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Risk assessment of the use of sub-optimal levels of weak-acid preservatives in the control of mould growth on bakery products

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Abstract

The hurdle technology approach was used to prevent fungal growth of common contaminants of bakery products including isolates belonging to the genera *Eurotium*, *Aspergillus* and *Penicillium*. Several levels (0.003%, 0.03% and 0.3%) of calcium propionate, potassium sorbate and sodium benzoate were assayed on a model agar system in a full-factorial experimental design in which the other factors assayed were pH (4.5, 6 and 7.5) and a_w (0.80, 085, 0.90 and 0.95). Potassium sorbate was found to be the more suitable preservative to be used in combination with the common levels of pH and a_w in Spanish bakery products. Sub-optimal concentrations (0.003% and sometimes 0.03%) led to an enhancement of fungal growth. None of the preservatives had a significant inhibitory effect at neutral pH.

Keywords: *Eurotium*; *Aspergillus*; *Penicillium*; Bakery products; Preservatives

1. Introduction

Mould growth on bakery products during storage is a serious economic problem. Because of the water activity of these products (0.75–0.90 a_w), spoilage moulds are commonly xerophilic, *Eurotium* and *Aspergillus* species. Moulds are destroyed during baking and contamination arises from mould spores derived from atmosphere or from surfaces during the cooling, finishing and wrapping procedures (Seiler, 1988).

In the last few years, the bakery products and flour confectionery sector has witnessed intense technological progress which has brought many changes in process innovation, and in commercial and qualitative characteristics of the products. Usually, bakery products are packaged in plastic films after baking and cooling, and they are consumed within 1 or 2 months (Ponte and Tsen, 1987).

Hurdle technology is now widely used in the design of new food products (Leistner, 1992). Hurdle technology combined with predictive microbiology may be the most suitable tool to develop new and safe products. The main variables to be considered in predictive microbiology for bakery products design must include a_w and pH, as well as others such as atmosphere composition in the package, concentration of preservatives, storage temperature, etc.

Weak organic acids such as propionic, benzoic and sorbic are used as hurdles in food preservation. The antimicrobial activity of these weak acids is mainly dependent upon the undissociated molecule (Eklund

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1983, 1985; Pethybridge et al., 1983). A reduction of pH from 6.0 to 5.0–5.2 results in a large relative increase in the proportion of the undissociated form (Chirife and Favetto, 1992). However, the modern trend is to reduce the concentration of preservatives in foodstuffs due to consumer demands.

The range of microorganisms used in this study represents the typical mycoflora of Spanish bakery products consisting of xerophilic species of *Eurotium*, *Aspergillus* and *Penicillium*. The a_w of these products varies between 0.70 and 0.85 approximately. Many *Aspergillus* species are xerophilic and capable of growing on media containing high concentrations of salt or sugar (Pitt and Hocking, 1997). Most xerophiles grow best between 22 and 25 °C (Beuchat and Hocking, 1990). Bakery products are kept at room temperature, conditions under which xerophiles grow optimally.

Spanish bakery products have pH values ranging from 4.3 (ensaimada) to 8.8 (magdalenas) (Abellana et al., 1997). The optimal pH for growth of most xerophiles is from 6.5 to 6.8 (Beuchat and Hocking, 1990). Some products spoil before the expiry date, and the most likely cause is condensation of moisture on the surface of the cakes after packing, if they have not been cooled enough. A recent study on bakery products (Abellana et al., 1999a) highlights the importance of a_w and storage temperature on the growth of *Eurotium* species.

The objectives of the present work were (i) to study the suitability of using both standard and sub-optimal concentrations of weak-acid preservatives to prevent the growth of common bakery products contaminants (the hurdle technology concept was applied by combining pH, a_w and preservatives as hurdles) and (ii) to develop predictive models from the data as tools for product design.

2. Material and methods

2.1. Fungal strains

Five isolates, *Eurotium amstelodami* (3.205), *E. herbariorum* (3.209), *E. rubrum* (3.228), *Aspergillus flavus* (3.226) and *A. niger* (3.227), from Spanish bakery products were used. These isolates are held in the culture collection of the Food Technology Department of Lleida University, Lleida, Spain. Isolates of two other species, *E. repens* (IBT 18 000) and *Penicillium corylophilum* (IBT 6978), were kindly provided by the Department of Biotechnology, Technical University of Denmark, Lyngby, Denmark and had been isolated from Danish bakery products.

