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Combined effects of weak acid preservatives, pH and water activity on growth of *Eurotium* species on a sponge cake

M.E. Guynot, A.J. Ramos*, D. Sala, V. Sanchis, S. Marín

Food Technology Department, Lleida University, UTPV-CeRTA, Rovira Roure 177, 25198 Lleida, Spain

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Abstract

The combined effects of weak acid preservatives (sorbate, benzoate and propionate), pH (6.0, 7.5) and water activity (a_w) levels (0.80, 0.85, 0.90) on growth of four *Eurotium* species isolated from bakery products on a sponge cake analogue were studied. Even though it is universally known that these preservatives are much more effective at lower pH values, we chose a 6–7.5 level to correlate with the pH of the Spanish cake product studied. In general, 0.3% doses of all three preservatives were effective only when they were applied at pH 6.0 and at 0.80–0.85 a_w. Potassium sorbate was clearly the most effective in inhibiting growth of all isolates. Under the conditions tested, application of all three preservatives added at 0.03% acted as growth promoter of all isolates rather than having a preservative effect.

Keywords: *Eurotium* spp.; Bakery product; Preservatives

1. Introduction

Microbial spoilage is the major problem causing deterioration in bakery products. It is caused mainly by moulds and yeasts and occasionally by bacteria (Earle and Putt, 1984). 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).

A study of different Spanish bakery products carried out by Abellana et al. (1997a) showed that the water activity (a_w) of these intermediate moisture products ranges from 0.71 to 0.79, with pH values between 4.26 and 8.82.

As products of intermediate moisture and slightly basic pH, cakes are susceptible to spoilage by xerophilic moulds (Seiler, 1988; Beuchat and Hocking, 1990; Pitt and Hocking, 1997; Fustier et al., 1998). Most filamentous xerophiles grow best in a range of 22–25 °C and the optimal pH is 6.5–6.8 (Beuchat and Hocking, 1990). The most widespread and probably most important moulds, in terms of biodeterioration of bakery products, are species of *Eurotium*, *Aspergillus* and *Penicillium* (Abellana et al., 1997b). *Eurotium* species as xerophiles are capable to grow below 0.85 a_w and are not fastidious in nutritional requirements (Beuchat and Hocking, 1990).

Most fungal spores are destroyed during baking by thermal inactivation (Earle and Putt, 1984; Legan, 1993). Post-process contamination by mould spores from the atmosphere or from surfaces during the cooling, finishing and wrapping procedures is unavoidable, however (Seiler, 1988; Fustier et al., 1998).

* Corresponding author. Tel.: +34-973-702-811; fax: +34-973-702-596.

E-mail address: ajramos@tecal.udl.es (A.J. Ramos).

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Seiler (1988) suggested that different levels of contamination have a small effect on mould-free shelf life. A tenfold reduction in inoculation level increased mould-free shelf life by only 10–20%, so attention to hygiene alone is unlikely to provide a large increase in mould-free shelf life, but it offers a simple way to reducing spoilage problems (Legan, 1993).

The addition of appropriate antimicrobial preservatives is used to reduce microbial spoilage and to increase shelf life of finished bakery products (Earle and Putt, 1984). In recent years, the use of chemical compounds in food products has come under increased criticism (Sofos and Busta, 1981). An option to enhance preservative effect and satisfy the consumers demands for reduced use of preservatives is to control factors affecting fungal growth, such as temperature, a_w, pH and atmospheric conditions (Buchanan, 1993; El Halouat and Debevere, 1997; Abellana et al., 1999a,b, 2000). This is the basic concept of the combined methods, which consists of a combination of various parameters (also called hurdles) that may act synergistically to inhibit or retard microbial growth resulting in stable products at room temperature (Chirife and Favetto, 1992; Leistner, 1992). Previous studies suggest that the effectiveness of weak acid preservatives is strongly linked with the pH and a_w (Earle and Putt, 1984; Seiler, 1988; Stratford and Anslow, 1998).

This study was carried out to determine the effect of different levels of weak organic acid preservatives, i.e. propionic, benzoic and sorbic acids, at different levels of a_w and pH, on the growth of different species of *Eurotium* on a sponge cake analogue medium, representative of a Spanish bakery product.

