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Modelling the inhibition of sorbic and benzoic acids on a native yeast cocktail from table olives

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ABSTRACT

The single and combined effects, in a synthetic medium at selected pH values, of sorbic and benzoic acids on a yeast cocktail (*Saccharomyces cerevisiae*, *Pichia anomala*, *Issatchenkia occidentalis*, and *Candida diddensiae*, isolated from table olives) have been studied. Applying the checkerboard method the minimum inhibitory concentration (MIC) obtained for the respective individual preservatives (expressed as undissociated acid) were: sorbic acid, 5.94, 3.85 and 3.19 mM at pH of 4.5, 4.0, and 3.5, respectively; and benzoic acid, not detected (at total 20.5 mM), 10.40 and 6.83 mM, respectively, for the same pH levels. The estimated fractional inhibitory concentrations (FIC) indexes showed additive effects between inhibitors. Fractional area (fa), modelled by the (extended) Lambert and Lambert [2003. A model for the efficacy of combined inhibitors. J. Appl. Microbiol. 95, 734–743] equation (ELPM), also showed additives of both preservatives but different shapes in the dose–response curves; the individual MIC (as undissociated acid) deduced from this method were: 5.60, 3.31, and 3.26 mM for sorbic acid at pH of 4.5, 4.0, and 3.5, respectively; and 29.65 (extrapolated), 10.00, and 6.25 mM for benzoic acid at the same pH levels. Mixtures above the curves connecting the limits (MIC) at each pH were also inhibitory. There was agreement between MIC values from FIC and ELPM, although the last one provided further information on the inhibition behaviour. *I. occidentalis* was the most resistant yeast of the cocktail to sorbic and benzoic acids.

1. Introduction

Yeasts are well known for their importance in food and beverage production. However, they are also significant as spoilage microorganisms, especially in food and beverages with a low pH, high salt concentrations and low temperatures (Querol and Fleet, 2006).

Table olive packaging can suffer spoilage due to yeasts in spite of the habitual low pH obtained in the final products (Garrido Fernández et al., 1997). Yeasts can grow if a residual sugar concentration is present in the packed olives, where they can reach population levels of $\approx 6 \log_{10}$ colony forming units (cfu)/ml (Arroyo López et al., 2005). Panagou (2004) reported that untreated green olives packed in acidified brines under aerobic conditions or modified atmospheres showed a slight increase in yeast counts of about 1.0–1.5 $\log_{10}$ cfu/g. The effect of sorbic or benzoic acids and their salts on yeasts and their use to extend the shelf life of foods is documented in the scientific literature (Lück, 1990; Praphailong and Fleet, 1997; Querol and Fleet, 2006). Several works have been carried out to determine the influence of sorbate and benzoate on olive yeast populations during fermentation (Turantas et al., 1999) or packing (Arroyo López et al., 2006a, 2007a). Other studies on the use of sorbic and benzoic acids to stabilize table olives were reported by Rodriguez de la Borbolla y Alcalá et al. (1961) and Marsilio and Cichelli (1992). The single or combined use of these weak acids (or their salts) in table olives is authorized by the Trade Standards for Table Olives issued by the International Olive Oil Council (IOOC). The limits are established in 500 and 1000 mg/kg flesh expressed as sorbic and benzoic acids, respectively (IOOC, 2004). However, information on the effects of these preservatives on table olive yeasts is still scarce, especially with respect to their combined effect.

To discover enhanced or synergistic mixtures of inhibitors, the checkerboard method, interpreted according to the summed fractional inhibitory concentrations ($\sum$FIC) procedure is usually applied (Bonapace et al., 2000). In a mixture, when no growth is observed, the ratio between the concentration of an individual inhibitor (in presence of other inhibitor) divided by its own minimum inhibitory concentration (MIC) is termed its FIC. For mixtures, where there are no interactions between the inhibitors, addition is defined when the observed MIC for a mixture is equivalent to the sum of FIC of the individual components, being equal to 1, i.e.,

$$\sum \mathrm{FIC} = \frac{x}{\mathrm{MIC}_X} + \frac{y}{\mathrm{MIC}_Y} = 1 \quad (1)$$

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where MIC_x and MIC_y are the MIC concentrations of the inhibitors *X* and *Y*, and *x* and *y* their respective concentrations in the mixture. The basic assumption used in the ∑FIC method is that all inhibitors in a mixture have identical dose–response. However, this behaviour is not observed in some preservatives.

Lambert and Pearson (2000) developed a simple mathematical model called the Lambert–Pearson model (LPM), to fit the observed dose–response profile of an inhibitor. From the curves fitted with this method, the MIC (concentration of the inhibitor above which no growth is observed) and NIC (non-inhibitory concentration or concentration above which the inhibitor begins to have a negative effect on growth) can be obtained. Inhibitor concentrations above a threshold concentration (NIC) are required to obtain a level of inhibition. The inhibitory strength can be related to the steepness of the slope between the NIC and the MIC values (Lambert and Pearson, 2000). Later, the dose–response profile method was expanded to include antimicrobial combinations (Lambert and Lambert, 2003). In this work, this new model is termed ELPM.