2.2. Experimental design

A full four factorial design with three replicates was applied. The factors assayed were three different weak-acid preservatives: calcium propionate (Fluka, Switzerland); potassium sorbate (Panreac, Spain); and sodium benzoate (Probus, Spain) and a_w (0.80, 085, 0.90 and 0.95), pH (4.5, 6 and 7.5) and concentrations of preservatives (0%, 0.003%, 0.03% and 0.3%). The responses recorded were colony diameters and time to visible growth (lag phase).

2.3. Media preparation

The basic medium used in this study was a 2% wheat flour agar (20 g of commercial wheat flour in 1 l of medium). Various amounts of glycerol were added to the basic medium in order to achieve the desired a_w levels (210, 320, 440 and 520 g for 0.95, 0.90, 0.85 and 0.80 a_w, respectively). Previously, a curve was obtained by adding different amounts or glycerol to a certain amount of medium and measuring the resulting a_w levels. Simultaneously, pH was varied by substituting water in the media by McIlvaine's buffer consisting of 0.1 M citric acid and 0.2 M Na_2HPO_4. Agar (2%) and the required amount of preservative were added to the media before autoclaving. Molten media (15 ml) was poured into 9-cm sterile Petri plates. The a_w and pH of the final media were checked with an AquaLab instrument (Decagon, Pullman, WA), and a Crison micropH2000 pH meter (Crison, Barcelona), respectively.

2.4. Inoculation, incubation and measurement

For each isolate, a conidial spore suspension of 10^6 spores ml^{-1} was prepared and Petri plates were point-inoculated in the centre. Plates were incubated at 25 °C in sealed polyethylene bags in order to maintain a constant relative humidity value.

The Petri plates were examined daily or as required and the diameter of the growing colonies measured in

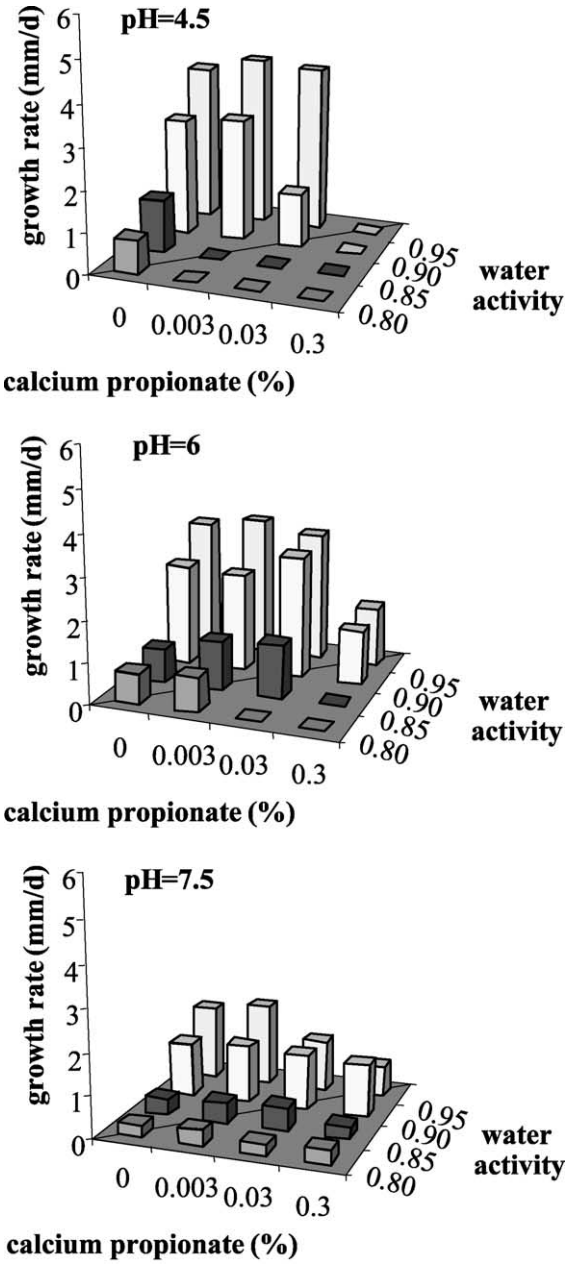

Fig. l. Combined effect of calcium propionate, pH and a_w on growth rates (mm day^{-1}) of *E. rubrum*.

two directions at right angles to each other. Measurements were carried out for a maximum of 60 days because shelf life for bakery products ranges from 4 to 8 weeks. Lag phase was defined as the time until 5 mm of diameter colony was visible.

