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Effects of pH and sugar concentration in *Zygosaccharomyces rouxii* growth and time for spoilage in concentrated grape juice at isothermal and non-isothermal conditions

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ABSTRACT

The effect of pH (1.7–3.2) and sugar concentration (64–68 °Brix) on the growth of *Zygosaccharomyces rouxii* MC9 using response surface methodology was studied. Experiments were carried out in concentrated grape juice inoculated with *Z. rouxii* at isothermal conditions (23 °C) for 60 days. pH was the variable with the highest effect on growth parameters (potential maximum growth rate and lag phase duration), although the effect of sugar concentration were also significant. In a second experiment, the time for spoilage by this microorganism in concentrated grape juice was evaluated at isothermal (23 °C) and non-isothermal conditions, in an effort to reproduce standard storage and overseas shipping temperature conditions, respectively. Results show that pH was again the environmental factor with the highest impact on delaying the spoilage of the product. Thereby, a pH value below 2.0 was enough to increase the shelf life of the product for more than 60 days in both isothermal and non-isothermal conditions. The information obtained in the present work could be used by producers and buyers to predict the growth and time for spoilage of *Z. rouxii* in concentrated grape juice.

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1. Introduction

Grape juice and its by-products represent an important part of the food industry in the world. Argentina grape production is mainly industrialized, where wine and concentrated grape juices are the two mayor types of commercial products. Mendoza and San Juan provinces (West of Argentina) are the main manufacturers of concentrated grape juices in the country, with 75% of their production mainly exported to United States, Japan, Russia and México (Bruzone, 1998; INV, 2013). Concentrated grape juice represents a critical additive in several mass consumption products. Due to their natural qualities, concentrated grape juice is employed to manufacture baby foods, pharmaceutical products, foods and drinks (Bruzone, 1998). Concentrated grape juices are microbiologically more stable than other fruit products due to the high sugar concentration and usually are stored at room temperature without any additional treatment (ICMSF, 1980; Splittstoesser, 1987). However, these products are not free of microbiological spoilage problems. The combination of high concentration of sugar and low pH still support the development of a reduced number of microorganism species. Osmophilic yeasts represent the primary spoilage cause in high sugar food and drink industries, with the genus *Zygosaccharomyces* as the most frequent described spoilage microorganism (ICMSF, 1980; Deák and Beuchat, 1993; Worobo and Splittstoesser, 2005; Martorell et al., 2007).

The genus *Zygosaccharomyces* has a long history of spoilage in the food industry. Three *Zygosaccharomyces* species, *Z. bailii*, *Z. bisporous*, and *Z. rouxii*, have been associated with the spoilage of grape must, concentrated grape juice and wine (Loureiro and Malfeito-Ferreira, 2003; Fugelsang and Edwards, 2007; Deák, 2008). Spoilage by *Zygosaccharomyces* species can be categorized

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into two groups: i) visible growth on the surface of the product, and ii) fermentative spoilage manifested by alcoholic, esteric or other types of odours and/or visible evidence of gas production, leading to bubbling of the product and/or packaging expansion (Legan and Voyset, 1991; Smith et al., 2004).

In a previous study from our group, the osmotolerant and osmophilic yeast population in concentrated grape juice from Argentina was characterized, with *Z. rouxii* being the only yeast species isolated from spoilt products. Moreover, in other samples without visible evidence of spoilage, *Z. rouxii* was also frequently isolated representing 76% of the total yeast population (Combina et al., 2008). The unique physiological characteristics of *Z. rouxii* are largely responsible for their ability to cause spoilage. These include resistance to low-acid preservatives, extreme osmotolerance, ability to adapt to high glucose concentrations, or low activity water (a_w) values and high temperatures, ability to ferment glucose, and ability to growth at low pH values (Emmerich and Radler, 1983; James and Stratford, 2003; Martorell et al., 2007).

Few studies have been carried out to assess the effect of limiting factors on the growth *Z. rouxii*. The vast majority of these studies were performed in culture media and some of them assessed each variable independently (Kalathenos et al., 1995; Praphailong and Fleet, 1997; Membré et al., 1999). Conversely, the response surface (RS) methodology is a very useful tool which has been previously applied to estimate the combined effects of different environmental variables on yeast growth (Arroyo-López et al., 2006; D'Amato et al., 2006). The RS methodology has been widely used in predictive microbiology as a secondary polynomial model to predict the microorganism response as a function of environmental changes determining at the same time the interaction among them (McMeekin et al., 1993).

In this work, the combined effect of the two limiting factors (pH and sugar concentration) on the growth parameters of a native strain of *Z. rouxii* (MC9) previously isolated from spoilt concentrated grape juice was assessed. This task was accomplished using RS methodology, as secondary model. In an effort to provide useful and practical considerations to producers and buyers, the time for spoilage (TFS) was also determined in natural substrate under the standard storage (isothermal) and shipping (non-isothermal) temperature conditions normally found in Argentinean concentrated grape juices.

2. Materials and methods

2.1. Yeast strain

The strain *Z. rouxii* MC9, previously isolated from spoilt concentrated grape juices, was used in the present study. The strain was identified by molecular sequencing of the D1/D2 domain of the 26S ribosomal gene and registered at the Wine Research Centre Microorganism Collection from INTA, Argentina (GenBank Accession Number KF002711). This strain was selected from a previous study among several native *Z. rouxii* strains because of its better adaptation to concentrated grape juice and fast growth (Rojo et al. unpublished data).