2. Material and methods

2.1. Fungal isolates

Single isolates of four species were used in this study: *Eurotium amstelodami* (3.205), *E. herbariorum* (3.209) and *E. rubrum* (3.228), isolated from bakery products (Abellana et al., 1997b). These isolates belong to the Food Technology Department microorganisms collection of the Lleida University. The other isolate, *E. repens* (18,000), was kindly provided by the Department of Biotechnology of the Technical University of Denmark and had been isolated from a Danish bakery product.

2.2. Preparation of the analogue

The medium used in this study was a Spanish sponge cake analogue described by Abellana et al. (1999b). 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. Ingredients were mixed and placed on aluminium plates. Dough was baked in an oven (P-Selecta, 210) at 160 °C for 30 min. Cooking foil was also put in the oven for sterilisation. After baking, plates were covered with the sterile cooking foil and transferred to the laminar flow bench. The cakes were exposed to UV light for 10 min to eliminate surface contaminants. They were then cut into 5×5 cm square pieces, which were aseptically placed into 9-cm sterile petri dishes.

The dough, after baking, had a pH of about 7.5, and its initial a_w was near 0.75. To reach the required pH (6.0), citric acid was added to the solid ingredients before cooking, whereas a_w was adjusted to 0.80, 0.85 and 0.90 by addition of sterile distilled water after cooking. The pH value was adjusted to 6 in order to avoid a big substantial change of the organoleptic features due to low pH. Previously, two curves were made. One to determine the amount of citric acid necessary to achieve the desired pH and another to determine the distilled water needed to increase the a_w of the dough.

Because of their high solubility, salts of each weak acid were used (Thakur et al., 1994). Calcium propionate, sodium benzoate and potassium sorbate were added to the solid ingredients before baking to give final concentrations of 0.03% and 0.3% on a weight basis.

Before inoculation, cake treatments were allowed to equilibrate at 25 °C for 48 h, in a closed container with beakers containing glycerol–water solutions of the same a_w as the sponge cake, in order to create an atmosphere with the same equilibrium relative humidity (ERH). Finally, a_w values were confirmed using a water activity meter (AquaLab, Decagon Devices, Pullman, WA, USA) and the pH by a pH meter (Crison, micropH 2001, Crison Instruments, Alella, Spain). The initial checked values obtained for a_w were 0.80 ± 0.03, 0.85 ± 0.02, 0.90 ± 0.01, and for pH, they were 6 ± 0.15 and 7.5 ± 0.15.