2.5. Statistical treatment of the results

Analysis of variance was carried out for the whole set of data in order to find significant differences

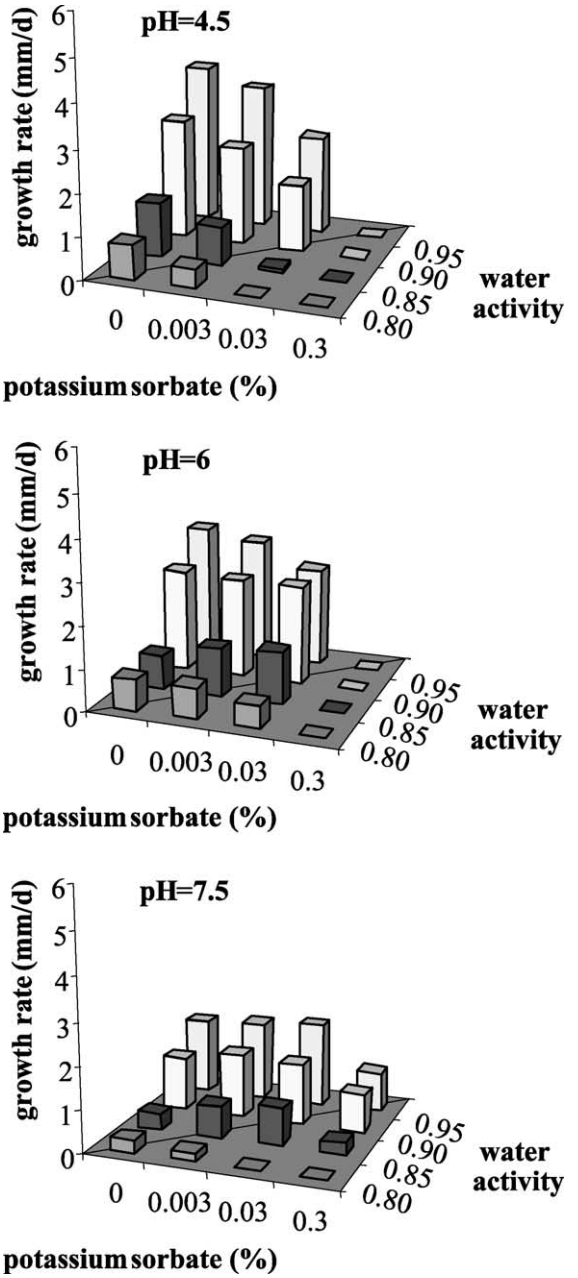

Fig. 2. Combined effect of potassium sorbate, pH and a_w on growth rates (mm day^{-1}) of *E. rubrum*.