The aim of this work was to study the effect of sorbic and benzoic acids and their mixtures on a yeast cocktail (representative of table olive yeast populations) at selected pH levels, considering the possible synergistic or antagonistic effect of the preservative combinations. Information about the more resistant yeast species to these weak acids was also obtained. These results can be useful to the industry for optimizing the concentrations of both preservatives in table olive packing.

2. Materials and methods

2.1. Yeast cocktail preparation

Four yeast species, *Saccharomyces cerevisiae*, *Pichia anomala*, *Issatchenkia occidentalis* and *Candida diddensiae*, previously isolated and identified from table olive elaborations (Arroyo López et al., 2006b) were used for the cocktail preparation. The use of a yeast cocktail in this experiment was chosen to mimic the situation in olive brines where, usually, there is no a single but a coexistence of several yeast species.

Prior to each experiment, the four species were inoculated separately in 5 ml of a Yeast–Malt–peptone–glucose broth medium (YM, Difco™, Becton and Dickinson Company, Sparks, USA) and incubated at 30 °C for 48 h. Then, the tubes were centrifuged at 9000*g* for 15 min and the pellets re-suspended separately in 5 ml of sterile peptone water (0.1% wt/vol). To form the yeast cocktail, 3 ml from each suspension sample were combined to reach a total inoculum level of $7.30(\pm 0.20)\log_{10}$ cfu/ml, which was confirmed by surface spread on YM agar plates.

2.2. Growth medium preparation and experimental design

The basal medium selected for all experiments was YM broth. This medium was adjusted with HCl (0.5 M) to different pH values (4.5, 4.0 and 3.5) and sterilized at 121 °C for 15 min. Subsequently, the media were modified with different concentrations of potassium sorbate and sodium benzoate, using a 20% (wt/vol) sterile stock solution of each preservative. Transformation of potassium sorbate and sodium benzoate concentrations into sorbic and benzoic acid levels was achieved by multiplying by 0.746 and 0.847 (ratios of the molecular weight of the acids to the molecular weight of their salts), respectively. A complete factorial experimental design was used for each pH level, using the concentrations shown in Table 1. Concentrations are expressed as mg/l for similarity with the units required by the Trade Standard for Table Olive (IOOC, 2004). So the number of treatments was 132 for pH 4.5, 110 for pH 4.0 and 81 for pH 3.5.

Table 1
Sorbic and benzoic acid concentrations, at different pH values, in YM broth

pH	Sorbic acid (mg/l)	Benzoic acid (mg/l)
4.5	0, 26, 50, 75, 100, 150, 250, 375, 500, 750, 1000	0, 50, 100, 150, 200, 300, 500, 750, 1000, 1500, 2000, 2500
4.0	0, 26, 50, 75, 100, 150, 250, 375, 500, 750	0, 50, 100, 150, 200, 300, 500, 750, 1000, 1500, 2000
3.5	0, 26, 50, 75, 100, 150, 250, 375, 500	0, 50, 100, 150, 200, 300, 500, 750, 1000

A complete factorial experimental design was used for each pH level.

2.3. Optical density (OD) measurements

Growth (OD data) was recorded in Bioscreen C equipment (Labsystems, Helsinki, Finland) at 30 °C, using a wide band filter (420–580 nm) and pre-shaking for 10 s. Measurements were taken every 2 h for 7 days. The inocula were obtained from the first decimal dilutions of the initial cocktail suspension. The wells of the Bioscreen plate were filled with 0.05 ml of the diluted inocula and 0.35 ml of YM broth medium (modified with the diverse pH values and preservative concentrations), reaching an inoculum level of $5.76 \pm 0.07\log_{10}$ cfu/ml. The inocula were just above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. All experiments were carried out in duplicate. Simultaneously, two uninoculated wells for each experiment were also included in the plate. When the experiments were over, randomly selected wells (which included growth and no growth samples) were spread on YM agar plates and their counts estimated. In addition, colony and cell morphology of these plates were studied to visually detect the species more resistant to the preservatives.

2.4. Estimation of dissociated and undissociated acids

The relationship between the undissociated and dissociated weak acids present at a specific pH value is given by the Handerson–Hasselbalch (HH) equation:

$$\mathrm{pH} = \mathrm{p}K_a + \log_{10}\left(\frac{\mathrm{Anion}}{\mathrm{Acid}}\right) \quad (2)$$

From it, the fraction of undissociated acid was estimated from the equation:

$$\alpha = \frac{1}{1 + 10^{\mathrm{pH}-\mathrm{p}K_a}} \quad (3)$$

The estimation of the undissociated (α*total acid content) and dissociated ($(1-\alpha)$*total acid content) acids from the total concentration of weak acid is then straightforward. The changes of the undissociated sorbic and benzoic acid fractions in YM broth within the pH limits used in this study are shown in Fig. 1.

2.5. Study of the inhibitory effects of the mixtures by the FIC procedure

This procedure can be applied directly to the data (checkboard method) by observing the concentration of the individual preservatives and their mixtures at which inhibition is noticed. The MIC for an individual inhibitor or for the binary mixtures of inhibitors was defined as the minimal required concentration of preservatives tested that inhibited yeast growth as assessed by the corresponding fa (see below for definition). The limit for no