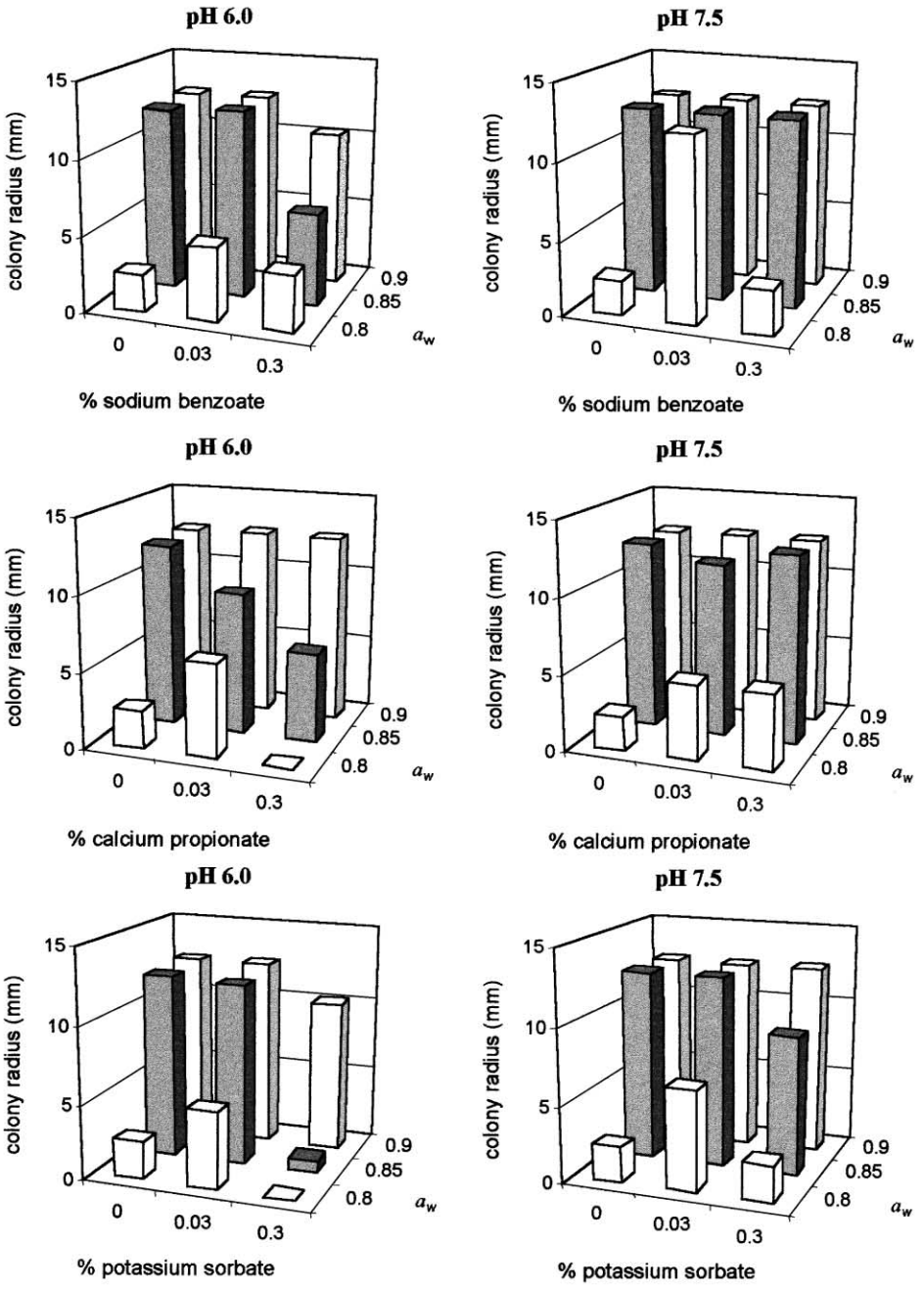

Fig. 1. Combined effect of different preservatives (sodium benzoate, calcium propionate, and potassium sorbate), pH, and water activity (a_w) on growth of *E. amstelodami* after 14 days of incubation at 25 °C.

2.3. Inoculation, incubation and growth assessment

Fourteen-day-old cultures of the isolates were used for experiments. Cake analogues were inoculated with a needle at four points using conidial suspensions of 10^6 spores/ml. Plates containing the cake analogue were then placed in the sealed containers described above in order to maintain the desired ERH. Containers were incubated at 25 °C for 28 days.

Diameters of the growing colonies were measured after 7, 14, 21 and 28 days, with the aid of a binocular magnifier. At the end of the experiment, after 4 weeks, cake analogues were analysed for fungal populations (CFU g^{-1}) by spread plating of serial dilutions using a suitable medium for xerophiles, Dichloran 18% glycerol agar (DG18) (Beuchat and Hocking, 1990; Pitt and Hocking, 1997). A ground portion of each cake analogue (approximately 5 g) was dried in an oven at

