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Evaluation of different chemical preservatives to control *Zygosaccharomyces rouxii* growth in high sugar culture media

M.C. Rojo [a], F.N. Arroyo López [b], M.C. Lerena [a, c], L. Mercado [c], A. Torres [a, d], M. Combina [a, c, *]

[a] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917 (C1033AAJ), Ciudad Autónoma de Buenos Aires, Argentina
[b] Food Biotechnology Department, Instituto de la Grasa (CSIC), Av. Padre García Tejero 4, 41012, Seville, Spain
[c] Oenological Research Center, Estación Experimental Agropecuaria Mendoza, Instituto Nacional de Tecnología Agropecuaria (EEA Mza INTA), San Martín 3853 (5507) Luján de Cuyo, Mendoza, Argentina
[d] Microbiology and Inmunology Department, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional 36, KM 601, Córdoba, Argentina

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ABSTRACT

Zygosaccharomyces rouxii is an osmophilic yeast responsible for a large amount of economic loss in high sugar food production. Statistical modelling techniques were used in the present study to assess the individual effects of different chemical preservatives (potassium sorbate, sodium benzoate, dimethyldicarbonate, vanillin, ferulic, *p*-coumaric and caffeic acids) to control the growth of a cocktail of five yeast strains belonging to this species and isolated from spoilt concentrated grape juices. None of the preservatives assayed were able to completely inhibit the *Z. rouxii* growth. However, the mathematical models obtained in a high sugar culture media showed that especially four preservatives (potassium sorbate, sodium benzoate, dimethyldicarbonate and vanillin) were the best options to control the growth of this microorganism, obtaining a maximum reduction on yeast growth of approximately 40%. On the contrary, *p*-coumaric and caffeic acids were the preservatives with the lower effects, which only showed a maximum growth reduction percentage of approximately 15%. Results obtained in this paper could be very useful for industry for a better control of this spoilage yeast in concentrated grape juice.

1. Introduction

Osmotolerant and osmophilic yeasts are the most common spoilage agents of sugar-rich foods, where water activity (a_w) is the main limiting factor for microbial growth (Deak & Beuchat, 1994). Among all yeast species recognized, only a small fraction (about 10 yeast species), are responsible for major losses in processed foods around the world (Pitt & Hocking, 1997). The importance of these spoilage yeasts is increasing, because in the modern world a great proportion of foods are being processed, preserved in some form, and stored or transported over long distances before consumption. Spoilage yeasts most frequently described in sugar food and drink industries are those belonging to *Zygosaccharomyces* genus. Spoilage resulting from growth of the yeast *Zygosaccharomyces* is widespread and has caused considerable economic losses in the food industry (Fleet, 2011). Food containing high concentrations of sugar (40–70%) includes sugar cane, sugar syrups, honey, concentrate fruit juices, jams, jellies and dried fruits. Their spoilage by yeasts is not uncommon, with *Zygosaccharomyces rouxii* being most frequently implicated because of its unique ability to tolerate the high osmotic stresses and low a_w conditions of these products, and also due to its resistance to different preservatives (Fleet, 2011; Martorell, Stratford, Steels, Fernández-Espinar, & Querol, 2007; Stratford et al., 2013). Occasionally, other yeasts are also found and these include *Zygosaccharomyces bailii*, *Zygosaccharomyces bisporus*, *Zygosaccharomyces mellis*, *Schizosaccharomyces pombe*, *Torulasopora delbrueckii* and various *Candida* species (Combina et al., 2008; Martorell et al., 2007; Stratford, 2006). Spoilage activity by *Zygosaccharomyces* can lead to excessive gas production in foods. The amount of CO_2 generated can be sufficient to distort packaging, and break cans or kegs (Grimbaum, Ashkenazi, Treister, Goldschmied-Reouven, & Block, 1994).

In a previous study, we have characterized the osmophilic and osmotolerant yeast species present in Argentinean concentrated

* Corresponding author. Estación Experimental Agropecuaria Mendoza. Instituto Nacional de Tecnología Agropecuaria (EEA Mza INTA). San Martín 3853 (5507) Luján de Cuyo, Mendoza, Argentina. Tel.: +54 261 4963020x295.
E-mail addresses: maricombina@yahoo.com, combina.mariana@inta.gob.ar (M. Combina).

