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Effects of temperature, pH and sugar concentration on the growth parameters of *Saccharomyces cerevisiae*, *S. kudriavzevii* and their interspecific hybrid

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ABSTRACT

The effects of temperature, pH and sugar concentration (50% glucose + 50% fructose) on the growth parameters of *Saccharomyces cerevisiae* T73, *S. kudriavzevii* IFO 1802^T and the hybrid strain *S. cerevisiae* × *S. kudriavzevii* W27 were studied by means of response surface methodology based in a central composite circumscribed design. Lag phase could not be properly modelled in the wine model system, where yeasts started the fermentation in few hours after inoculation. In the case of the maximum specific growth rate (μ_{max}), the temperature was the most important variable for three yeasts, although the effects of sugar concentration (in T73 and W27) and pH (W27 and 1802) were also significant ($p<0.05$). The only retained interaction was between the variables temperature and pH for yeast 1802. The polynomial equations built for μ_{max} were used both to assess the behaviour of yeasts as a function of the factors and to predict their growth. In the case of temperature, the profiles obtained by the equations showed that response of the hybrid W27 was similar to T73 and different to 1802. When pH was the factor under study, the response of the hybrid W27 was closer to 1802 than yeast T73. For sugar concentration, the response of the hybrid W27 was similar to T73 but different to 1802. To the best of our knowledge, this is the first time that predictive models are used to assess and compare the response of a hybrid strain with respect to its parental species. The information obtained could also be useful to estimate the possible effect of climatic change on yeast growth.

1. Introduction

Yeasts play a prominent role in wine fermentations, which can strongly affect the quality and flavour of the final product (Querol and Fleet, 2006). Among several yeasts, *Saccharomyces cerevisiae* and *S. bayanus* var. *uvarum* are the most important species present during the fermentation process (Pretorius, 2000; Querol and Fleet, 2006). Recently, interspecific hybrid strains between *Saccharomyces* species have been described as involved in wine fermentations. González et al. (2006) described wine yeast hybrids between the species *S. cerevisiae* × *Saccharomyces kudriavzevii* and *S. cerevisiae* × *S. bayanus*. Several strains selected as commercial wine yeasts also resulted to be *Saccharomyces* hybrids (González et al., 2006; Bradbury et al., 2006), for instance the hybrid *S. cerevisiae* × *S. kudriavzevii* Lalvin W27. Therefore, hybrid strains appear as well adapted to the stress conditions (low pH, high sugar concentration and ethanol content) occurring during wine fermentations (Belloch et al., 2008), and their enological characterization confirmed their interesting properties according to the new trends in winemaking (González et al., 2007).

Different factors can affect the course of the fermentation, influencing the ecology and adaptation of the microbiota present. The temperature is a variable that directly affects the growth rate of the microorganisms (Charoenchai et al., 1998), and the final composition of wine (Torija et al., 2003). Another significant variable is the concentration of fermentable sugars in grape musts, ranging between 125 and 250 g/L (Fleet and Heard, 1993). It is likely that the initial concentrations of glucose and fructose (main grape sugars) will selectively influence the species and strains of yeast present during fermentation. Must pH, ranging from 2.75 to 4.25, is also considered an important factor for the survival and growth of yeasts (Fleet and Heard, 1993). Due to climatic change, glucose and fructose are increasing their concentrations in grapes meanwhile the acidity decreases, affecting the global wine quality (Jones et al., 2005). This fact originates musts with a higher initial amount of fermentable sugars and higher pH. Hence, these factors must be studied with more detail, especially the interactions between them and their influence on microorganisms.

Several studies have modelled the wine fermentation process (Malherbe et al., 2004; Colombié et al., 2005; Coleman et al., 2007), whereas other works focused on studying the influence of environmental variables on the microorganisms involved in wine fermentations. In this context, Charoenchai et al. (1998) reported the effects of

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temperature, pH, and sugar concentration on the growth rates and cell biomass of several wine yeasts such as *Kloeckera apiculata*, *Torulaspora delbrueckii*, *Pichia anomala*, *S. cerevisiae* and various *Candida* species. Medawar et al. (2003) modelled the lag phase of *Brettanomyces intermedius* i100 as a function of ethanol content in laboratory medium, whereas D'Amato et al. (2006) showed the influence of temperature, ammonium and glucose concentrations on *S. cerevisiae* growth in a synthetic must. Finally, Serra et al. (2005) studied the effects of temperature and pH on the growth of *S. bayanus* var. *uvarum* P3, *S. cerevisiae* VL3C and their interspecific hybrid in order to use hybrids as active dry yeasts. However, more information is still necessary to understand the behaviour of yeasts during wine fermentation. The prediction of the kinetic of the fermentation process in relation to the properties of the grape must is of great interest for wine industry (D'Amato et al., 2006).