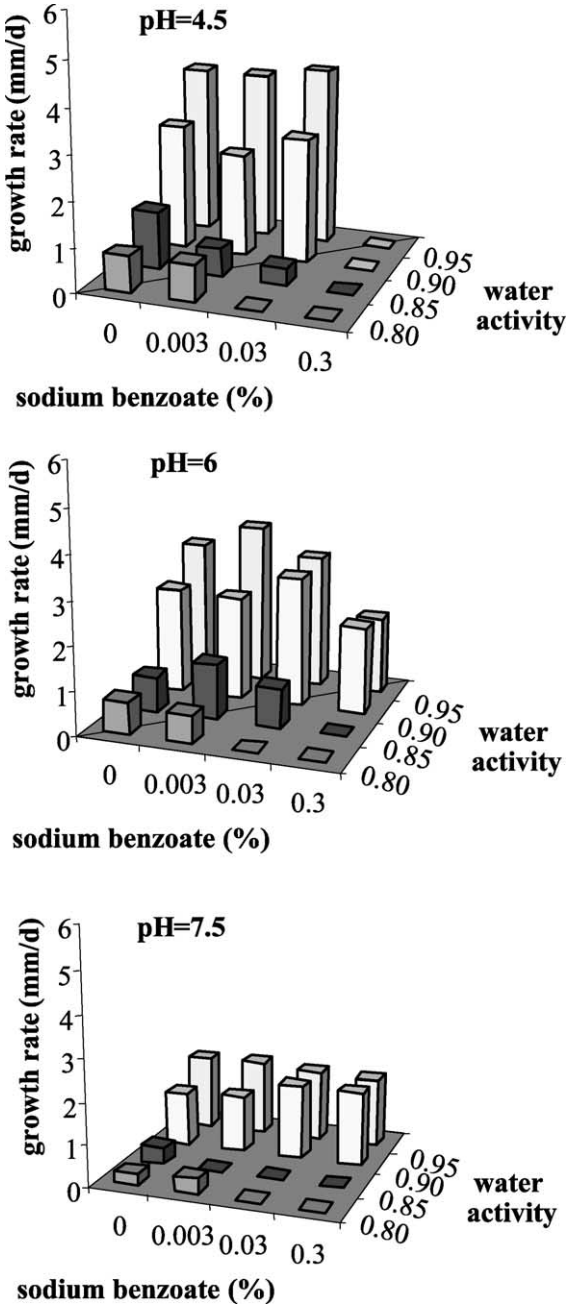

Fig. 3. Combined effect of sodium benzoate, pH and a_w on growth rates (mm day^{-1}) of *E. rubrum*.

between the levels of factors assayed and significant interactions between factors. For this purpose, Statistical Analysis System package (SAS, version 6.12) was used. Time was present in the analysis as a covariate. Colony radius was plotted against time to obtain growth rates (mm day^{-1}) under each set of treatment conditions. Finally, both growth rates and lag phases were analysed by projection to latent

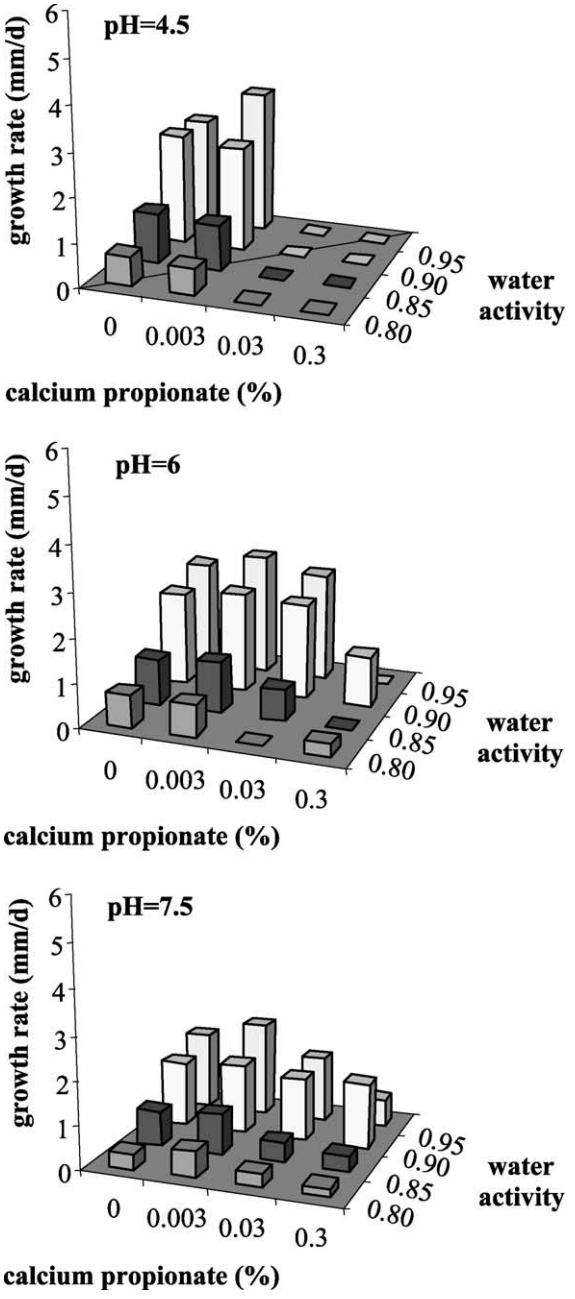

Fig. 4. Combined effect of calcium propionate, pH and a_w on growth rates (mm day^{-1}) of *E. amstelodami*.