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grape juice. *Z. rouxii* was the only yeast species isolated from spoiled concentrated grape juices and it was the main yeast species present in unspoiled samples followed by lower proportions of other yeast species (Combina et al., 2008). We have also shown that the spoilage depends on the size of the initial inoculum as well as the strain ability to grow under certain conditions (Combina et al., 2008). We confirmed that the visual sign of spoilage become noticeable when yeast growth reaches approximately 10^4–10^5 CFU/g and are clearly evident at 10^6–10^7 CFU/g (Combina et al., 2008; Fleet, 2011). In a recent work performed by our group, the microbiological stability of the concentrated grape juice according their pH and sugar concentration was established. Results allowed us to propose a model system to determine the intrinsic stability of the product and to define the juice parameters (pH and a_w) for increasing microbial stability (Rojo et al., 2014). However, when the product has a high population of osmophilic yeast, the adjustment of these parameters is not enough to inhibit microbial growth and to extend the shelf life long enough to allow the exportation of the product. Thus, it is necessary to develop other technological tools to obtain a reduction in this population, without affecting the sensorial characteristics of the concentrated grape juice.

The control of spoilage yeasts is one of the most important aspects in food preservation. Factors such as low temperature, reduce a_w, addition of preservatives and low pH, are all used to inhibit yeast species and other microorganisms (Hidalgo Togores, 2002; Marechal, Martinez de Marnanon, Poirier, & Gervais, 1999). Moreover, the addition of preservatives in foods has been used for centuries. The most commonly used food preservatives are weak acids, such as sorbic, benzoic, propionic, and acetic acids and sulfur dioxide (sulphite), some of which occur naturally in foods (fruits and vegetables) (Piper, 2011). Weak acid preservatives are widely used in sugar containing low-pH foods such as fruit juices, beverages, wine, dressings and sauces, in which spoilage is most often caused by yeasts, moulds and lactic acid bacteria (Beuchat, 1982; Vermeulen et al., 2008). Yeast resistance and even metabolism of such preservatives raises problems for the food industry; causing a requirement for increased preservative levels in low-pH foods to prevent yeast spoilage (Piper, 2011).

Dimethyldicarbonate (DMDC) is a chemical preservative that has recently been approved for the control of spoilage yeasts in wines (Martorell et al., 2007; OIV, 2013). It has been demonstrated that more than 3 mM DMDC is necessary for a complete inhibition of alcoholic fermentation conducted by different yeast species (Costa, Barata, Malfeito-Ferreira, & Loureiro, 2008; Delfini et al., 2002). Also, Renouf, Strehaiano, and Lonvaud-Funel (2008) have suggested that DMDC should not be used as a preventive agent but only as curative agent against unwanted populations already present in wine.

Nowadays, modern consumers prefer high quality foods that are more natural, minimally processed and preservative free. This latter along with stricter legislation regarding current preservatives has challenged the food industry leading to increased research into the use of "naturally derived" antimicrobials. Over the last two decades, other preservatives from plant, animal and microbial origins have been intensely investigated for practical applications (Pozo-Bayón, Monagas, Bartolomé, & Moreno-Arribas, 2012). Thereby, vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major constituent of vanilla beans and is the main flavouring compound used in numerous foods such as ice cream, chocolate and confectionary products. Moreover, recent reports have shown that vanillin can be an effective inhibitor of yeasts and moulds when tested in fruit purees and fruit based agar systems (López-Malo, Alzamora, & Argaiz, 1995; López-Malo, Alzamora, & Argaiz, 1998). Additionally, different studies have demonstrated the potential application of phenolic extract as antimicrobial and antioxidant agents in order to prevent food spoilage and to prolong the shelf life of final products (García-Ruiz et al., 2012). Hydroxycinnamic acids (HCAs) are endogenous components of grapes and are considered natural food preservatives (Ou & Kwok, 2004; Smid & Gorris, 1999). The most abundant HCAs in grapes are caffeic, *p*-coumaric and ferulic acids, in decreasing order (Flanzy, 2000). HCAs have been reported to inhibit growth of a variety of organisms including fungi and bacteria (Campos, Couto, & Hogg, 2003; Ravn, Andary, Kovacs, & Moelgaard, 1989; Stead, 1993; Van Sumere, Cottenie, De Gref, & Kint, 1971; Walker, Bais, Halligan, Stermitz, & Vivanco, 2003). In particular, the growth of a number of yeast species is inhibited by HCAs in concentrations over 1 mM, with ferulic and *p*-coumaric acids being generally found to be the most inhibitory (Harris, Jiranek, Ford, & Grbin, 2010; Ou & Kwok, 2004; Pastorkova et al., 2013).

