growth need to be quantified (Cuppers et al., 1997). The medium and method developed in the present study can be used in future studies in bakery products to improve their self life.

Acknowledgements

The authors are grateful to the European Union FAIR PL98-4075 and to the Catalonian Government (CIRIT, Comisió Interdepartamental de Recerca i Innovació Tecnològica).

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