2.2. Media and growth conditions

Z. rouxii MC9 was previously grown on YPD broth (40 g/L glucose, 5 g/L bacteriological peptone, 5 g/L yeast extract, 20 g/L agar) during one day at 28 °C. Before inoculation in natural substrate (concentrated grape juice), the strain was adapted to osmotic shock by growing in a medium (MYGF) with an intermediate concentration of sugar (195 g/L glucose, 195 g/L fructose, 20 g/L malt extract, 5 g/L yeast extract) with pH adjusted to 4.5 by the addition of citric acid. This last medium was incubated during three days at 28 °C without shaking until the highest possible population was reached (10^7 CFU/mL) right at the end of exponential growth phase. Experiments were finally performed in concentrated grape juice provided by a local company located in Mendoza (Argentina).

Table 1
Biological growth parameters of *Zygosaccharomyces rouxii* MC9 (μ_{max}, potential maximum growth rate; λ, lag phase duration) obtained at isothermal conditions (23 °C) for the different treatments (combinations of pH and °Brix) included in the experimental design.

Run	°Brix	pH	μ_{max} ($\log_{10}$ CFU/mL $days^{-1}$)	λ (days)
1	64	1.7	0.000	>60
2	64	1.7	0.000	>60
3	64	1.7	0.000	>60
4	64	2.5	0.360	2.23
5	64	2.5	0.530	1.39
6	64	2.5	0.199	3.07
7	64	3.2	0.417	0.00
8	64	3.2	0.404	0.00
9	64	3.2	0.434	0.00
10	66	1.7	0.000	>60
11	66	1.7	0.000	>60
12	66	1.7	0.000	>60
13	66	2.5	0.287	4.14
14	66	2.5	0.285	1.99
15	66	2.5	0.262	6.29
16	66	3.2	0.455	0.88
17	66	3.2	0.392	1.59
18	66	3.2	0.422	0.17
19	68	1.7	0.000	>60
20	68	1.7	0.000	>60
21	68	1.7	0.000	>60
22	68	1.9	0.058	17.38
23	68	1.9	0.085	17.28
24	68	1.9	0.066	17.33
25	68	2.1	0.140	11.97
26	68	2.1	0.132	8.42
27	68	2.1	0.115	15.52
28	68	2.5	0.181	6.73
29	68	2.5	0.165	12.1
30	68	2.5	0.168	9.37
31	68	3.2	0.185	0.95
32	68	3.2	0.182	2.29
33	68	3.2	0.192	0.00

Note: Values were obtained with the Baranyi and Roberts' model (1994) using DMfit 2.1. program. In the case of no growth, μ_{max} and λ were set to 0.000 $\log_{10}$ cfu/mL $days^{-1}$ and 60 days, respectively, for modelling purposes.

2.3. Experimental design

The different runs (a total of 66) were carried out in 1-L of concentrated grape juice placed in sterile bag-in-box with a Vitop® valve. Bags were placed in metal containers to reproduce the standard storage (isothermal) and overseas shipping (non-isothermal) conditions and monitored for 60 days. The experimental design was obtained from the combination of two variables (pH and sugar concentration) with 3 levels for each variable (Table 1). Variables levels were established taking into consideration a range of conditions usually found in Argentinean concentrated grape juices. The pH values were 1.7, 2.5 and 3.2 and the sugar concentration (expressed as °Brix) were 64 °Brix (779 g/L reducing sugar; a_w: 0.778 ± 0.003), 66 °Brix (810 g/L; a_w: 0.767 ± 0.003) and 68 °Brix (842 g/L; a_w: 0.744 ± 0.003). Two intermediate pH values (1.9 and 2.1) were also evaluated at the sugar concentration condition more frequently required by the market (68 °Brix), yielding a total of eleven different treatments, each performed in triplicate. To achieve the different pH values, grape juices were passed through ion exchange column to obtain the desired pH prior to concentration in order to reproduce industrial

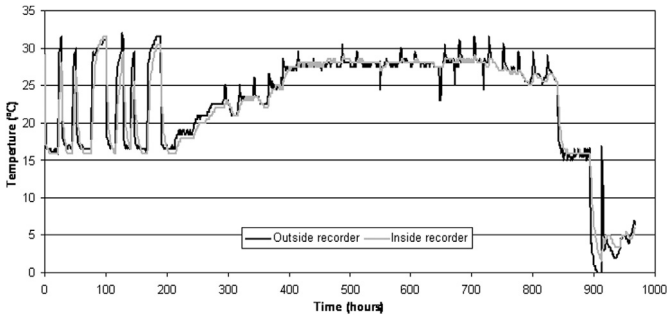

Fig. 1. Inside and outside temperature profiles (non-isothermal conditions) recorded during the experiment to reproduce overseas shipping of concentrated grape juice from the south Hemisphere (summer time) to north Hemisphere (winter time).