In the present work, seven chemical preservatives were assessed to control the growth of *Z. rouxii* in a high sugar culture media, in order to find a new strategy for preservation of concentrated grape juices. The study was carried out by using a modelling approach, obtaining diverse mathematical equations useful to determine the percentage of growth reduction of this microorganism as a function of preservative concentration.

2. Material and methods

2.1. Yeast strains

Five strains belonging to *Z. rouxii* species (MR4, MT6, MC8, MC9 y MC10) previously isolated from spoilt Argentinean concentrated grape juices were used in the present study (Combina et al., 2008). Strains were previously identified by molecular sequencing of the D1/D2 domain of 26S ribosomal gene and registered at the Wine Research Centre Microorganism Collection from INTA (Mendoza, Argentina).

2.2. Yeast cocktail preparation

First, the five *Z. rouxii* strains were independently grown during 24 h at 28 °C on YPD broth (40 g/L glucose (Biopack Co.), 5 g/L bacteriological peptone (Britania Co.), 5 g/L yeast extract (Britania Co.) and 20 g/L agar (Britania Co.). Then, a high sugar culture media MYGF 195 g/L glucose (Biopack Co.), 195 g/L fructose (Biopack Co.), 20 g/L malt extract (Britania Co.), 5 g/L yeast extract (Britania Co.) adjusted to pH 4.5 by citric acid addition) was prepared and inoculated with the different strains to form a cocktail of approximately 10^4 CFU/mL. Finally, yeasts were incubated in this high sugar culture media during 48 h at 28 °C without shaking until the yeast population reached 2×10^7 CFU/mL.

2.3. Modelling of the individual effects of preservatives on *Z. rouxii* growth

The effect of preservatives on yeast growth cocktail was studied using MYGF as basal medium supplemented with different doses of the following chemical compounds: potassium sorbate (Sigma–Aldrich Co., St. Luis, USA), sodium benzoate (Sigma–Aldrich Co.), dimethyldicarbonate (Velcorin®), vanillin (Sigma–Aldrich Co.) and different hidroxy-cinnamic acids such as ferulic, *p*-coumaric and caffeic acids (Sigma–Aldrich Co.). The first three preservatives were selected considering the products approved by the International Organization of Vine and Wine (OIV) and the National Viticulture Institute (INV, Argentina), whereas the last preservatives were selected by their natural character and its promising antimicrobial activities in food industry. Chitosan (Lallemand Co.) was also evaluated, but no effect on *Z. rouxii* growth was detected (data not

shown). Thus, it was not included in the present study. Table 1 shows the ranges tested for each inhibitory compound used and their respective stock mother solutions.

The assays were conducted in microtiter plates using an automatic spectrophotometer model Bioscreen C (Labsystem, Finland). This spectrophotometer uses two microtiter plates with 100 wells each one. Therefore, 200 assays were conducted simultaneously. The wells of the microplate were filled with 350 μL of MYGF medium supplemented with the different chemical preservatives (Table 1) and 20 μL of inoculum, reaching an initial inoculum level of 1×10^6 CFU/mL. The inocula were always above the detection limit of the apparatus, which was determined by comparison with a calibration curve previously established. Un-inoculated wells for each experimental series were also included in the microtiter plate to determine, and consequently subtract, the noise signal. Therefore, a total of 273 growth curves (13 levels of each preservative × 7 preservatives × 3 replicates) were obtained and analysed. Assays were conducted at 28 °C and optical density (OD) measurements were performed after a pre-shaking of 20 s every 2 h during 7 days.

The inhibitory effect microorganism growth increases with higher preservative concentrations. This effect on the growth is manifested by a reduction in the area under the OD–time curve relative to the positive control (absence of preservative). The areas under the OD–time curves were calculated by integration using OriginPro 7.5 software (OriginLab Co.). The relative growth for each preservative concentration, denoted as the fractional area (fa), was obtained using the ratios of the test area ($area_{test}$) and the positive control of the yeast ($area_{cont}$, without preservative), according to the following formula:

$$fa = (area_{test})/(area_{cont})$$

Then, growth reduction (Gr) percentage was estimated according to the following formula:

$$Gr = (1 - fa)*100$$

This parameter has the premise that in the absence of preservatives the growth of microorganism is the maximum and the fa is 1. Thus, in this case, the Gr consequently will be 0. The plot of the Gr versus preservative concentration produced a sigmoid–shape curve that was fitted with the reparameterized Gompertz equation proposed by Zwietering, Jongenburger, Rombouts, and Van't Riet (1990), which has the following expression:

$$Gr = A*\exp\left(-\exp\left\{(r*e*(c-x))/A + 1\right\}\right)$$

where x is the preservative concentration assayed (mM), A is the maximum Gr reached (%), r is the maximum rate of change (mM^{-1}) and c is the preservative concentration (mM) above which Gr begins. These parameters were obtained by a nonlinear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, i.e. loss function (observed − predicted). This task was accomplished using the nonlinear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of variance explained by the model (R^2) with respect to experimental data.