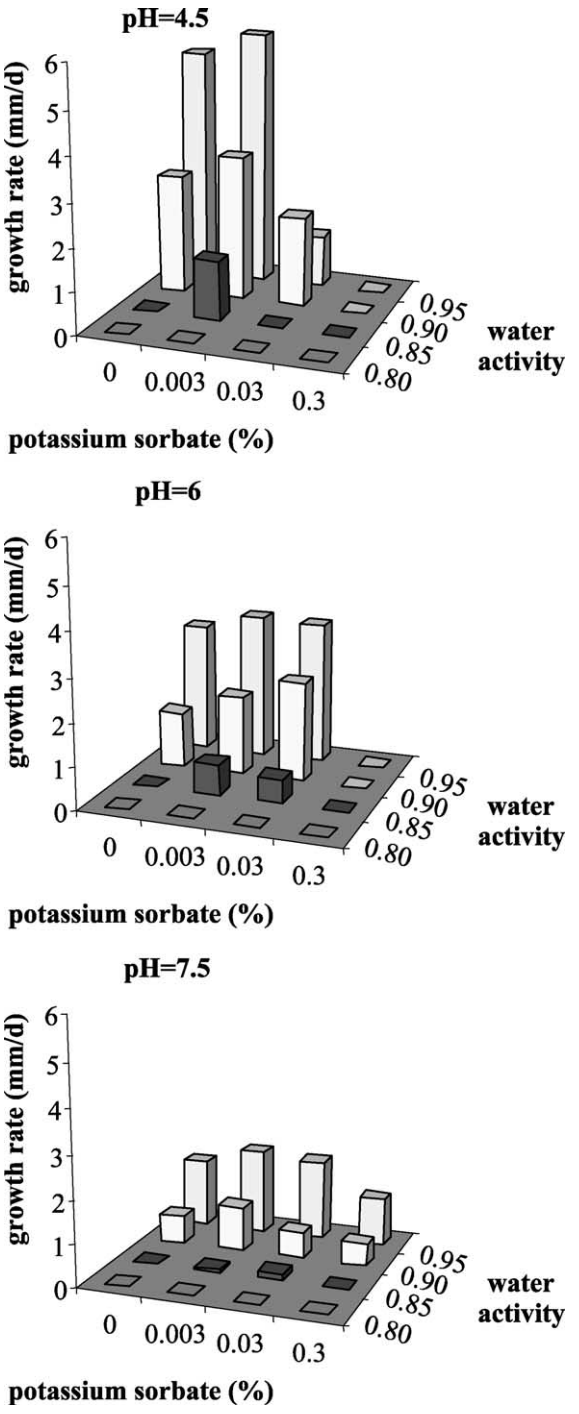

Fig. 5. Combined effect of potassium sorbate, pH and a_w on growth rates (mm day^{-1}) of *A. niger*.

structures (PLS, by means of partial least squares analysis) in order to establish suitable predictive models. PLS is a regression extension of PCA which is used when it is of interest to connect the information in two blocks of variables, *X* and *Y*, to each other (Eriksson et al., 1999). The latter analyses were performed with Simca-P version 7.01 software (Umetrics, Sweden).

3. Results

3.1. Impact of weak-acid preservatives on growth rates

Under optimum conditions, *Aspergillus* species grew faster (maximum growth rates of 7 mm day^{-1}) than *Eurotium* isolates (maximum growth rates of 4 mm day^{-1}) and *P. corylophilum* (maximum growth rate of 2 mm day^{-1}). *Eurotium* isolates, mainly *E. amstelodami* and *E. rubrum*, were able to grow at low a_w (e.g., 0.80 a_w), with decreasing growth rates from 0.95 to 0.80 a_w, whereas the *Aspergillus* and *Penicillium* isolates barely grew at 0.85 a_w. In the absence of preservatives, growth rates always decreased with decreasing a_w values. Growth of all isolates was faster at pH 4.5 and slowest at 7.5.

According to the analysis of variance, all single factors had a significant effect on colony radiuses and interacted significantly among them. However, there was no general trend for the efficacy of the different preservatives (benzoate, sorbate and propionate) tested: their controlling ability depended on the other factors. For example, the different preservatives and concentrations significantly affected growth rates, under all a_w levels. For some of the isolates, no

Table 1
Goodness of fit (R^2) and prediction (Q^2) of the three-component models fitted for each preservative

Component	Calcium propionate		Potassium sorbate		Sodium benzoate	
	R^2	Q^2	R^2	Q^2	R^2	Q^2
1	0.603	0.548	0.619	0.578	0.551	0.472
2	0.679	0.594	0.741	0.683	0.628	0.523
3	0.741	0.643	0.786	0.704	0.672	0.523

R^2, goodness of fit.
Q^2, goodness of prediction.