conditions. The °Brix of the concentrated grape juices were confirmed using a digital hand-held refractometer (Atago PAL-2, Japan) and the pH was determined using a digital pH meter (Altronix, United States). Each treatment was inoculated with $1.2 \pm 0.2 \times 10^2$ CFU/mL of the strain *Z. rouxii* MC9, which represent the maximum limit accepted for fungi and yeasts count by the local buyers. Un-inoculated bags for each experimental series were also introduced as a negative control. The experiments were conducted at two different temperature systems, namely isothermal condition (23 ± 0.5 °C), to simulate the most frequent storage temperature, and non-isothermal condition, to reproduce the overseas shipping temperature. This last temperature profile was established using as a model the data recorder during wine shipping to destinations in the Northern Hemisphere (Leinberger, 2006; Hartley, 2008). Internal and external temperatures were monitored by *iButton*® temperature data logger placed inside the bag and outside of the metal containers in the control treatments. The recorded non-isothermal profile is shown in Fig. 1, in which three different regions can be observed. The first section of the graph shows the temperature behaviour of the container on the dockside in Santiago (Chile) harbour in summer. The middle section shows a hypothetical sea journey from Santiago (Chile) to the Asian continent (e.g. Japan). The third section of Fig. 1 shows the temperature in the containers on the dockside in the destination harbour in winter. The simulated shipping time was set to 45 days, after which the treatments were maintained at room temperature (23 °C) until completion of the experiment (60 days).

2.4. Modelling yeast growth and time for spoilage

Concentrated grape juice samples at isothermal (23 °C) and non-isothermal conditions were aseptically taken every two days to follow *Z. rouxii* MC9 growth. Samples were decimal diluted in 30% (w/v) glucose to prevent osmotic shock and to allow the recovery of sublethally injured cells. Dilutions were then spread into two culture media. Selective high sugar media: MY50G (a_w 0.89) (Beuchat, 1993) and TGY media (Beuchat et al., 2001) were chosen to detect *Z. rouxii* growth in the concentrated samples. Plates were incubated during 3–5 days at 28 °C before counting. Growth parameters (μ_{max}, potential maximum growth rate; λ, lag phase duration) were calculated from each treatment contemplated in the experimental design by directly fitting plate count ($\log_{10}$ CFU/mL) *versus* time (days) using the Baranyi and Roberts model (1994). For this purpose, DMfit Software 2.1 was used.

In addition, bags at isothermal and non-isothermal conditions were also daily examined in order to detect signs of fermentative activity such as gas production, bubbling or packaging expansion. Also, the time required for spoilage (TFS, days) was recorded. This spoilage was correlated with a *Z. rouxii* population level in the concentrated grape juice, which was always higher than 10^6 CFU/mL (data not shown).

Table 2
Regression coefficients estimated by means of the ANOVA analysis for the biological growth parameters (μ_{max} and λ) of *Zygosaccharomyces rouxii* MC9 at isothermal conditions (23 °C).

Regression coefficient	Value	Standard deviation
Model for μ_{max}		
β_0 (Mean/Inter)	−50.035	23.086
β_1 °Brix (L)	1.409	0.696
β_2 °Brix (Q)	−0.010	0.005
β_3 pH (L)	3.765	0.654
β_4 pH (Q)	−0.162	0.037
β_5 °Brix*pH	−0.041	0.009
R^2	0.902	
Lack of fit	0.181	
Pure error	0.061	
Model for λ		
β_0 (Mean/Inter)	−2221.430	683.381
β_1 °Brix (L)	85.390	20.607
β_2 °Brix (Q)	−0.700	0.155
β_3 pH (L)	−419.81	19.365
β_4 pH (Q)	45.270	1.101
β_5 °Brix*pH	2.45	0.285
R^2	0.928	
Lack of fit	0.000	
Pure error	53.940	

Note: Significant coefficients can be determined from their respective Pareto chart.

In the secondary modelling step, growth parameters (μ_{max} and λ) and TFS of *Z. rouxii* MC9 in both temperature systems (IC, isothermal conditions; NIC, non-isothermal conditions) were adjusted to an RS equation as follows:

$$Y = \beta_o + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \varepsilon \quad (1)$$

where Y is the parameter modelled (μ_{max}, λ, TFS-IC or TFS-NIC), β_o is the mean/intercept term, β_i are the coefficients to be estimated during the RS fitting (β_1 is the coefficient for the linear effect of X_1, β_{11} for the quadratic effect of X_1, β_{12} for the interaction between variables X_1 and X_2, and so on), ε is the term for error and X_1 and X_2 are the environmental variables under study (pH and sugar concentration (°Brix), respectively). For the main effects, regression coefficients can be interpreted as the increase or decrease (depending of the positive or negative coefficient sign) in the response when the factor changes one unit. Analysis of the RS was made using the Experimental Design module of the Statistica 7.0 software package, using the pure error, derived from repetitions of experiments, as option in the corresponding ANOVAs. Model fitting was also checked by the lack of fit test and the coefficient of determination, R^2.

3. Results

The combined effect of pH and sugar concentration on the growth parameters of the spoilage yeast *Z. rouxii* MC9 was evaluated in concentrated grape juice at isothermal temperature. Moreover, the TFS of concentrated grape juice inoculated with this microorganism was modelled at isothermal and non-isothermal conditions, obtaining representative data of the product shelf life under experimental conditions.

