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Efficacy of sorbates on the control of the growth of *Eurotium* species in bakery products with near neutral pH

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Abstract

The effects of sorbic acid and potassium sorbate on growth of different *Eurotium* isolates when added to a bakery product analogue were tested under different environmental conditions. Water activity of the products was adjusted to values in the range of 0.75–0.90, and storage temperatures were in the range of 15–30 °C. Preservatives were added in concentrations ranging from 0.025% to 0.2%. It was observed that 0.025% and 0.05% concentrations always enhanced the isolates growth, while 0.1% had little preservative effect. Finally, even the highest concentration (0.2%) was not suitable as it only controlled fungal growth under certain water activity and temperature levels. It was concluded that these weak-acid preservatives are not useful when added to bakery products with near to neutral pH.

Keywords: *Eurotium*; Bakery products; Preservatives; Sorbates; Fungal spoilage

1. Introduction

Fungal spoilage of bakery products before their expiry date is an important concern for manufacturers in the summer season in Spain. Spoilage of bakery products is mainly due to species of *Eurotium*, *Aspergillus* and *Penicillium* (Abellana et al., 1997b). Losses due to mould spoilage vary between 1% and 5% of products depending on season, type of product and method of processing (Malkki and Rauha, 1978).

Usually, bakery products are packed in plastic films after baking and cooling, and they are consumed within 1 or 2 months (Fustier et al., 1998). Fungal spoilage, however, sometimes arises before the expiry dates of the bakery products, because the humidity on their surface is higher than it should be. The reason may be the difference of temperature between the product and the environment once packed. Moulds are destroyed during baking, but contamination arises from mould spores derived from atmosphere or from surfaces during the cooling, finishing and wrapping procedures (Seiler, 1988).

Preservation of bakery products commonly involves the use of preservatives such as propionates and sorbates, and sometimes benzoates. They are commonly added in concentrations that do not exceed 0.3% (Davidson and Juneja, 1990). Although weak-acid preservatives have no toxicological implications at the applied concentrations, at present there is a general consumer pressure towards decreasing con-

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centrations or using as less preservative as possible. Sorbic acid is known to inhibit many enzymes in baked goods applied in concentrations ranging from 0.1% to 0.3% (Lück, 1980); its inhibitory effect is unlikely to be due to inhibition of a single enzyme. The points of attack in the cell may well differ in bacteria, yeasts and molds (Liewen and Marth, 1985). For food preservation, the undissociated proportion of the sorbic acid has by far the most effective action. Owing to its low dissociation constant of 1.73×10^{-5}, sorbic acid, unlike other preservative acids, can also be employed for preserving weakly acidic foodstuffs with a relative high pH (Lück, 1980). The higher solubility of potassium sorbate renders it a preferred form of sorbic acid in foods. In oils, however, sorbic acid is more soluble than the potassium salt (Thakur et al., 1994).

Previous in vitro studies have shown that under certain conditions, the use of these preservatives enhances the growth of some fungal isolates in bakery products (Marín et al., 2002a).

The objective of this work was to assess the convenience of using sorbic acid and potassium sorbate to control spoilage of bakery products by *Eurotium* species.

2. Materials and methods

2.1. Fungal isolates

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

Table 1
pH values of cake analogues as affected by the addition of sorbic acid and potassium sorbate

	0%	0.025%	0.05%	0.1%	0.2%
Sorbic acid	6.9	7.0	6.8	6.5	6.2
Potassium sorbate	6.9	7.0	7.1	7.2	7.3

Table 2
Analysis of variance of the effects of sorbic acid and potassium sorbate added at different concentrations (c) to cake analogues kept at different a_w and temperature levels (T) on growth of six *Eurotium* strains (s)