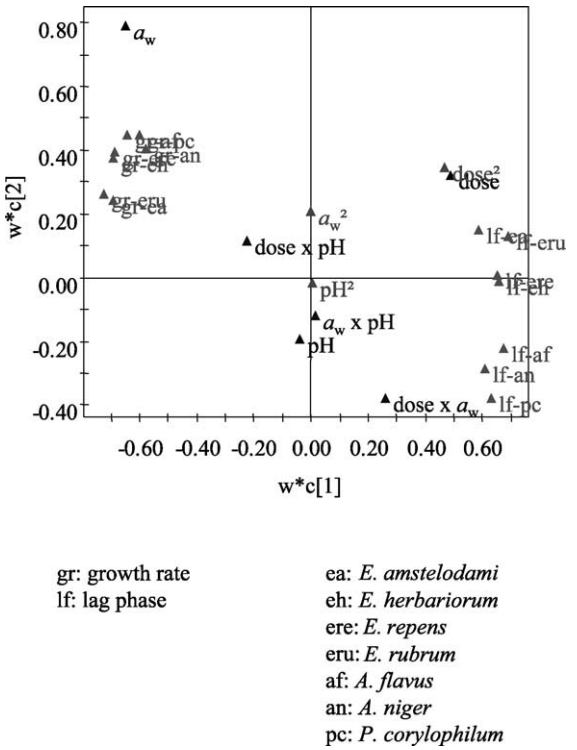

Fig. 6. Loadings plot obtained for the two first components of the model fitted for potassium sorbate.

differences were observed between types of preservatives at pH 6. For *A. flavus* and *E. amstelodami*, calcium propionate appeared to be the more useful, but *E. repens* and *E. rubrum* were somewhat better controlled by potassium sorbate. Figs. 1–3 show the complete set of growth rates obtained for *E. rubrum*.

The lower concentrations of preservatives enhanced growth under most of the conditions. There was a very significant interaction between pH and preservative concentration. In general, for *E. amstelodami*, *E. herbariorum* and sometimes for *E. rubrum* the 0.003% concentration increased the growth rate under all pH levels, while the 0.03% concentration was only effective at pH 4.5. The 0.3% concentration controlled growth at both 4.5 and 6 pH values, but it was much more effective at 4.5 (Fig. 4). *E. repens* and *Aspergillus* and *Penicillium* isolates were not significantly inhibited by a 0.003% nor a 0.03% concentration of any of the preservatives, while the 0.3% concentration had an effect similar to that for the other isolates (Fig. 5).

In summary, calcium propionate and sodium benzoate would be only suitable for the whole a_w level range tested at 0.3% concentration and pH 4.5, while a 0.3% concentration of potassium sorbate inhibited totally the growth of all the isolates at both 4.5 and 6.

3.2. Impact of weak-acid preservatives on lag phases

Experiments lasted a maximum of 60 days, consequently this value is the maximum lag phase recorded for many treatments. A similar, but inverted pattern to the one observed for growth rates was obtained.

For *Eurotium* isolates at 0.90–0.95 a_w, increases in the lag phases due to the two lower preservative concentrations were almost negligible (3–4 days at most). By contrast, no growth was observed at the 0.3% concentration at pH 4.5, and sometimes pH 6, depending on the isolate considered. At 0.80–0.85 a_w, longer lag phases were found in the range from 2 to 60 days.

With few exceptions, *Aspergillus* and *Penicillium* isolates had lag phases of more than 60 days at 0.80–

Table 2
Model coefficients obtained for each isolate using potassium sorbate as preservative for prediction of lag phases

	A. flavus	*A. niger*	*P. corylophilum*	*E. rubrum*	*E. repens*	*E. herbariorum*	*E. amstelodami*
Constant	1.200	1.372	1.556	0.949	1.020	1.044	0.926
Conc.	0.243	0.186	0.174	0.358	0.296	0.294	0.315
a_w	− 0.656	− 0.677	− 0.746	− 0.401	− 0.481	− 0.505	− 0.319
pH	− 0.118	− 0.144	− 0.072	− 0.221	− 0.229	− 0.236	− 0.227
Conc.^{2}	0.214	0.154	0.143	0.336	0.270	0.266	0.296
a_w^2	0.065	0.086	0.020	0.169	0.171	0.176	0.179
pH^2	− 0.006	− 0.009	− 0.009	− 0.015	− 0.016	− 0.017	− 0.017
Conc.×a_w	0.238	0.239	0.239	0.104	0.135	0.143	0.068
Conc.×pH	− 0.233	− 0.243	− 0.243	− 0.212	− 0.231	− 0.239	− 0.189
a_w×pH	− 0.050	− 0.069	− 0.069	− 0.114	− 0.121	− 0.124	− 0.121

0.85 a_w. At 0.90–0.95 a_w, lag phases were shorter than 30 days when no preservative was used or at 0.003% concentration. Higher concentrations led to longer lag phases at pH 4.5 and 6 (>60 days). In general, lag phases were either quite short (<9 days) or >60 days.