3.1. Modelling yeast growth at isothermal conditions

Experiments were carried out under storage temperature at 23 °C (isothermal), which allowed to build growth curves and to calculate the growth parameters for the different experimental

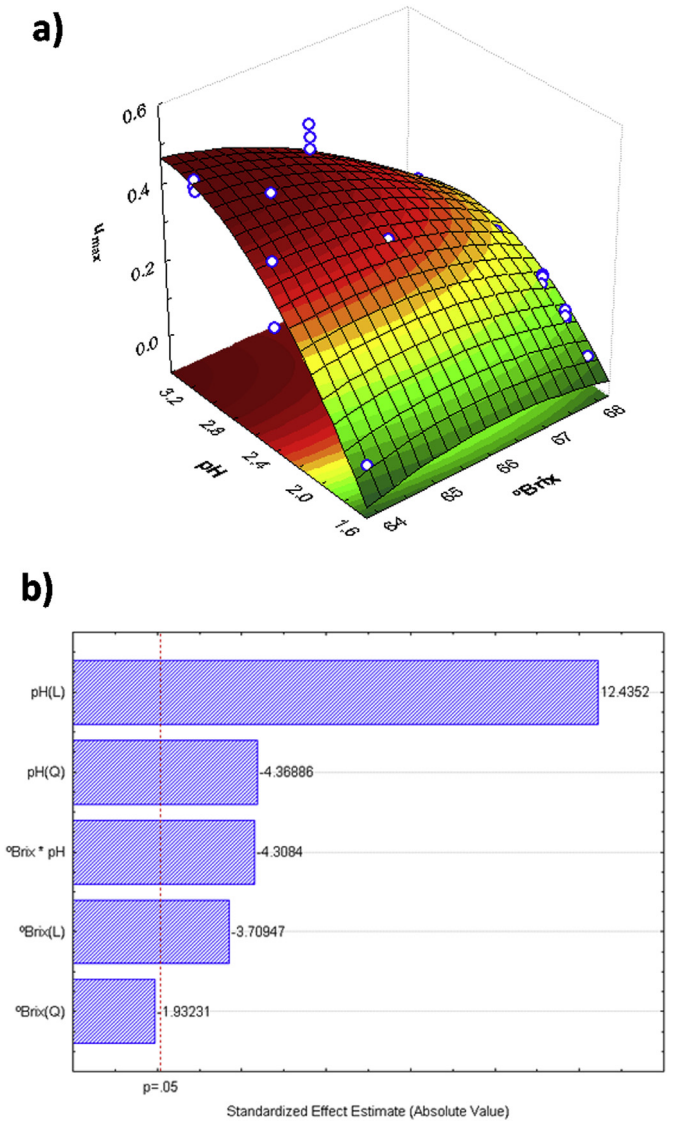

Fig. 2. Response surface (a) and Pareto chart standardized estimate effect of the regression coefficients (b) for potential maximum growth rate (μ_{max}, $\log_{10}$ cfu/mL $days^{-1}$) of *Zygosaccharomyces rouxii* MC9 as a function of pH and sugar concentration (°Brix) at isothermal conditions (23 °C) in concentrated grape juice.

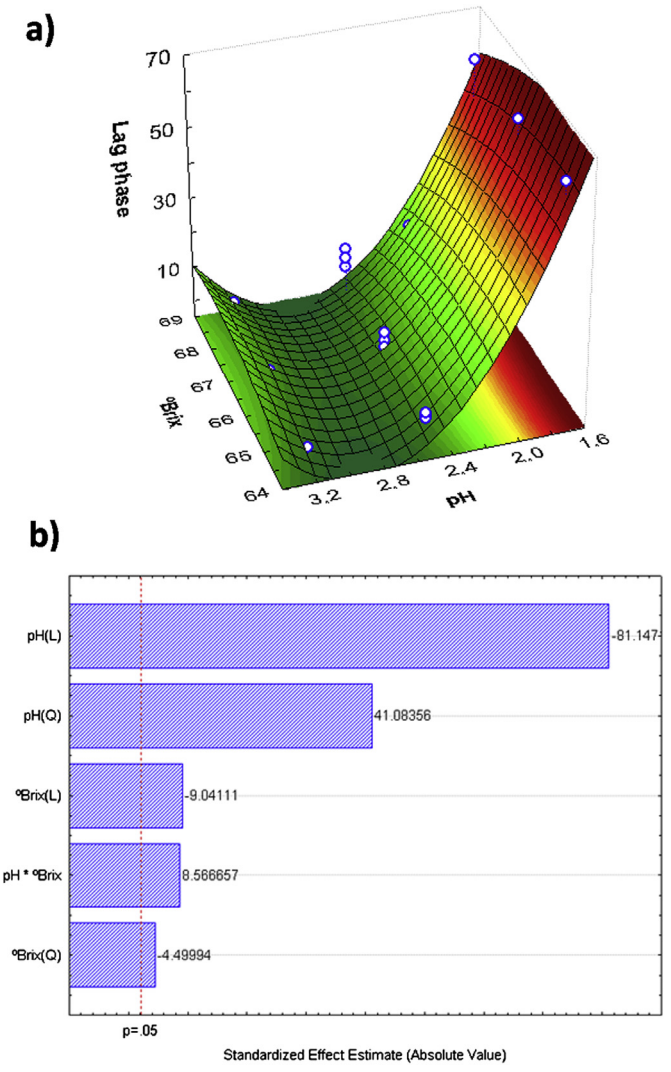

Fig. 3. Response surface (a) and Pareto chart standardized estimate effect of the regression coefficients (b) for lag phase duration (λ, days) of *Zygosaccharomyces rouxii* MC9 as a function of pH and sugar concentration (°Brix) at isothermal conditions (23 °C) in Argentinean concentrated grape juice.