Factor	*df*	Sorbic acid		Potassium sorbate	
		MS	*F*	MS	*F*
s	5	31,554.1	195.6*	336,474.1	199.9*
a_w	2	239,215.2	3708.2*	217,669.9	2982.4*
T	3	1,781,143.1	1841.0*	168,624.0	1540.3*
c	4	27,743.7	215.0*	8007.3	54.9*
$s \times a_w$	10	1453.9	4.5*	3143.4	8.6*
$s \times T$	15	20,656.1	42.7*	22,747.0	41.6*
$s \times c$	20	2246.9	3.5*	2846.8	3.9*
$T \times c$	12	4886.3	12.6*	9915.3	24.7*
$a_w \times T$	6	12,899.2	66.6*	29,035.6	132.6*
$a_w \times c$	8	5651.0	21.9*	4203.0	14.4*
$s \times a_w \times T$	30	4098.5	4.2*	4767.8	4.4*
$s \times a_w \times c$	40	4438.8	3.4*	3570.9	2.4*
$T \times c \times a_w$	18	9577.6	16.5*	6487.7	10.5*
$s \times T \times c$	60	6499.4	3.4*	3909.4	1.9*
$s \times T \times c \times a_w$	82	5732.6	2.2*	4524.2	1.5*

df, degrees of freedom; MS, mean squares; *F*, calculated *F*-ratio.
* Significant, $P < 0.0001$.

2.2. Preparation of the cake analogues

The medium used in this study was a Spanish sponge cake analogue (similar to Madeira cakes). Madeira cakes and their modifications are among the most commonly consumed cakes with no fermentation involved. Laboratory analogues were used instead of actual cakes, because this allowed getting sterile cakes without other interfering moulds on them. They were 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. When preservatives were used, they were mixed with the eggs and added to the mix of ingredients in amounts of 0.25, 0.5, 1 and 2 g kg^{-1} dough. Ingredients were mixed and placed on aluminium plates. Dough was baked in the oven at 160–170 °C for 15–20 min; cooking foil was also put in the oven to sterilize it. After baking, plates were covered with sterile cooking foil to transfer them to the laminar flow bench. The cakes were exposed to UV light for 10 min to eliminate surface contamination. 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 an Aqualab (Decagon, Pullman, USA). The pH was measured with a pH meter (Crison, micropH 2001,

Crison Instruments, Alella, Spain) and a penetration electrode for solids. Table 1 shows the mean pH values obtained for each preservative treatment.

Petri plates (9-cm diameter) with two compartments (Bibby Sterilin, 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, 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) were used. Agar plugs (5-mm diameter) taken from the growing margins of the colonies were aseptically placed in the centre of each Petri plate next to the wall, 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 15 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.

A full-factorial design with three replicates was applied. Factors were preservative, its concentration, a_w, temperature and isolates. The a_w levels studied

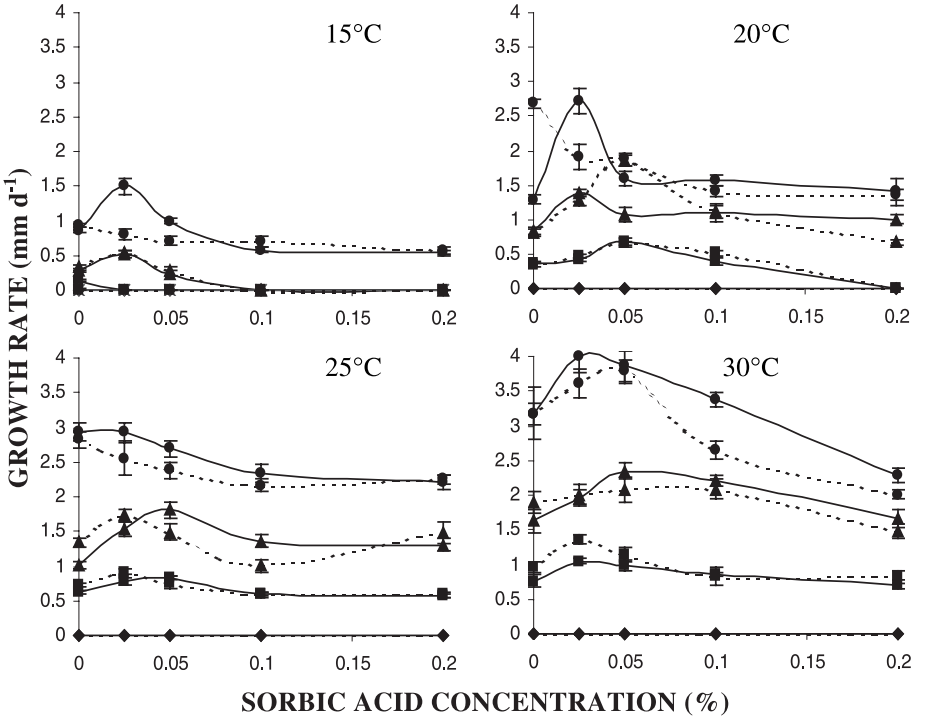

Fig. 1. Effect of sorbic acid on growth rates of *E. amstelodami* A1 (—) and A2 (- - -) isolates on cake analogues under different water activities (●, 0.90; ▲, 0.85; ■, 0.80; ♦, 0.75) and temperature levels.