It was interesting to note that, in most cases a slow growth rate did not necessary relate to a long lag phase. For example, *Eurotium* species grew slowly at 0.80–0.85 a_w but lag phases were similar to those at 0.90–0.95 a_w. Similarly, although growth rates were low at pH 7.5, lag phases were similar to those recorded for the other pH levels.

3.3. Modelling of the results

Initially, an overview of the results was carried out by principal component analysis (PCA) on *Y*-variables. This overview led to the exclusion of one outlying observation from the initial set of results. After that a two-component fit was carried out. The first component highlighted the negative correlation between growth rates and lag phases and the positive correlation among growth rates themselves (for different isolates) and lag phases themselves. The second component explained the difference between lag phases of *Eurotium* species and the other isolates.

Both PCA and projections to latent structures (PLS) are multivariate methods. The ability to predict response variables (*Y*) from an assembly of predictor variables (*X*) is perhaps the most intriguing and important objective in multivariate data analysis (Eriksson et al., 1999). The PLS analysis led to a three-component model. In this model, the different weak-acid preservatives were considered as different classes. The cumulative R^2 and Q^2 values obtained for the whole model and separately by classes are shown in Table 1. R^2 and Q^2 were excellent for propionate and sorbate, but not as good for benzoate. Growth rates generally had higher R^2 and Q^2, although *Aspergillus* and *Penicillium* isolates had also good fits for lag phases. Fig. 6 shows the loadings plot for the first two components for sorbate, although plots for all three preservatives were very similar. From this picture, it can be seen the high influence of a_w and concentration in the first component, the positive correlation between a_w and growth rates and between concentration and lag phases, and the negative corre-