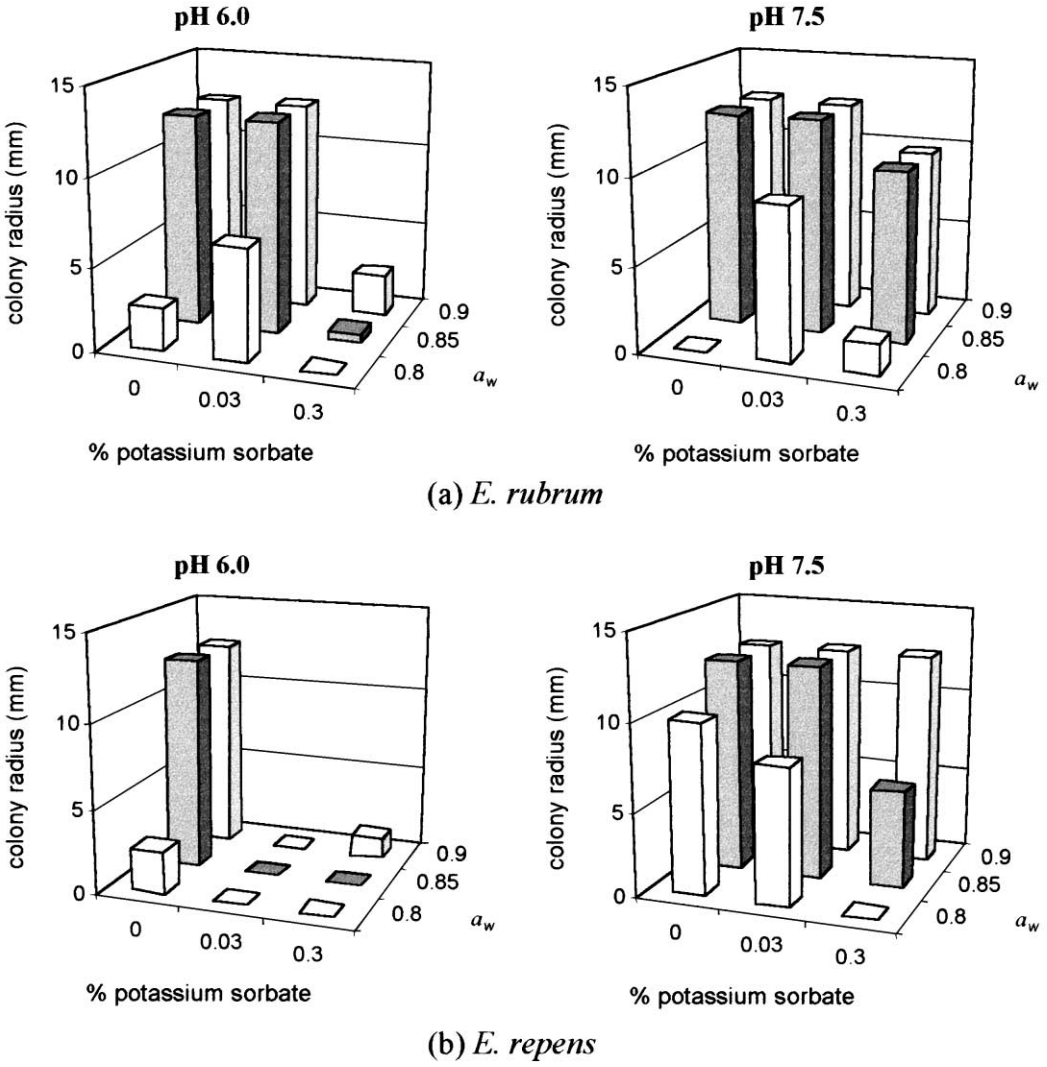

Fig. 2. Combined effect of potassium sorbate, pH, and water activity (a_w) on growth of two isolates: (a) *E. rubrum* and (b) *E. repens* after 14 days of incubation at 25 °C.

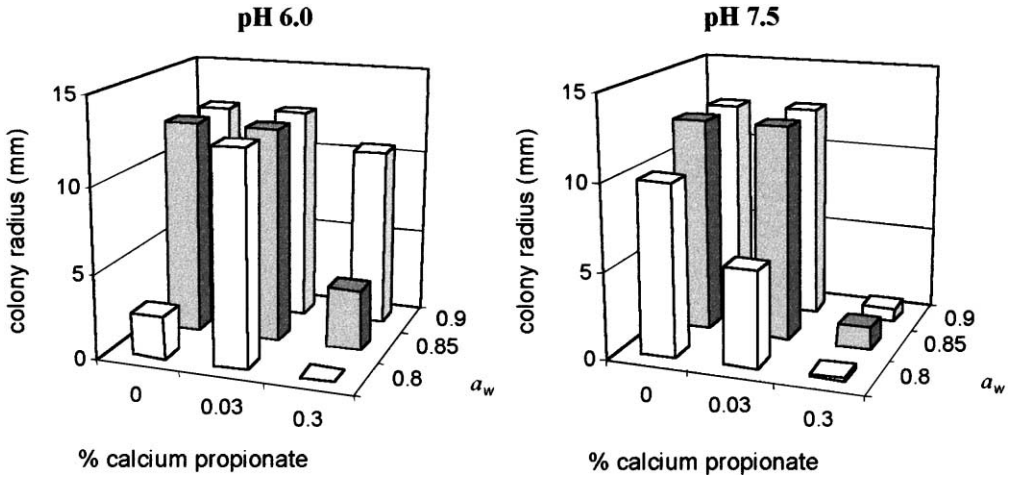

Fig. 3. Combined effect of calcium propionate, pH, and water activity (a_w) on growth of *E. repens* after 14 days of incubation at 25 °C.