Finally, an analysis of variance was performed by means of the one-way ANOVA module of Statistica 7.1 software to check for significant differences among fitted parameters obtained from the different preservatives. For this purpose, a post-hoc comparison was applied by means of the Fisher LSD test.

Table 1
Concentration ranges of the chemical preservatives assayed in the present study.

Preservatives[a]	Tested concentrations (mM)
Potassium sorbate	0, 0.3, 0.7, 1.3, 2.0, 2.7, 3.3, 4.0, 4.7, 5.3, 6.0, 6.7, 10.0
Sodium benzoate	0, 0.3, 0.7, 1.4, 2.1, 2.8, 3.5, 4.2, 4.9, 5.6, 6.2, 6.9, 10.4
Dimethyldicarbonate (DMDC)	0, 1.9, 2.7, 4.0, 5.3, 6.7, 8.0, 9.3, 10.6, 12.0, 13.3, 14.6, 16.0, 17.3
Vanillin	0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 25.0, 30.0
Caffeic acid	0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 22.0
Ferulic acid	0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 22.0
p-coumaric acid	0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 14.0, 16.0, 18.0, 20.0, 22.0

[a] A stock solution of each preservative was prepared, except for dimethyldicarbonate (99.9% w/v purity) which was added directly from a mother solution of 9.3 M. For potassium sorbate and sodium benzoate the mother solution was 120 mM in sterile water, for vanillin 0.7 M in ethanol, while for hydrocinamic acids was 0.35 M in ethanol.

3. Results

In the present study, the individual effect of seven different chemical preservatives on the growth of a *Z. rouxii* cocktail was evaluated in an automated spectrophotometer by analysing a total of 273 OD growth curves obtained in a high sugar culture media. In this work the main results obtained after mathematical modelling of experimental data, which always had a R^2 value above 0.90, are shown.

Within the concentration range tested, none of the preservatives assayed was able to completely inhibit the growth of the *Z. rouxii* cocktail. However, they were able to partially inhibit yeast growth in different percentages. Fig. 1 shows as an example, the fit with the reparameterized Gompertz equation for one of the three series obtained for Gr experimental data of *Z. rouxii* cocktail as a function of potassium sorbate and sodium benzoate concentrations. Clearly, the whole sigmoid-shaped curve can be divided into three sections: i) a first section corresponding to preservative concentrations with Gr around 0, ii) preservative concentrations above which Gr progressively increased, and iii) a third section where maximum Gr was obtained. As can be deduced from Fig. 1, the yeast behaviour was different depending of the preservative assayed, with a curve shifter to the right (lower inhibitory effect) in the case of sodium benzoate.

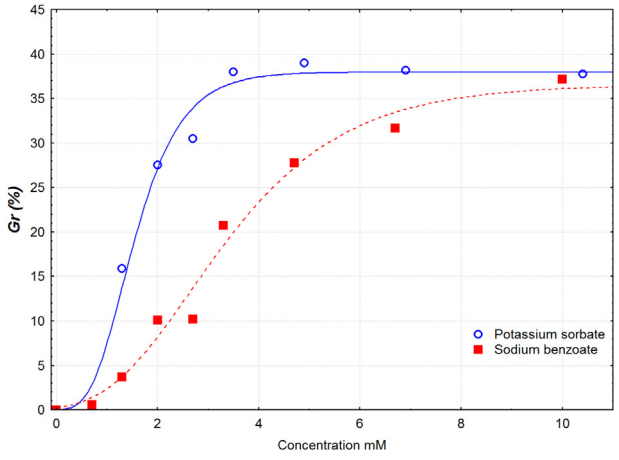

Fig. 1. Fit with the reparameterized Gompertz equation for the growth reduction (Gr) experimental data deduced from the fa obtained for potassium sorbate and sodium benzoate concentrations.