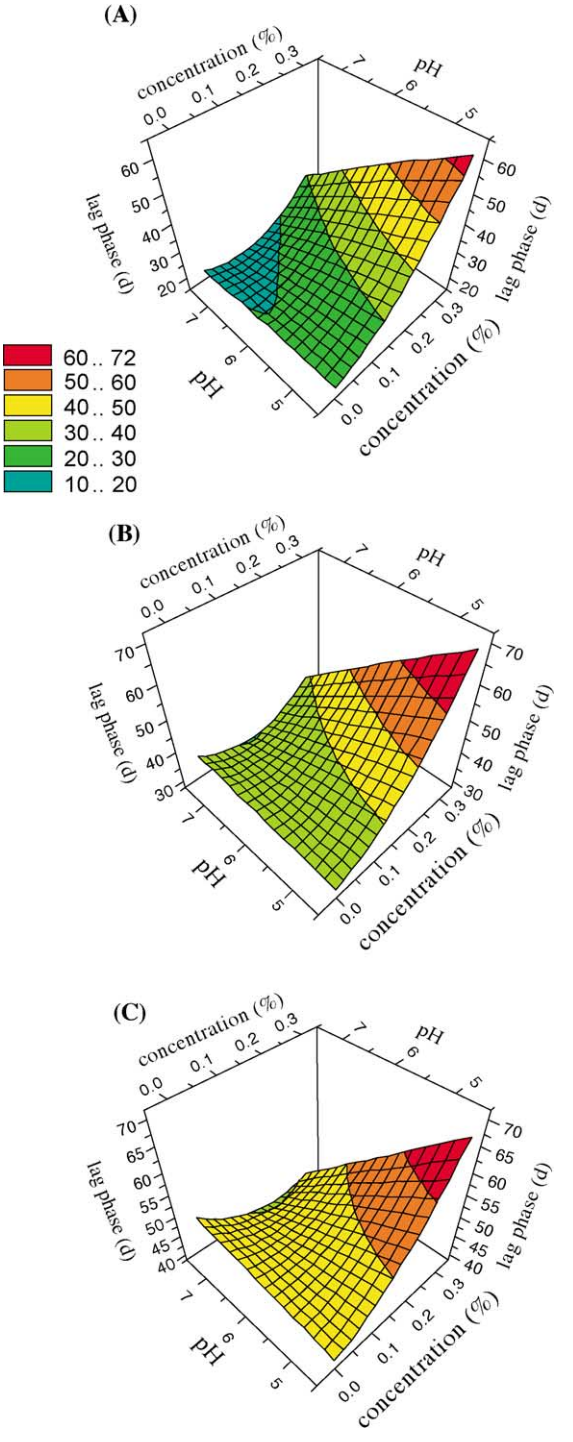

Fig. 7. Response surface models for (A) *E. herbariorum*, (B) *A. flavus*, and (C) *P. corylophilum* obtained at 0.85 a_w and using potassium sorbate as preservative.

lation between a_w and lag phases and between concentrations and growth rates. On the other hand, a_w, concentration, pH and concentration $\times$ a_w were the major factors affecting the second component, while the third component represented mainly the pH effect.

Table 2 shows the coefficients for the model using potassium sorbate as an example for each variable and Fig. 7 represents some of these models. The coefficients give a quick idea of the importance given by the model to each factor involved.

4. Discussion

Results from this work were obtained in an agar medium so the conclusions cannot be directly extrapolated to actual products. The results provide a general picture of the responses of these microorganisms to the environmental conditions which occur in these products. It should be noted that effective levels of preservatives might differ significantly in foods compared with culture media.

In this study, the combined impact of preservatives and other important growth factors such as a_w and pH have been assayed. Fungal growth can be prevented by adding preservatives but there is increasing pressure to reduce use of preservatives (Sofos and Busta, 1981). Growth may also be controlled by manipulating physical factors (El Halouat and Debevere, 1997). Earle and Putt (1984) pointed out that by using the appropriate pH and ERH to maximise the effect of sorbic acid, the amount of the acid needed to produce a specific increase in shelf life can be minimised. However, the decrease in preservative concentrations must be carefully carried out and other factors, such as pH of products must be taken into account if spoilage is to be avoided. A 0.003% concentration may be bad at any pH level, while 0.03% would be only useful at pH 4.5 but have a negative effect at 6–7.5.

Preservatives have no effect on the shelf life of bakery products of high pH (near 7.0). Many Spanish bakery products are of this pH, and contain useless preservatives that should be removed from their recipes. In such products, low a_w is the main preservation factor.

In the treatments where some growth was reported, no differences were observed on the effectiveness of the preservatives used here. Potassium sorbate, however, totally inhibited growth over a wider range of conditions. Consequently, this would be the most suitable weak-acid preservative for use in bakery products as it prevented fungal growth at both pH 4.5 and 6, regardless of a_w levels (0.80–0.95 a_w). This is very important as the other preservatives tested would be only useful for certain acid bakery products such as croissants or ensaimadas, and only at the 0.3% level. Sodium benzoate has the advantage of low cost, but is mainly useful in foods with a pH value from 4.0 to 4.5 or lower (Chichester and Tanner, 1972). Sorbic acid is less pH-dependent as it can be effective at pH 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). Sorbic salt was used in the experiment because it is known that sorbic acid cannot be used in baked goods as it sublimes upon heating (Chichester and Tanner, 1972).

It must be taken into account, however, that a wide a_w range has been used in this study (0.80–0.95 a_w). Most common bakery products have a_w values in the range 0.70–0.85, which restricts fungal growth. Under low a_w conditions, the situation is more optimistic as a total control of growth may he achieved at pH 4.5 by using concentrations of both 0.03% and 0.3% and at pH 6 with a 0.3% concentration.

The results for the different isolates tested, indicated that *Aspergillus* and *Penicillium*, although commonly found in cakes do not represent an important spoilage risk if the industrial process is well-controlled and the a_w is below 0.80. *E. amstelodami* and *E. rubrum* were the species with a major potential to cause spoilage.

In the last few years, predictive microbiology has become an important research area. Most of the studies, deal with bacteria and yeasts, with few researchers working with moulds, due mainly to the fact that it is difficult to find a right quantification method. Abellana et al. (1999b) predicted both lag phases and germination rates in relation to a_w and temperature for some *Eurotium* species on a flour wheat–sucrose agar media. Models such as the examples given in this paper may be an important tool to help food technologists to predict shelf life of products or may give an

idea of the suitability of a certain change in the product formulation. Although the use of models such as these obtained from an in vitro experiment are only indicative, they may save time and money invested in screening at pilot or industrial scale.

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