130 °C for 1 h, to determine the moisture content and calculate the CFU on a dry matter basis.

2.4. Statistical analysis of results

Analyses of variance were calculated for colony radii after 7, 14, 21, 28 days and CFU g^{-1} by using Statistical Analysis System (SAS) program (SAS Institute, Cary, NC, USA). CFU data were transformed prior to analysis, by $y = \log(\text{CFU g}^{-1})$, to homogenise variance.

3. Results

3.1. Effect of preservatives on colony radius of spoilage fungi on cake analogues

The factors studied, as well as their interactions, have a significant effect on the growth of all isolates tested. Comparing the different days of measurement at the beginning of the incubation, the colony diameters were too small and, at the end, almost all analogues were totally colonised. The most effective days

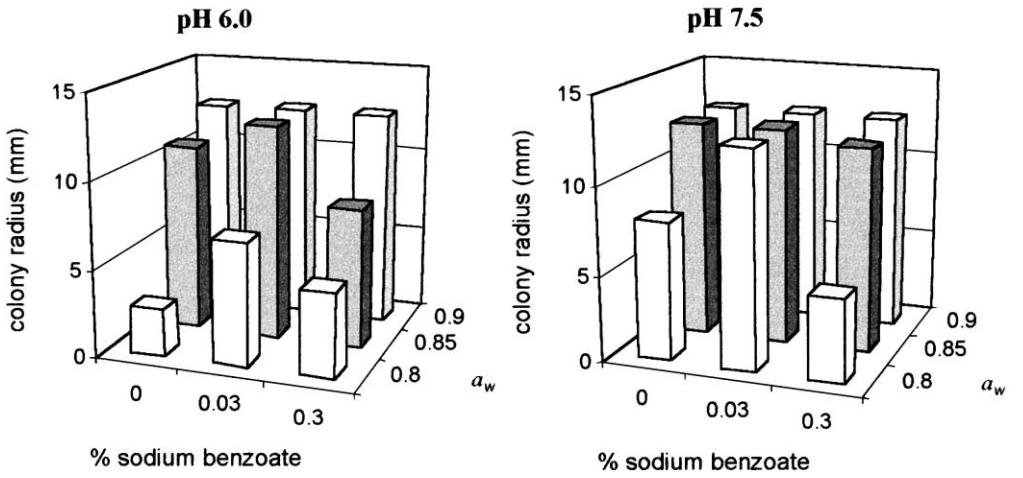

Fig. 4. Combined effect of sodium benzoate, pH, and water activity (a_w) on growth of *E. herbariorum* after 14 days of incubation at 25 °C.

for the statistical analyses were 14 and 21 days after inoculation, which gave comparable results.

The growth of all species was quite similar and, as was expected, growth was faster at 0.90 a_w and slower at the reduced water activity. In the absence of preservatives, all isolates grew better at pH 6.0 than at 7.5.

In general, the three preservatives were effective only at pH 6.0 and at 0.80–0.85 a_w. Potassium sorbate was clearly the most effective at inhibiting growth of all isolates. Fig. 1 shows the growth of *E. amstelodami* under all conditions tested. The combined effect of high doses of preservatives, low values of a_w and pH 6.0 had an additive effect in the inhibition of fungal growth. The best inhibition was found when 0.3% of potassium sorbate was used at pH 6.0 and 0.80–0.85 a_w for all isolates. At pH 6.0 and 0.90 a_w, *E. repens* and *E. rubrum* were also sensitive to sorbate and their growth decreased significantly (Fig. 2).

The efficacy of calcium propionate depended on the species, the most sensitive isolate being *E. repens*. It was inhibited at 0.3% at both pH tested in the range of 0.80–0.90 a_w. The efficacy on the other species was restricted to pH 6.0 and low levels of a_w (0.80–0.85) (Figs. 1 and 3).

All species reacted similarly to sodium benzoate. Contrary to what was expected, the inhibitory effect at pH 6.0 was higher at 0.85 than at 0.80 a_w. As an example, Fig. 4 shows the growth of *E. herbariorum* at all conditions tested. Sodium benzoate was also effective at 0.3%, pH 7.5/0.80 a_w, as well as pH 6.0/0.85 a_w.