conditions. Table 1 shows the μ_{max} and λ values obtained for the different conditions included in the experimental design. It can be observed in Table 1, λ and μ_{max} at 23 °C changed as the pH and sugar concentration were modified. Specifically, μ_{max} ranged from 0.000 (experiments with pH 1.7) to 0.530 ($\log_{10}$ CFU/mL $days^{-1}$) (pH 2.5 and 64 °Brix), while λ ranged from 0.000 (experiments with the highest pH at 3.2) to >60 days (experiments with the lowest pH value 1.7).

Table 2 shows the regression coefficients obtained from the ANOVA analysis for the growth parameters μ_{max} and λ of *Z. rouxii* MC9 as a function of environmental factors. The variance in the response that was explained by both models was 90.2% for μ_{max} and 92.8% for λ. Therefore, both models can be considered suitable to describe the effects of environmental variables under study (pH and sugar concentration) on yeast growth.

The RS equations that predict the response of both growth parameters as a function of the environmental variables can be deduced from Table 2 by replacing the appropriate coefficients in the general Equation (1). These polynomial equations can be used by the industry to predict the behaviour of *Z. rouxii* as a function of diverse combinations of pH and sugar concentration within the studied experimental range (interpolation region). Figs. 2a and 3a show the graphical representation of these equations for μ_{max} and λ, respectively. Both RSs show a clear curvature effect along the pH axis. As pH decreased, the μ_{max} value decreased (positive correlation) while λ increased (negative correlation), suggesting an inhibitory effect of this factor on *Z. rouxii* growth. Figs. 2b and 3b show the Pareto chart for the standardized effect of the studied environmental factors for μ_{max} and λ, respectively. With the exception of the quadratic effect of °Brix for μ_{max}, all regression coefficients were significant at a p-value <0.05. As it can be observed in Fig. 2b, the highest effects for both growth parameters were observed for pH (linear and quadratic effect), followed by the interaction °Brix*pH and the linear effect of °Brix (in the case of μ_{max}) and the linear effect of °Brix, interaction °Brix*pH, and the quadratic effect of °Brix (in the case of λ). The comparative lower inhibitory effect of sugar concentration on the growth of *Z. rouxii* compared to that of pH was probably due the good adaption of this yeast to high osmotic pressures.

Table 3
Time for spoilage (TFS) of *Zygosaccharomyces rouxii* MC9 in concentrated grape juice at isothermal (IC, 23 °C) and non-isothermal (NIC, variable temperatures) conditions for the different treatments (combinations of pH and °Brix) included in the experimental design.

Run	°Brix	pH	TFS-IC (days)	TFS-NIC (days)
1	64	1.7	>60	>60
2	64	1.7	>60	>60
3	64	1.7	>60	>60
4	64	2.5	15.00	18.00
5	64	2.5	14.00	17.00
6	64	2.5	14.00	17.00
7	64	3.2	13.00	12.00
8	64	3.2	14.00	13.00
9	64	3.2	14.00	12.00
10	66	1.7	>60	>60
11	66	1.7	>60	>60
12	66	1.7	>60	>60
13	66	2.5	26.00	21.00
14	66	2.5	26.00	22.00
15	66	2.5	27.00	21.00
16	66	3.2	14.00	15.00
17	66	3.2	15.00	14.00
18	66	3.2	14.00	14.00
19	68	1.7	>60	>60
20	68	1.7	>60	>60
21	68	1.7	>60	>60
22	68	1.9	>60	>60
23	68	1.9	>60	>60
24	68	1.9	>60	>60
25	68	2.1	48.00	41.00
26	68	2.1	49.00	42.00
27	68	2.1	48.00	42.00
28	68	2.5	35.00	25.00
29	68	2.5	36.00	24.00
30	68	2.5	35.00	24.00
31	68	3.2	26.00	24.00
32	68	3.2	27.00	23.00
33	68	3.2	26.00	24.00

Note: Experiments were followed for a maximum of 60 days. For this reason, when spoilage was not detected after this time, TFS was set at 60 days for model fit.

3.2. Modelling time for spoilage at isothermal and non-isothermal conditions

TFS of *Z. rouxii* MC9 in concentrated grape juice was modelled at both isothermal (IC, 23 °C) and non-isothermal (NIC) conditions. Table 3 shows the TFS values obtained for the different conditions included in the experimental design. As it can be easily seen in Table 3, TFS changed as a function of the pH and the sugar concentration evaluated. Specifically, TFS-IC (23 °C) ranged from 13.66 days (experiments with pH 3.2 and 64 °Brix) to >60 days (experiments with pH 1.7), while TFS-NIC (variables temperatures) ranged from 12.66 days (pH 3.2 and 64 °Brix) to >60 days (runs with the lowest pH value). In general, the treatments conducted at non-isothermal conditions showed a slight lower microbial stability (evidenced in lower TFS values) than those conducted at isothermal conditions (see Table 3). In the most favourable pH value for growth (3.2), increasing the sugar concentration from 64 to 68 °Brix doubled the microbial stability of the product for both temperature conditions.