were 0.75, 0.80, 0.85 and 0.90, and the experiments were carried out at 15, 20, 25 and 30 °C. Two sources of sorbates were used: potassium sorbate and sorbic acid added at concentrations of 0.025%, 0.05%, 0.1% and 0.2%. In all cases, observations were carried out daily or less often depending on the treatments, 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 were carried out for a maximum of 2 months. Growth rates were estimated by linear regression of colony radius against time (mm day^{-1}).

2.4. Statistical analyses of the results

One-way analysis of covariance was used to analyze colony radius, so effects of single factors (a_w, temperature, isolate, preservative and concentration) as well as their interactions could be assessed for statistically significant differences. The SAS System (version 6.12, SAS Institute) statistical package was used for analysis of variance.

3. Results

3.1. Sorbic acid

All single factors assayed as well as their interactions had a significant effect on growth of *Eurotium* isolates (Table 2). No growth was found at 0.75 a_w on the cake analogues, while the most important slowing of growth was observed when a_w was decreased from 0.90 to 0.85 a_w (Figs. 1 and 2). Similarly, growth was slowed down when decreasing temperatures from 30 to 15 °C in 5 °C intervals. The different isolates behaved in a significantly different manner. Similarities were found, however, between the isolates belonging to the same species. It was seen that *E. chevalieri* isolates grew slower than *E. herbariorum* ones.

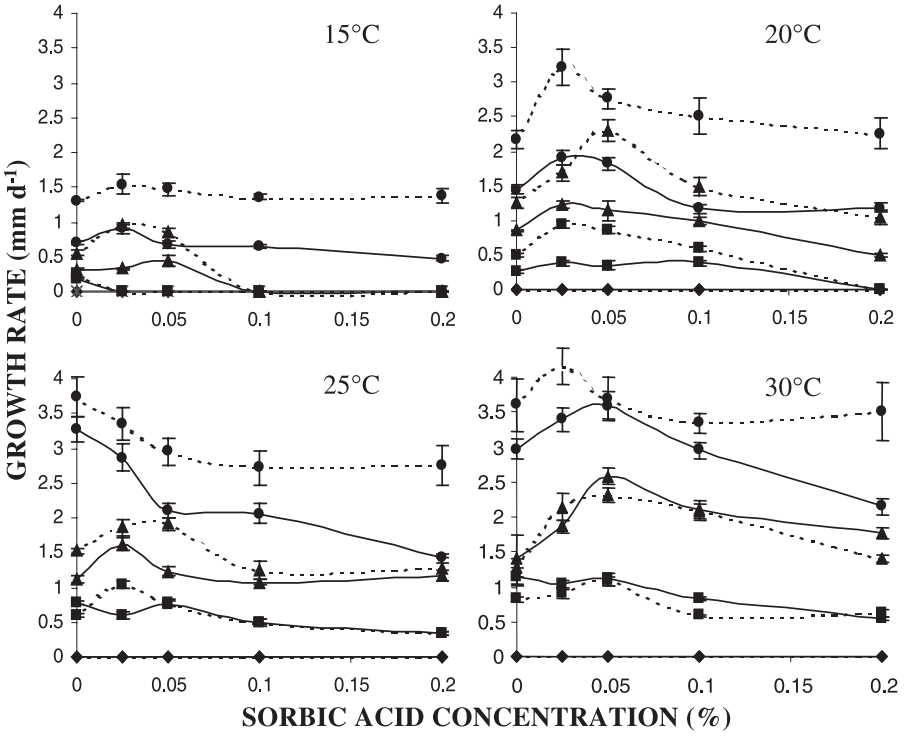

Fig. 2. Effect of sorbic acid on growth rates of *E. chevalieri* C1 (—) and *E. herbariorum* H1 (- - -) on cake analogues under different water activities (●, 0.90; ▲, 0.85; ■, 0.80; ◆, 0.75) and temperature levels.