An important observation is that, under some conditions, the use of low doses of all three preservatives led to a quicker spoilage of the cake. A larger colony radius was observed at 2 weeks when 0.03% preservative was applied (Figs. 1–4).

3.2. Effect of preservatives on populations (CFU) of spoilage fungi on cake analogues

Contrary to colony radius results, in this case, no good correlation was found. It has to be taken into account that CFU depend on the degree of sporulation. Only potassium sorbate was effective against all species. The best inhibition was obtained when a 0.3% dose was used at pH 6.0 and at 0.80 a_w. At higher values of water activity, the effectiveness of the

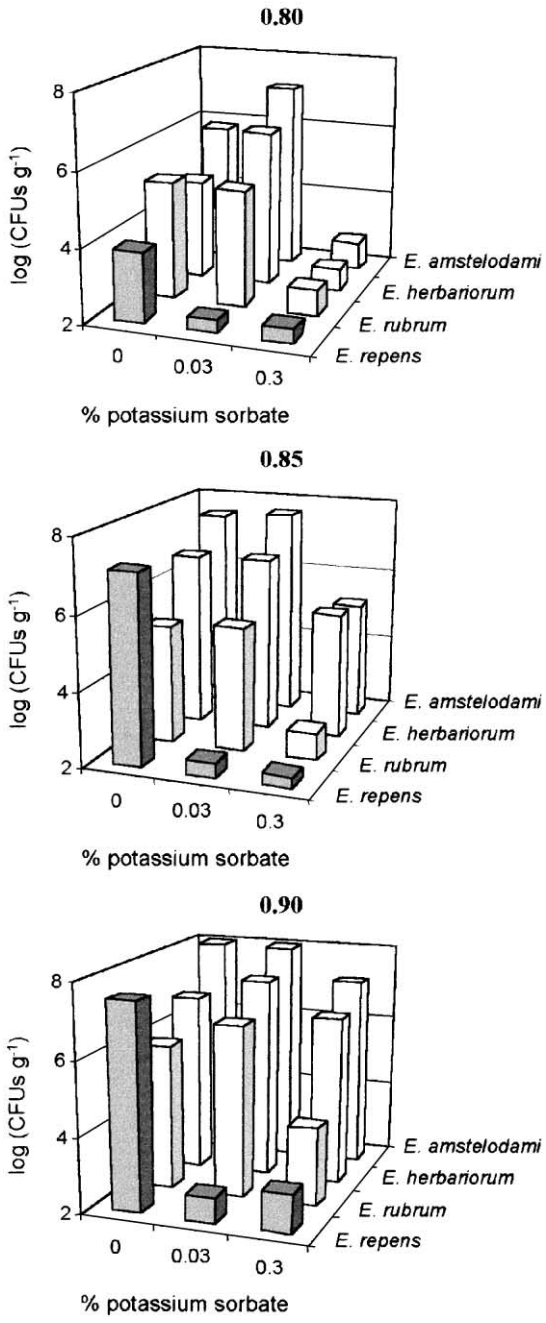

Fig. 5. Effect of potassium sorbate on the CFUs (colony forming units) on the different isolates at pH 6.0 and all water activity (a_w) tested after 14 days of incubation at 25 °C.

preservative depended on the species. Fig. 5 shows the growth of all isolates, in terms of the log of colony forming units (CFU), versus doses of potassium sorbate at pH 6.0 and different conditions of water activity. *E. repens* and *E. rubrum* were the more sensitive to 0.3% potassium sorbate at high a_w.

Only *E. repens* was sensitive to the low dose (0.03%) of potassium sorbate at pH 6.0. Growth of the remaining isolates was enhanced when 0.03% doses were used (data not show).

4. Discussion

Fungal species used in our study belong to the most widespread and important genus in biodeterioration of bakery products (Williams, 1990). A preliminary study on wheat flour agar was carried out by Marín et al. (submitted for publication), checking the effectiveness of the same preservatives on the growth of these *Eurotium* species and some *Aspergillus* and *Penicillium* species. They tested doses from 0.003% to 0.3% at different levels of pH (4.5, 5.0, 6.0 and 7.5) and water activity (0.80, 0.85, 0.90 and 0.95). Results showed that a 0.003% dose was completely useless at any pH level, while a total control of growth was achieved at pH 4.5 by using doses of both 0.03% and 0.3%, and at pH 6.0 with a 0.3% dose. The sponge cake used in our study has a pH near to 7.0 so the pH levels tested were 6.0 and 7.5.