Table 2 shows the parameters obtained after the mathematical fit of the different preservatives. Parameter *A*, related to the maximum percentage of Gr obtained, ranged from 14.9% (*p*-coumaric acid) to 39.9% (vanillin), parameter *r* (rate of change) ranged from 0.595 mM^{-1} (caffeic acid) to 190.950 mM^{-1} (DMDC), while parameter *c* (preservative concentration above which inhibition begins) ranged from 0.106 mM (DMDC) to 6.67 mM (caffeic acid).

From fitted parameters, it is possible to get the mathematical equations to estimate how *Z. rouxii* reduces its growth as a function of the different preservative concentrations. Graphical representations of these equations are shown in Fig. 2. Among the legally approved preservatives, it is clearly deduced that DMDC was the most effective compound (with one of the highest A and *r* values and the lowest *c* value), with the curve most shifted to the left. As also shown in Fig. 2, the sigmoid-curve for potassium sorbate was clearly above sodium benzoate, which is indicative of the higher inhibitory effect of the former preservative. Among "naturally derived" preservatives, vanillin was clearly the most effective preservative to partially inhibit *Z. rouxii* growth (Fig. 2). Specifically, among HCAs preservatives, ferulic acid was the most effective inhibitor, reaching the maximum Gr percentage at lower doses compared to the other two HCAs tested. The minimal inhibitory effect was observed for caffeic acid, where doses higher than 40 mM were necessary to achieve the maximum Gr (Fig. 2).

Finally, Fig. 3 shows the ANOVA analysis carried out for the maximum Gr obtained (*A*) for the different chemical preservatives. Clearly, *p*-coumaric and caffeic acids showed the lowest inhibitory effect, without significant differences between them, obtaining only a 15% of yeast Gr. Ferulic acid exhibited about 30% of maximum Gr, while sodium benzoate, potassium sorbate, vanillin and DMDC formed a statistically homogeneous group with the highest Gr values reached (around 40%).

4. Discussion

OD measurements are usually used in predictive microbiology to assess microbial response as a function of environmental variables and chemical compounds by using diverse methodologies such as time to turbidity (Cuppers & Smelt, 1993) or fa (Bautista Gallego, Romero Gil, Garrido Fernández, & Arroyo López, 2012). Three of the preservatives assayed (potassium sorbate, sodium benzoate and DMDC) are chemical compounds approved to be used in grape must and wine, using in this work the maximum dose range allowed by the International Organization of Vine and Wine and the National Viticulture Institute (Argentina). The others four compounds (vanillin and hidroxycinnamic acids as ferulic, *p*-coumaric and caffeic acids) and their doses were selected due to their natural character and because they show some antimicrobial activities previously demonstrated in different works (Martorell et al., 2007).

Table 2
Fitted parameters *A* (maximum yeast Gr obtained), *r* (maximum rate of change) and *c* (preservative concentration above which growth reduction begins) obtained from the reparameterized Gompertz equation for the different chemical preservatives assayed. Standard deviations are shown in parentheses.

Preservative (mM)	*A* (%)[a]	*r* (mM^{-1})	*c* (mM)
DMDC	38.93 (1.61)a	190.950 (65.790)a	0.106 (0.045)a
Sodium benzoate	35.77 (1.88)a	11.190 (3.110)b	1.180 (0.235)a,b
Potassium sorbate	39.08 (1.54)a	32.250 (19.680)b	0.602 (0.190)a
Ferulic acid	29.45 (2.89)b	3.586 (0.910)b	1.23 (1.18)a,b
Caffeic acid	16.49 (6.40)c	0.595 (0.049)b	6.67 (3.11)c
Vanillin	39.98 (1.69)a	2.813 (0.136)b	1.87 (1.72)a,b
p-coumaric acid	14.86 (1.97)c	2.205 (1.689)b	4.95 (4.31)b,c

[a] Values followed by different letters are significantly different according to a Fisher-LSD post-hoc comparison test.

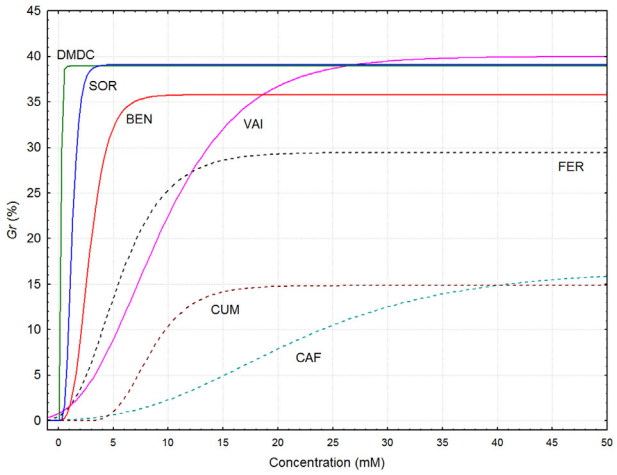

Fig. 2. Plot of the mathematical equations obtained from triplicate experiments for potassium sorbate (SOR), sodium benzoate (BEN), dimethyldicarbonate (DMDC), vanillin (VAI), ferulic (FEU), *p*-coumaric (CUM) and caffeic acids (CAF) for the estimation of the growth reduction (Gr) as a function of preservative concentration.