Response surface (RS) is a very useful tool which has been previously applied to estimate the effects of environmental variables on yeast growth (Sorensen and Jakobsen, 1997; D'Amato et al., 2006; Arroyo-López et al., 2006). This methodology has widely been used in predictive microbiology as a secondary model (McMeekin et al., 1993) to predict the microorganism response to environmental changes. Particularly, central composite designs with star points are very useful because they provide rotatability, high quality predictions over the entire design space and low number of experimental runs (Myers and Montgomery, 2002).

The aims of this paper are: a) to estimate the effects of temperature, pH and sugar concentration (50% glucose + 50% fructose) on the yeasts *S. cerevisiae* T73, *S. kudriavzevii* IFO 1802^T, and the hybrid strain *S. cerevisiae* × *S. kudriavzevii* W27, obtaining at the same time valuable information about the possible effect of climatic change on their growth, b) to use mathematical modelling obtained from RS methodology to predict the response of these microorganisms under different combinations of the environmental variables, and c) to assess and compare the response of the hybrid strain with respect to representative strains of its parental species.

2. Materials and methods

2.1. Yeast strains

The yeasts used in this study were *S. cerevisiae* Lalvin T73 (abbreviated as T73), *S. kudriavzevii* IFO 1802^T (hereafter 1802) and the hybrid strain *S. cerevisiae* × *S. kudriavzevii* Lalvin W27 (henceforth W27). T73 was selected years ago in our laboratory from a wine fermentation in Alicante, Spain (Querol et al., 1992), and is commercialised as Lalvin T73 (Lallemand Inc., Montreal, Canada). The hybrid W27 was also isolated from wine fermentations in Wädenswill, Switzerland (Schütz and Gafner, 1994), and is also commercialised (Lallemand Inc.). Finally, *S. kudriavzevii* has never been described in wine fermentation. For this reason we have used the type strain of this species, IFO 1802^T, isolated from decayed leaves in Japan (Naumov et al., 2000). The strains T73 and 1802 were used as representatives of the parental species involved in the formation of the hybrid W27, because as a natural hybrid their real parental strains are unknown.

2.2. Inoculum preparation

Single colonies from pure cultures of each species were inoculated separately into 5 mL of Yeast-Malt-peptone-glucose broth medium (YM, Difco™, Becton and Dickinson Company, Sparks, USA) and then incubated at 25 °C for 24 h. The initial pH and glucose concentration of the medium was 5.5 ± 0.2 and 10 g/L, respectively. After this period, tubes were centrifuged, the pellets washed with sterile saline solution (0.9% NaCl, wt/vol) centrifuged and re-suspended again in sterile saline solution to obtain a concentration of about 7 $\log_{10}$ CFU/mL.

2.3. Growth medium preparation

Fermentation experiments were carried out in a complex synthetic medium MS300 miming a standard natural must previously described by Bely et al. (1990). Natural musts show a variable composition among different years that can influence the yeast growth. For this reason, a defined synthetic must was chosen in this work as the most appropriate growth medium to overcome this variation. Firstly the sugar concentration (50% glucose + 50% fructose) was adjusted in distilled water according to the experimental design and heated at 100 °C for 15 min to prevent sugar caramelization. Stocks for the other components of the medium (mineral salts, vitamins, amino acids and anaerobic factors) were previously sterilized by filtration (0.2 µm) and then added to the basal medium in the appropriate concentration (Rossignol et al., 2003). Finally the pH was adjusted by aseptically adding tartaric acid (85%, wt/vol) according to the experimental design. We chose this organic acid because it is a compound normally found in grapes and wines and it is very rarely metabolized by ascomycetous yeasts. Sterile glass bottles (≈60 mL of volume) were filled with 55 mL of synthetic must and independently inoculated with 50 µL of the corresponding yeast saline suspension to reach an initial concentration of inoculum of about 4.50 ± 0.21 $\log_{10}$CFU/mL for T73, 4.36 ± 0.32 $\log_{10}$CFU/mL for the hybrid W27, and 4.15 ± 0.29 $\log_{10}$CFU/mL for 1802. Bottles were incubated without shaken.