It is important to note that we used an analogue of a Spanish bakery product so as to obtain results that are close to what could happen if this kind of product was contaminated by these species. We worked with pure cultures rather than mixtures in order to determine how the experimental condition alters the behaviour of each strain on the cakes. The interactions between species were not studied.

The usual a_w level of these products is 0.75–0.85, but it could be higher if product is wrapped without sufficient cooling, as this could result in condensation of water inside the packet. Consequently, the levels used for this study were 0.80, 0.85 and 0.90 a_w. Water activity is probably the most important environmental factor determining whether and at which rate a microorganism will grow on intermediate moisture food (Seiler, 1988; Fustier et al., 1998; Membré et al., 1999). In agreement with Leistner (1992) and with previous studies carried out in this laboratory by Abellana et al. (1999a,b), we found that reduction in the a_w of the analogues slowed deterioration.

Weak organic acid preservatives such as propionic, benzoic and sorbic acid are used as one of the numerous hurdles employed in food preservation (Chirife and Favetto, 1992; Legan, 1993; Thakur et al., 1994). Our study confirms the dependence of such type of preservatives upon the pH of the product, reported earlier by several studies (Earle and Putt, 1984; Seiler, 1988; Chirife and Favetto, 1992; Frías et al., 1996; Praphailong and Fleet, 1997; Fustier et al., 1998; Stratford and Anslow, 1998). We found a significant influence of a_w, pH and their interactions in growth inhibition. Preservative capacity was enhanced at low levels of pH (6.0) and a_w (0.80–0.85).

The currently accepted theory of the inhibitory mechanism action of this type of preservative suggests that they act via depression of internal pH, inhibiting metabolism, in particular, the enzymes of glycolysis (Russell, 1992). Praphailong and Fleet (1997) described the mechanisms by which some yeast species were tolerant to high concentrations of benzoate and sorbate. They had attributed the tolerance to weak acids with the ability of these microorganisms to pump out intracellular protons and anions. In agreement with previous studies (Earle and Putt, 1984; Legan, 1993), potassium sorbate was shown to be the most effective preservative against *Eurotium* species. In general, 0.3% was effective at pH 6.0 and 0.80–0.85 a_w for all isolates. However, at high a_w, some differences were found between species in their sensitivity to sorbate, with *E. repens* and *E. rubrum* being more affected. Stratford and Anslow (1998) suggested an inhibitory role for sorbic acid as a membrane-active compound since it acts at high pH where weak acid preservatives are not expected to be active. Chirife and Favetto (1992) reported that a_w influences the proportion of undissociated to dissociated propionic acid. They found that at a constant pH, the proportion of undissociated acid increased with decreasing a_w levels (from 1.00 to 0.73).

We observed the capacity of 0.03% doses to promote growth. This may be explained by the fact that certain microorganisms may be able to use this kind of products in their metabolism to obtain energy, if they are present, in subinhibitory concentrations (Thakur et al., 1994; Frías et al., 1996).

The direct measure of the colony diameter was clearly the most reliable method for analysing the effect of preservatives on fungal growth. Since sporulation and hyphal extension are not necessarily correlated, the analysis of fungal population (CFU g^{-1}) could under or overestimate the growth, depending on the degree of sporulation.

To conclude, the preservative with a bigger impact on the fungal growth was potassium sorbate. Its effect depended mainly on the pH, being only capable of inhibiting fungal growth at pH 6.0. Since a great number of bakery products (including sponge cakes) have a pH near 7.0, the use of these weak acids as preservatives is not enough to assure the microbiological safety. Another alternative in this hurdle technology approach could involve modified atmosphere packing (Abellana et al., 2000) or novel natural preservatives.

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References

Abellana, M., Torres, L., Sanchis, V., Ramos, A.J., 1997a. Caracterización de diferentes productos de bollería industrial: I. Estudio del pH y de la actividad de agua (a_w). Alimentaria 285, 75–77.

Abellana, M., Torres, L., Sanchis, V., Ramos, A.J., 1997b. Caracterización de diferentes productos de bollería industrial: II. Estudio de la micoflora. Alimentaria 287, 51–56.