Table 4 shows the regression coefficients obtained from the ANOVA analysis for the TFS-IC and TFS-NIC of *Z. rouxii* MC9 as a function of the environmental factors under study. The percentage of explained variance by the models was the highest, with 96.7 and 97.6% for TFS-IC and TFS-NIC, respectively. All the regression terms were retained in the mathematical equations, with very similar values for both temperature conditions. Moreover, it is possible to deduce from Table 4 the RS equations that predict the TFS of the concentrated grape juice as a function of pH and sugar concentration at storage and overseas shipping temperatures. By replacing the appropriate terms in the general Equation (1), the respective RS equations for TFS are:

Table 4
Regression coefficients estimated by means of the ANOVA for the Time for Spoilage (TFS) of *Zygosaccharomyces rouxii* MC9 in concentrated grape juice at isothermal (IC, 23 °C) and non-isothermal (NIC, variable temperatures) conditions.

Regression coefficient	Value	Standard deviation
Model for TFS-IC		
β_0 (Mean/Inter)	2849.592	201.003
β_1 °Brix (L)	−79.148	6.061
β_2 °Brix (Q)	0.596	0.045
β_3 pH (L)	−232.069	5.696
β_4 pH (Q)	20.771	0.324
β_5 °Brix*pH	1.537	0.084
R^2	0.967	
Lack of fit	0.000	
Pure error	4.670	
Model for TFS-NIC		
β_0 (Mean/Inter)	2771.561	210.003
β_1 °Brix (L)	−75.864	6.061
β_2 °Brix (Q)	0.573	0.045
β_3 pH (L)	−228.12	5.696
β_4 pH (Q)	27.544	0.324
β_5 °Brix*pH	0.956	0.084
R^2	0.976	
Lack of fit	0.000	
Pure error	4.670	

Note: Significant coefficients can be determined from their respective Pareto charts.

$$\mathrm{TFS-IC(days)} = 2849.592 - 79.148(^{\circ}\mathrm{Brix}) + 0.596(^{\circ}\mathrm{Brix})^2 - 232.069\ \mathrm{pH} + 20.771(\mathrm{pH})^2 + 1.537(^{\circ}\mathrm{Brix})^{*}(\mathrm{pH}) \quad (2)$$

$$\mathrm{TFS-NIC(days)} = 2771.561 - 75.864(^{\circ}\mathrm{Brix}) + 0.573(^{\circ}\mathrm{Brix})^2 - 228.12\ \mathrm{pH} + 27.544(\mathrm{pH})^2 + 0.956(^{\circ}\mathrm{Brix})^{*}(\mathrm{pH}) \quad (3)$$

Both equations allow the estimation of the time (in days) that *Z. rouxii* needs to produce visible spoilage in concentrated grape juice at isothermal and non-isothermal conditions.

A graphical representation of the combined effect of the limiting growth factors extending the microbial stability of the product at isothermal and non-isothermal conditions is shown in Figs. 4a and 5a, respectively. Both RSs had a very similar morphology. The highest effects were noticed for the linear and quadratic effects of pH (Figs. 4b and 5b). Specifically, a gradual decrease of pH was directly correlated with the increase in the microbial stability of the product, showing a greater difference in the highest concentrations of sugar evaluated. At pH values below 2.5, small variations in the pH caused a significant increase in product's shelf life (see Figs. 4a and 5a). Moreover, concentrated grape juice with pH adjusted below 2.0 allowed to increase the product's shelf life of the product for more than 60 days.

4. Discussion

It has been previously reported that the spoilage of various high-sugar products, such as honey, maple syrup, dried fruits, concentrated fruit juices, raw sugar cane, jams and jellies, is caused mainly by the metabolism of osmophilic yeasts (Deák and Beuchat, 1993; Tokuoka, 1993). Yeasts have been reported to be significant