Low concentrations of sorbic acid (0.025 and 0.05%) led always to an enhancement of growth regardless of a_w and temperature levels. Moreover, the 0.1% concentration enhanced growth rates under some of the conditions tested (mainly 0.85 a_w at 20–30 °C), while the effectiveness of the 0.2% concentration depended on temperature and a_w. Surprisingly, sorbic acid was more effective at the highest water activity (0.90), then at 0.80 and finally at 0.85 a_w. Referring to temperature, at 15 °C and under most of the a_w levels, a 0.2% concentration resulted in an inhibition of growth; the highest inhibition, however, was reported at 30 °C but only at 0.90 a_w.

3.2. Potassium sorbate

Similarly to the previous section, all single factors assayed as well as their interactions had a significant effect on growth of *Eurotium* isolates (Table 2). No growth was found at 0.75 a_w on the cake analogues, while increasing growth rates were found with increasing a_w levels (Figs. 3 and 4). Similarly, growth was slowed down when decreasing temperatures from 30 to 15 °C in 5 °C intervals. The different isolates behaved in a significantly different manner. Similarities were found, however, between the isolates belonging to the same species. It was seen that *E. chevalieri* isolates grew slower than the others.

Potassium sorbate was less effective than sorbic acid at inhibiting fungal growth, mainly at the lower temperatures (15 and 20 °C). At the highest concentration tested, the growth rates were still high (up to 3.45 mm day^{-1} at 30 °C and 0.9 a_w). Both 0.025% and 0.05% concentrations led to an enhancement of growth. The 0.1% concentration was just effective at 30 °C and at some of the other temperatures when a_w was low. Finally, the 0.2% concentration was mainly effective at 0.80 a_w and under certain temperatures at 0.85 a_w. Referring to temperature, there was not a clear improvement of preservative activity at any of the temperature levels, and finally, *E. herbariorum*

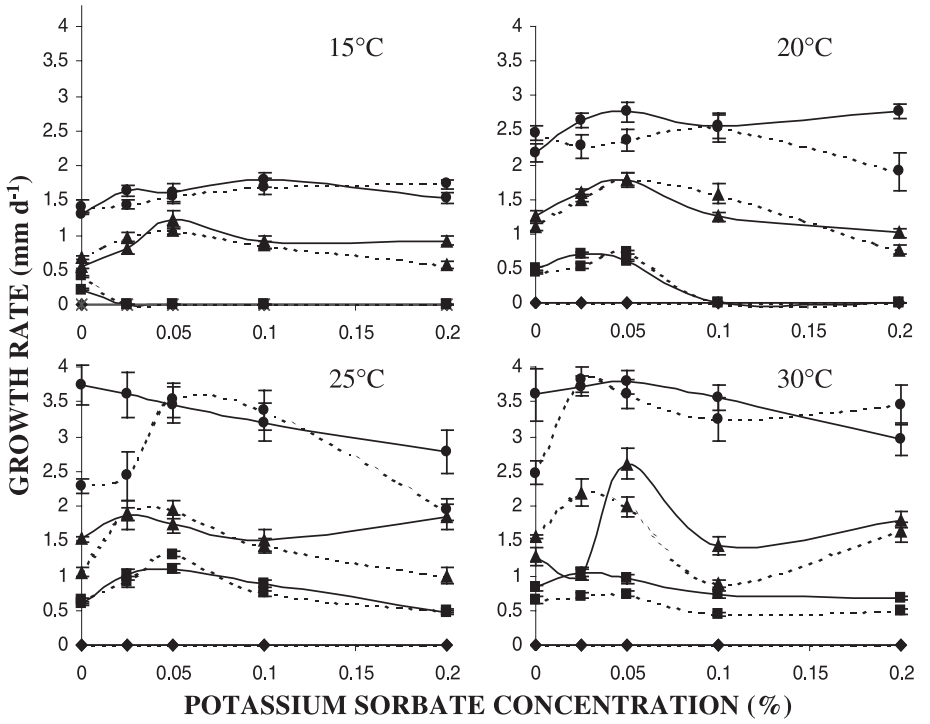

Fig. 3. Effect of potassium sorbate on growth rates of *E. herbariorum* H1 (—) and H2 (- - -) on cake analogues under different water activities (●, 0.90; ▲, 0.85; ■, 0.80; ◆, 0.75) and temperature levels.