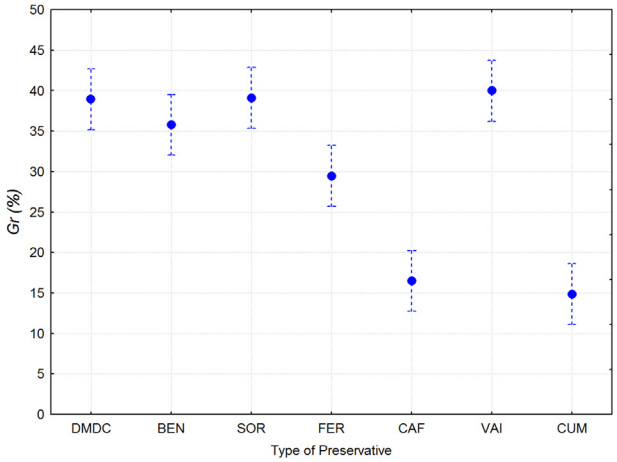

Fig. 3. One-way ANOVA analysis graphical representation of the growth reduction (Gr) obtained for *Z. rouxii* (dependent variable) as a function of the different preservatives (categorical variable). Mean values were obtained from triplicate experiments. DMDC, BEN, SOR, FER, CAF, VAI and CUM stand for dimethyldicarbonate, sodium benzoate, potassium sorbate, ferulic acid, caffeic acid, vanillin and *p*-coumaric acid, respectively.

Control of commodity spoilage by yeasts depends on the application and maintenance of conditions (during processing and storage) that either kill the cells or prevent their growth (Rojo et al., 2014). Many environmental factors (e.g., temperature, pH, a_w, physical matrix, etc.) determine the physical and chemical limits for survival and growth of yeasts. However the precise limits are difficult to define, since the impact of one factor may be influenced by others factors (Fleet, 2011). Concentrated grape juices represent a complex matrix where different stresses are combined (low pH and high sugar concentration). Therefore, our assay was performed in a culture medium that mimics the characteristics of the natural substrate (concentrated grape juice) in order to better extrapolate the results obtained (Praphailong & Fleet, 1997; Vermeulen et al., 2008). Yeasts rarely appear in food and beverage ecosystems as single species cultures. Exceptions occur in highly processes products where spoilage outbreaks by single and well-adapted species are known, such as *Z. rouxii* in high sugar products (Stratford, 2006). Moreover, it is widely known that the resistance level to preservatives is strain-dependent and it is also influenced

by the physiological status of the cells (Martorell et al., 2007). In this work, seven preservatives were individually assayed in a high sugar concentration culture media with low pH (4.5) using a cocktail of *Z. rouxii* strains isolated from concentrated grape juices naturally spoiled.

In contrast with previous reports, our results show that chitosan did not exhibit any antimicrobial activity against the *Z. rouxii* strain cocktail in high sugar concentration media. Even at the highest dose tested in this work (300 mg/L), which exceeds the maximum level allowed in wines (100 mg/L), no inhibition was observed.

Many organic acids preservatives such as sorbic and benzoic acids are usually added in a wide range of foods and beverages to control yeast growth. The effectiveness of these agents depends on their concentration and others food properties such as sugar concentration and pH (Fleet, 2011). The antimicrobial action of weak acid preservatives is pH-dependent, and it has been established that they are much more active in acidic environments. Low pH favours the undissociated form of the weak acid, which is membrane permeable, and consequently more toxic to the cell (Martorell et al., 2007). The hypothesis that undissociated acid molecules pass into the cell and inhibit yeast growth through acidification of the cytoplasm has been already demonstrated (Krebs, Wiggins, Stubs, Sols, & Bedoya, 1983; Stratford et al., 2013). Sorbic acid has a higher pK_a ($pK_a = 4.76$) than benzoic acid ($pK_a = 4.20$). As a result, the undissociated fraction of sorbic acid is always above that of benzoic acid. In our work medium, the undissociated form of sorbic and benzoic were 65% and 43% respectively. Albeit the maximum Gr reached with both compounds was statistically similar (39.1 vs 35.7%), a lower dose of potassium sorbate was necessary to reach the same inhibition percentage. Consequently potassium sorbate would be a more efficient inhibitor for concentrated grape juice yeasts than sodium benzoate. Similar results have been also found by Arroyo-López, Bautista-Gallego, Durán-Quintana, and Garrido-Fernández (2008) with a cocktail of table olive related yeasts.