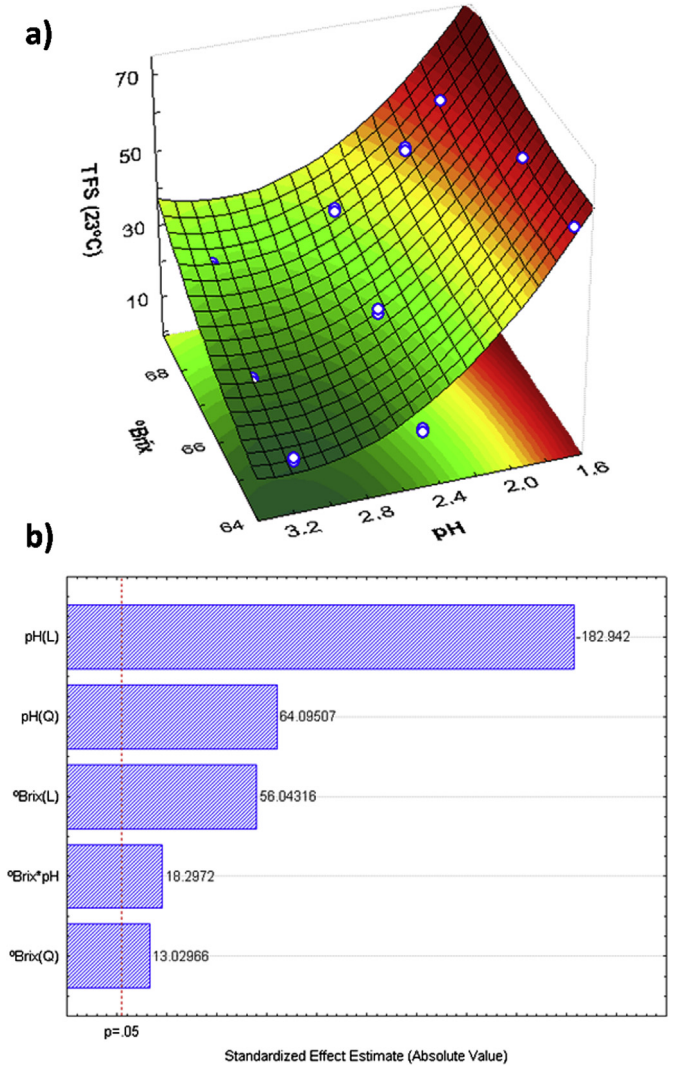

Fig. 4. Response surface (a) and Pareto chart standardized estimate effect of the regression coefficients (b) for time for spoilage (TFS, days) produced by *Zygosaccharomyces rouxii* MC9 as a function of pH and sugar concentration (°Brix) at isothermal conditions (IC, 23 °C) in concentrated grape juice.

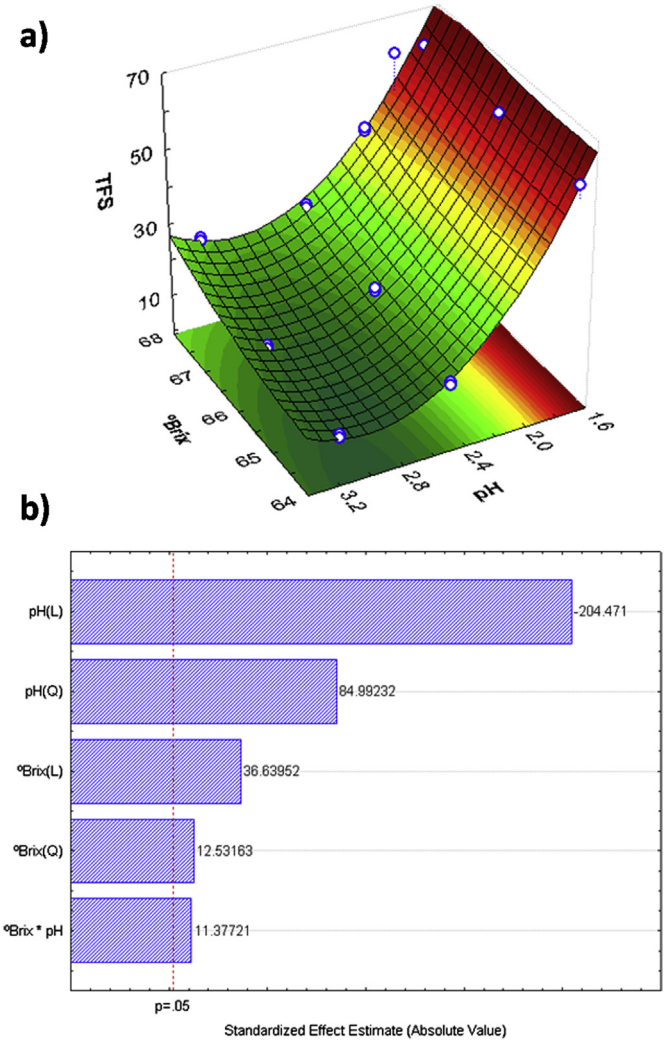

Fig. 5. Response surface (a) and Pareto chart standardized estimate effect of the regression coefficients (b) for time for spoilage (TFS, days) produced by *Zygosaccharomyces rouxii* MC9 as a function of pH and sugar concentration (°Brix) at non-isothermal conditions (NIC) in concentrated grape juice.

spoilage organisms, especially in food systems with low pH and high sugar concentrations. Also yeasts are responsible for spoilage in products containing sorbate and benzoate as preservatives as well as in the presence of alcohol where most bacterial species are inhibited (Praphailong and Fleet, 1997; Evans et al., 2004). Many environmental factors affect the yeast growth, but the response to each particular condition differ among the species (Praphailong and Fleet, 1997).

In order to design the adequate strategies to prevent spoilage, it is advantageous to know the identity of the spoilage microorganisms present in the products and in order to get an insight into the contamination source (Loureiro, 2000). In a previous study from our group, *Z. rouxii* has been described as the main microbiological agent responsible for the spoilage in concentrated grape juice in Argentina (Combina et al., 2008).