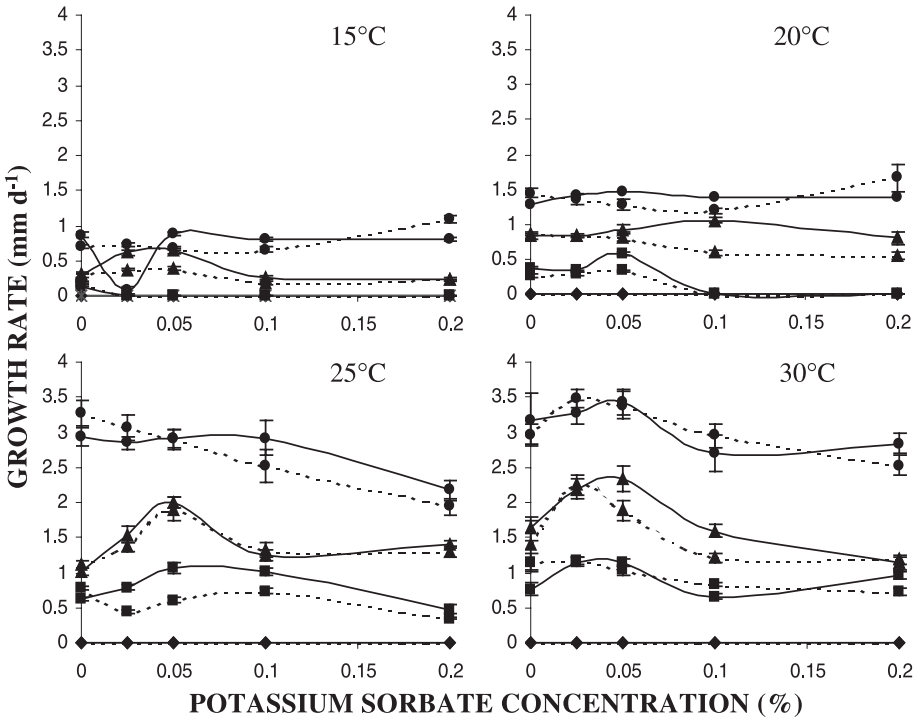

Fig. 4. Effect of potassium sorbate on growth rates of *E. amstelodami* A1 (—) and *E. chevalieri* C1 (- - -) on cake analogues under different water activities (●, 0.90; ▲, 0.85; ■, 0.80; ♦, 0.75) and temperature levels.

seems to be the less sensitive species to potassium sorbate.

4. Discussion

The typical mycoflora of Spanish bakery products consists of xerophilic species of *Eurotium*, *Aspergillus* and *Penicillium*; The range of microorganisms used in this study belong to the main genus: *Eurotium.* The a_w of these products varies between 0.70 and 0.90 approximately. A few fungi are capable of growing in environments with low water activity. Many *Aspergillus* species are xerophilic and are usually capable of growing on media containing high concentrations of salt or sugar (Thom and Raper, 1945). Xerophilic fungi exhibit no special temperature requirements for growth. Most filamentous xerophiles grow best in the 22–25 °C range (Beuchat and Hocking, 1990). Bakery products are kept at room temperature, under these conditions xerophiles may grow at their best. On the other hand, the optimal pH for growth of most xerophiles is 6.5–6.8; again, Spanish bakery products have been found to have pH ranging from 4.3 (ensaimada) to 8.8 (magdalenas) (Abellana et al., 1997a). It is known, however, that problems arise sometimes of spoilt products before the expiry date. The most likely cause is the condensation of moisture on the surface of the cakes once they are packed, if they have not been cooled enough and depending on the temperature in the packaging area. A recent work carried out by Abellana et al. (1999) highlights the importance of a_w and temperature in which bakery products are kept on the growth of *Eurotium* species.

Previous studies on moulds from bakery products have been carried out on wheat flour agar (Marín et al., 2002a). The present approach, however, may better simulate what happens on the real products. In those studies, potassium sorbate, compared to sodium benzoate and calcium propionate, showed a wider range of conditions where it totally inhibited fungal growth. It was concluded that this would be the most suitable weak-acid preservative to be used in bakery products as it was able to avoid fungal growth

at both pH 4.5 and 6, regardless of a_w levels (0.80–0.95 a_w).