Several resistance mechanisms to weak acids have been described in *Z. rouxii* and other related yeast species as *Z. bailii* and *Saccharomyces cerevisiae* (Piper, 2011; Stratford et al. 2013). Overall, the suggested resistance mechanisms are: i) degradation and metabolism of the preservative and ii) efflux pump removing preservatives (Piper, 2011; Stratford, Plumridge, & Archer, 2007; Warth, 1977, 1988). Recently, Stratford et al. (2013) proposed that extreme resistance to weak acid preservatives in *Z. bailii* is due to population heterogeneity, with a small proportion of cells having a lower intracellular pH. This reduces the level of accumulation of any weak acid in the cytoplasm, thus conferring resistance to all weak acids but not to other inhibitors. In our work, a complete inhibition of the *Z. rouxii* cocktail by sorbic and benzoic acid was not achieved, mainly because the susceptibility to these agents varies into the same yeast species, where some strains would be more resistant than others (Fleet, 2011).

The DMDC is used as antimicrobial agent for sterilizing fermented beverages. DMDC acts by inhibiting some glycolytic enzymes particularly the alcohol dehydrogenase and the glyceraldehyde 3-phosphate dehydrogenase by methoxycarbonylation of the nucleophilic residues (imidazoles, amines, thiols). Moreover, its effect is not directly pH dependent (Renouf et al., 2008). The effectiveness to DMDC to inhibit the several yeast species related to grape juice and wine has been already demonstrated (Delfini et al., 2002; Renouf et al., 2008; Siricururatana et al., 2013). In synthetic medium, the inhibitory activity of DMDC was yeast species and dose dependent, where 0.112 mM of DMDC were necessary to reach the complete inhibition of *Z. bailii* (Delfini et al., 2002). Moreover, *Z. bailii* isolated from syrups and candied fruit nougat were highly resistant to DMDC. Also, the minimum inhibitory concentration (MIC) recorded for *Z. rouxii* isolated from spoiled syrups evaluated in YPD (pH 4.0) was 0.065 mM (Martorell et al., 2007). On the contrary, Costa et al. (2008) found that the DMDC MIC to *Z. bailii* isolated from spoiled wines was as low as 0.007 mM. The most promising results were obtained with a combination treatment with DMDC (0.07 mM) and natamycin (0.015 mM) in grapes juices inoculated with a cocktail of *Dekkera*, *Kluyveromyces*, *Brettanomyces* and *Zygosaccharomyces* species, where the extension of self-life was similar to that of the positive control (3.5 mM and 3.3 mM of sodium benzoate and potassium sorbate, respectively) (Siricururatana et al., 2013). In our work, DMDC was not able to completely inhibit the growth of *Z. rouxii* cocktail in high sugar and low pH culture medium, even using higher doses than accepted. The maximum Gr obtained with DMDC was similar to sorbic and benzoic acids, in agreement with the previously mentioned works. Daudt and Ough (1980) established a semi-log correlation between the initial viable yeast population and the amount of DMDC required. When the initial viable cells increased 10-fold, an additional amount of approximately 0.04 mM of DMDC was required. In contrast, in our work, the maximum Gr was achieved with the minimal doses of DMDC assayed, and increasing doses did not produce further increase of the inhibitory effect. The hydrolysis rate of DMDC under various conditions has been studied and the half-life in the reaction with water is reported to be approximately 8 min at 30 °C, being completely hydrolysed within less than 1 h (Genth, 1979; Ough, 1983). Thus, the maximum antimicrobial effect would be expected to occur when the concentration is higher, soon after addition of the compound before degradation begins. Therefore, DMDC should not be used as preventive agent but only as curative agent against unwanted populations already presents in grape must.