Many yeast species are able to tolerate a wide range of pH, from pH 1.5 to 10.0. In fact, most yeasts prefer a slightly acidified medium, between 3.5 and 6.0, which is the pH found in most fruit juices, beverages and soft drinks. Water activity of foods is also a very important factor that limits yeast growth. While most yeasts will easily grow in 20% (w/v) glucose, only a limited number of yeast species are able to grow at low a_w produced by the presence of high sugar concentration (60% w/v) (Tilbury, 1980a,b). Moreover, *Z. rouxii* is able to grow in a wide range of pH values, such as pH 1.8 to 8.0 in the presence of high sugar concentration (Tokuoka, 1993) or pH 1.5 to 10.5 in 12% glucose medium (Restaino et al., 1983). In a well-documented review of spoilage yeasts, Fleet (1992) suggested that high sugar concentration may either increase or decrease the low-pH tolerance of yeasts and emphasized that a further study of these influences would be needed to clarify these discrepant observations. In this paper, we study the combined effects of pH and high sugar concentrations on *Z. rouxii* growth and TFS evaluated in natural substrate under two temperature conditions to mimic product storage and shipping overseas.

Membré et al. (1999) described the combined effects of pH and high sugar concentration on *Z. rouxii* growth rate in laboratory culture media. Our results suggest a partial agreement with these authors' observations. In their publication they found that increasing the sugar concentration from 300 (a_w: 0.957) to 800 g/L (a_w: 0.843) resulted in a reduction of the specific growth rate. Furthermore, they observed that the growth rate at high sugar

concentrations, such as 875 (a_w: 0.810) and 950 (a_w: 0.788) g/L, was very low. pH of 2.5 resulted in a 30% reduction in the growth rate, and no growth occurred at pH 2.0 at any sugar concentration assessed. In the present work, a pH of 1.9 allowed a slow growth of *Z. rouxii* in concentrated grape juice at 23 °C, whereas no growth was observed after 60 days at pH 1.7. The minimum pH value which allows the growth of *Z. rouxii* has been reported to be dependent on the strain, the culture medium employed and the compound used to acidified the medium (Splittstoesser, 1987; Tokuoka, 1993). For instance, Martorell et al. (2007) found that pH 2.2 was the minimal pH for growth of two *Z. rouxii* strains in a culture medium modified by adding HCl. On a separate report, Restaino et al. (1983) found that the growth of *Z. rouxii* was inhibited at pH 1.5 evaluated under similar conditions. However, when the pH value was adjusted with citric acid or other citrate/phosphate buffers, the minimal pH value to support *Z. rouxii* growth was 2.0 (Praphailong and Fleet, 1997; Membré et al., 1999). In the present study, the decrease in pH was obtained by treating the grape juice with an ion exchange column. This fact highlights the importance of evaluating the growth of spoilage yeast in natural substrates using acidification methods normally employed by the industry.

Praphailong and Fleet (1997) concluded that 700 g/L of glucose was the highest sugar concentration at which *Z. rouxii* proliferated. This value is not consistent with the results obtained in the present study, since increasing the sugar concentration in concentrated grape juice to levels as high as 842 g/L (68 °Brix) did not greatly affected *Z. rouxii* growth. Our results are in agreement with others authors' reports, who have evidenced the growth of *Z. rouxii* at low a_w levels, such as 0.650 (Legan and Voyset, 1991; Tokuoka, 1993). Moreover, Martorell et al. (2007) have reported that two *Z. rouxii* strains isolated from spoilt syrup were able to grow in medium containing 900 g/L of glucose.

In this study, we have shown by using RS methodology, that the main limiting factor that affect *Z. rouxii* growth was the pH, mainly when its value was below 2.1. In a recent work, Vermeulen et al. (2012) studied the influence of environmental stress factors on the growth/no growth boundary of *Z. rouxii*. The authors found that pH decrease from 7.0 to 3.5 had almost no effect on the detection time of the different *Z. rouxii* strains. Only pH values below 2.5 had a significant effect on the detection time with an increase of approximately 4–40 days. However, this pH value was over the relevant range of the target product (chocolate filling) and it was not included in the model design. Furthermore, even the strictest conditions assessed (pH 5.0 and a_w 0.76), could still support *Z. rouxii* growth (Vermeulen et al., 2012). These observations are in line with our results, where the increase in sugar concentration was not enough to inhibit the growth of this spoilage species in concentrated grape juice.

The decrease in the pH values in concentrated grape juice leads to an increase in the time required for spoilage. An extension of the self life for over 30 days represents a huge marketing advantage, since by that time the product should have arrived to the destination. Moreover, TFS was above 60 days when pH was below 2.1 units, independently of the sugar concentration and temperature conditions. Also, TFS of concentrated grape juice was slightly lower when a non-isothermal profile was applied. This may be due to the extreme and oscillating temperatures during the first week of this assay which produced water evaporation and subsequent condensation on the substrate surface, increasing a_w and promoting the onset of spoilage. It is also important to note that the concentrated grape juice was inoculated with $1.2 \pm 0.2 \times 10^2$ CFU/mL, which represent the maximum limit for fungi and yeasts count accepted by the buyers. This represents a worst-case scenario, because the concentrated grape juice contains the maximum yeast count tolerated by current industry standards. Therefore, the data presented here represents the minimum microbial stability period for this substrate.

5. Conclusions

The results of the mathematical modelling of the variables under study (pH and sugar concentration) suggest that it is advisable for producers and buyers to obtain pH values below 1.7 to achieve total inhibition of *Z. rouxii* MC9 in concentrated grape juices. However, this pH value could be difficult to achieve under industrial conditions. Alternatively, reducing the pH up to values of 2.0 may be enough to significantly increase the shelf life of the product at 68 °Brix for both storage and shipping overseas temperature conditions.

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