Sorbic acid has an activity less linked to pH than benzoic acid, it can be effective at pH levels of 6.5, although its activity increases as pH decreases (Lindsay, 1993). Besides inhibiting microorganisms as a weak-acid preservative, Stratford and Anslow (1998) suggested an inhibitory role for sorbic acid as a membrane-active compound. Sorbic acid acts at high pH where weak-acid preservatives are not expected to be active (Stratford and Anslow, 1996). The currently accepted theory of preservative action of these types of preservatives suggests that they act via depression of internal pH. Undissociated acid molecules are lipophylic and pass readily through the plasma membrane by diffusion. In the cytoplasm, approximately pH 7, acid molecules dissociate into charged anions and protons. These ions cannot pass across the lipid bilayer and accumulate in the cytoplasm, thus lowering cytoplasmic pH. This, in turn, inhibits metabolism by inhibiting the enzymes of glycolysis. The use of sorbic acid in baked goods creates no problems, provided that baking powder is used to raise the dough, and is added at 0.1–0.2% (Lück, 1980). It has not any residual taste, being its main drawback its high price (Calvo, 1991).

Bakery products are normally stored at room temperature, consequently a range from 15 to 30 °C was chosen for experiments. Referring to a_w, values as low as 0.70 were not used as none of the isolates tested could grow (Abellana et al., 1999), and then 0.75–0.90 was the range chosen, a bit higher than the typical products one. Finally, the legal limit for sorbic acid and sorbates in Spain is 0.2% in a dry matter basis; in the study we used a wet basis (so the highest value tested was a bit above the limit) and decreasing values from 0.2% to 0.025%.

No growth was observed at 0.75 a_w; although the minimum a_w for growth of *A. chevalieri* and *Aspergillus repens* has been established at 0.71 a_w in a malt extract agar (Ayerst, 1969), the nutrients are probably less easily assimilable in the food than in the agar medium. This would mean that bakery products with an initial a_w level less or equal to 0.75 would not require the use of preservatives at all, as long as the condensation of water on their surfaces is avoided by being wrapped when cool enough and avoiding wide temperature changes in the storage environment.

Significant differences were found between the isolates tested themselves and in relation to their responses to the different factors assayed; similarities were found, however, between the isolates belonging to the same species. In general, *E. chevalieri* and *E. amstelodami* isolates grew slower than *E. herbariorum* ones.

Other parameters such as colony-forming units (CFUs) and enzyme activity have been used to quantify the extent of fungal contamination on bakery products (Guynot et al., 2001; Marín et al., 2002b,c); however, the direct observation of the colonies on the surface of the products is, although time-consuming, the best one for research purposes.

The main conclusion drawn from this work is that sorbic acid and potassium sorbate are not useful at all at controlling spoilage in this kind of products. Previous studies have been carried out on cake analogues and *Eurotium* species (Guynot et al., 2001). A different methodology was used, however, to adjust a_w: sterile water was directly added to the pieces of cake analogues and left to equilibrate for 48 h at 25 °C. They concluded that potassium sorbate may only be useful at pH 6 but not at pH 7.5, which is nearer to the one in this study, so the conclusion is similar for both studies. They did not test different temperature levels, but the present work confirms that their results for 25 °C could be applied to the range 15–30 °C.

Consequently, bakery products with relatively high pH (7) may not need the use of preservatives as they do not have any effect on their shelf life. At the moment, many of these products are carrying useless preservatives which should be excluded from their recipes. There is a wide range of Spanish bakery products with these pH levels. In these products a suitable low a_w would be the main preservation factor.

On the other hand, 0.025%, 0.05% and 0.1% concentrations almost always led to an increase in colony radius; this means that the actual trend of reducing concentrations of preservatives could be counterproductive and cause negative effects on some foods.

The present work only shows the poor efficacy of sorbates in a single kind of bakery product (it could be a good example for high pH cakes, as the recipes are commonly quite similar). If other products such as croissants had been studied instead of Madeira cakes, better efficacy for preservatives would have

been found as these products have lower pH (around 5).

Another alternative to enhance preservative effects without increasing the concentrations is controlling other factors affecting fungal growth, such as temperature, a_w, pH and atmospheric conditions (Buchanan, 1993; El Halouat and Debevere, 1997). Water activity and pH, however, are too linked to organoleptic qualities of products, and they cannot be easily changed without altering them.

It can be concluded that for bakery products with pH near to 7, other alternatives must be considered. More investigation is needed in novel/natural preservatives, and modified atmosphere packaging for preservation of these products.

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