Abellana, M., Benedí, J., Sanchis, V., Ramos, A.J., 1999a. Water activity and temperature effects on germination and growth of *Eurotium amstelodami*, *E. chevalieri* and *E. herbariorum* isolates from bakery products. J. Appl. Microbiol. 87, 371–380.

Abellana, M., Magrí, X., Sanchis, V., Ramos, A.J., 1999b. Water activity and temperature effects on growth of *Eurotium amstelodami*, *E. chevalieri* and *E. herbariorum* on a sponge cake analogue. Int. J. Food Microbiol. 52, 97–103.

Abellana, M., Sanchis, V., Ramos, A.J., Nielsen, P.V., 2000. Effect of modified atmosphere packaging and water activity on growth of *Eurotium amstelodami*, *E. chevalieri* and *E. herbariorum* on a sponge cake analogue. J. Appl. Microbiol. 88, 606–616.

Beuchat, L.R., Hocking, A.D., 1990. Some consideration when analyzing foods for the presence of xerophilic fungi. J. Food Prot. 53, 984–989.

Buchanan, L., 1993. Predictive food microbiology. Trends Food Sci. Technol. 4, 6–11.

Chirife, J., Favetto, J., 1992. Some physico-chemical basis of food preservation by combined methods. Appl. Technol. 25, 389–396.

Earle, M.D., Putt, G.J., 1984. Microbial spoilage and use of sorbate in bakery products. Food Technol. N. Z. 19, 25–36.

El Halouat, A., Debevere, J.M., 1997. Effect of water activity, modified atmosphere packing and storage temperature on spore germination of moulds isolated from prunes. Int. J. Food Microbiol. 35, 41–48.

Frías, I., Alvarez, R., Sierra, A., Hardisson, A., 1996. Aspectos bromatológicos y toxicológicos de los conservantes benzoico y sórbico. Alimentaria 273, 109–114.

Fustier, P., Lafond, A., Champagne, C.P., Lamarche, F., 1998. Effect of inoculation techniques and relative humidity on the growth of moulds on the surfaces on yellow layer cakes. Appl. Environ. Microbiol. 64, 192–196.

Legan, J.D., 1993. Mould spoilage of bread: the problem and some solutions. Int. Biodeterior. Biodegrad. 32, 33–53.

Leistner, L., 1992. Food preservation by combined methods. Appl. Technol. 25, 151–158.

Malkki, Y., Rauha, O., 1978. Mould inhibition by aerosol. Baker's Dig. 52, 47.

Marín, S., Guynot, M.E., Neira, P., Bernadó, M., Sanchis, V., Ramos, A.J., 2002. Risk assessment of using suboptimal doses of weak acid preservatives for preventing bakery products mould contaminants from growing. Int. J. Food Microbiol., submitted for publication.

Membré, J., Kubaczka, M., Chéné, C., 1999. Combined effects of pH and sugar on the growth rate of *Zygosaccharomyces rouxii*, a bakery product spoilage yeast. Appl. Environ. Microbiol. 65, 4921–4925.

Pitt, J.I., Hocking, A.D., 1997. Fungi and Food Spoilage, 2nd edn. Aspen Publishers, Gaithersburg, MD, USA.

Praphailong, W., Fleet, G.H., 1997. The effect of pH, sodium chloride, sucrose, sorbate and benzoate on the growth of food spoilage yeasts. Food Microbiol. 14, 459–468.

Russell, J.B., 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. 73, 363–370.

Seiler, D., 1988. Microbiological problems associated with cereal-based foods. Food Sci. Technol. Today 2, 37–41.

Sofos, J.N., Busta, F.F., 1981. Antimicrobial activity of sorbate. J. Food Prot. 44, 614–622, 647.

Stratford, M., Anslow, P.A., 1998. Evidence that sorbic acid does not inhibit yeast as a classic weak acid preservative. Lett. Appl. Microbiol. 27, 203–206.

Thakur, B.R., Singh, R.K., Arya, S.S., 1994. Chemistry of sorbate — a basic perspective. Food Rev. Int. 10 (1), 71–91.

Williams, A.P., 1990. *Penicillium* and *Aspergillus* in the food microbiology laboratory. In: Samson, R.A., Pitt, J.I. (Eds.), Modern Concepts in *Penicillium* and *Aspergillus* Classification. Plenum, New York, pp. 67–71.