Vanillin is currently used as a principle-flavouring agent and it is also a “naturally derived” promising additive for inhibiting yeasts in some fruit products. The levels added to food products such as ice cream and sweets are typically 20 and 26 mM respectively (Hocking, 1997). Previous studies have shown that certain yeasts are able to convert vanillin to its respective alcohol and acid derivate (Edlin, Narbad, Dickinson, & Lloyd, 1995). However, it has not been established if such bioconversion process could adversely affect vanillin's antimicrobial activity (Fitzgerald, Stratford & Narbad, 2003). Cerrutti and Alzamora (1996) showed that growth of *S. cerevisiae*, *Z. rouxii*, *Z. bailii* and *Debaryomyces hansenii* was inhibited in culture media and apple pure containing 13 mM of vanillin. Additionally, vanillin was inhibitory to *Z. rouxii* and other yeasts at concentrations of 20 mM, but lower concentrations were also effective when combined with other harsher conditions such as lower temperatures and low pH (Fitzgerald, Stratfors, & Narbad, 2003). Moreover, Matamoros-León, Argaiz & López-Malo (1999) have shown that inhibitory concentrations of vanillin ranged from 7.3 mM to 8.6 mM to control different *Penicillium* spp in culture media. Synergistic effects on mould inhibition when vanillin and potassium sorbate were added in combination have been also demonstrated. In our work, a wide range of vanillin concentrations was tested (2–30 mM) reaching only 40% Gr of *Z. rouxii* in a high sugar culture media for the maximum vanillin concentration assessed. In our work, the culture media conditions (390 g/L glucose + fructose and pH 4.5) represent the most favourable conditions to *Z. rouxii* development due to osmophilic character of this yeast species and these could be acting as protective conditions against preservative effect.

Hydroxicinnamic acids and their derivate are present in plants and fruits, providing a natural protection against infection by pathogenic microorganisms (Chambel, Viegas, & Sá-Correia, 1999). The use of antimicrobial active compounds naturally occurring in

grapes (e.g. HCAs and other phenols) offers scope for a new approach in juice and wine preservation. Due to the ability to inhibit growth of food spoilage bacteria as well as their relatively safe status, these compounds are increasingly becoming subject of study for their potential multipurpose use in food preservation techniques (Rodríquez Vaquero, Alberto, & Manca de Nadra, 2007). Cinnamic compounds uncouple the energy transducing cytoplasmic membrane in bacteria and this effect on the bioenergetics status of the membrane may contribute to their antimicrobial action (Mirzoeva, Grishanin, & Calder, 1997). In fact, it is expected that these compounds may lead to the stimulation of the passive influx of protons across the plasma membrane by causing the non-specific increase of the membrane permeability and consequently induce the stimulation of plasma membrane proton-ATPase activity (Sá-Correia, Salgueiro, Viegas, & Novais, 1989). Chambel et al. (1999) reported that *S. cerevisiae* cells grown at pH 4, in the presence of growth inhibitory concentrations of cinnamic acids exhibited a more active plasma membrane proton ATPase than control cells. A previous study has also found that sensitivity to HCAs depends on the organisms as well as the specific HCA under study (Campos et al., 2003). Moreover, others authors have proposed that HCAs were fungistatic rather than fungicidal against *Dekkera* spp. and the antimicrobial properties of grape phenols significantly depend on differences in their chemical structures (Harris et al., 2010). The results presented here show that ferulic acid was the most effective HCAs in preventing the growth of *Z. rouxii* under high sugar concentration and low pH media conditions. This latter is in agreement with the results reported by Harris and colaborators (2010) who found that the minimum inhibitory concentration (MIC) for *Dekkera bruxellensis* and *Dekkera anomala* in culture media was 4 and 8 mM ferulic acid and 8 and 10 mM *p*-coumaric acid respectively. Recently, Pastorkova et al. (2013) demonstrated that *p*-coumaric and ferulic acids exhibited selective inhibitory effects on *Z. rouxii* with MICs higher or equal to 1.55 mM and 1.30 mM for *p*-coumaric and ferulic acids respectively. In contrast, caffeic acid did not show any anti-yeast or anti-bacterial activity. In our work, the maximum Gr reached by HCAs additions was about 15–30%, the MIC were not determined because total inhibition of *Z. rouxii* was not achieved at the maximal concentration of HCAs assayed.

5. Conclusions

The results obtained in this work show the great resistance of *Z. rouxii* to all chemical preservatives assayed in a high sugar culture media. Only a maximum Gr around 40% was obtained for DMDC, sodium benzoate, potassium sorbate and vanillin, while caffeic and *p*-coumaric acid had the lowest inhibitory effect (around 15%). Further research should be performed to estimate the combined effect of the highest inhibitory compounds to achieve complete *Z. rouxii* inhibition.

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