2.4. Experimental design

The effects of sugar concentration (50% glucose + 50% fructose), temperature, and pH on yeasts growth were tested using a central composite circumscribed design with three repetitions in the centre to account for pure error. The design was generated with Statistica 6.0 software package (StatSoft, Tulsa, OK, USA) and environmental variables levels were established into a range of conditions usually found in wine fermentations. Star points were situated at ± 1.68 from the centre to account for rotatability, which permits to extract the same amount of information (and consequently make predictions with the same precision) in all directions of the fitted surface (Myers and Montgomery, 2002). The full central composite circumscribed design, based on three basic principles of an ideal experimental design, primarily consists of (1) a complete 2^n factorial design, where n is the number of test variables, (2) n_0 number of centre points and (3) start points on the axis of each design variable at a distance of ± 1.68 from the design centre. Hence, the total number of design points was $N = 2^n + 2n + n_0$, in our case 17 treatments. For statistical calculations the variables were coded according to the equation:

$$\text{Coded variable} = [\text{physical value} - 1/2*(\text{high value} + \text{low value})] / [1/2*(\text{high value} - \text{low value})] \quad (1)$$

Relationships between coded and physical levels are shown in Table 1. All experiments were randomly performed in duplicate. The full experimental design was independently run for each yeast and monitored for at least two weeks.

Table 1
Relationship among physical and coded values of environmental variables used in the central composite circumscribed design.

Point	Coded values				
	Star	Low	Centre	High	Star
	−1.68	−1.00	0.00	+1.00	+1.68
Temperature (°C)	13.9	18.0	24.0	30.0	34.1
pH	2.24	2.75	3.50	4.25	4.76
Sugar concentration[a] (g/L)	116	150	200	250	284

[a] (50% glucose + 50% fructose).

2.5. Enumeration of cells

Samples were taken from the fermentations at variable time intervals, diluted in sterile saline solution and plated onto YM agar plates. Sampling times were established according to the yeast growth conditions so that the total number of samples ranged from 8 to 15 and were distributed through the different phases of the yeast growth (lag, exponential and stationary). All plates were incubated aerobically at 25 °C for 48 h. Counts were expressed as CFU/mL.

2.6. Primary model

Growth parameters (maximum specific growth rate and lag phase duration) were calculated from each treatment by directly fitting plate count versus time to the reparametized Gompertz equation proposed by Zwietering et al. (1990):

$$y = D^{*}\exp\{-\exp[((\mu_{max}{*}e)/D){*}(\lambda - t)) + 1]\} \quad (2)$$

where $y = \ln(N_t/N_0)$, N_0 is the initial population (CFU/mL) and N_t is the population at time t; $D = \ln(N_\infty/N_0)$ is the maximum population value reached with N_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h). Growth data from each treatment and yeast were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e., loss function (observed − predicted)^2. This task was accomplished using the non-linear module of the Statistica 6.0 software package and its Quasi-Newton option.

2.7. Secondary model

Lag phase (λ) and maximum specific growth rate (μ_{max}) were subsequently adjusted to a response surface of the general form:

$$Y = ß_o + ß_1X_1 + ß_{11}X_1^2 + ß_2X_2 + ß_{22}X_2^2 + ß_3X_3 + ß_{33}X_3^2 + ß_{12}X_1X_2 + ß_{13}X_1X_3 + ß_{23}X_2X_3 + \varepsilon. \quad (3)$$

where Y is the growth parameter modelled, $ß_o$ is the mean/intercept term, $ß_i$ are the coefficients to be estimated during the RS fitting ($ß_1$ is the coefficient for the linear effect of X_1, $ß_{11}$ for the quadratic effect of X_1, $ß_{12}$ for the interaction between variables X_1 and X_2, and so on) and ε is the term for error. X_1, X_2, and X_3 are the environmental variables (temperature (T), pH and sugar concentration (S), 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 6.0 software package, using the pure error, derived from repetitions in the centre, as option in the corresponding ANOVAs. Model performance was checked by the lack of fit test and the determination coefficient R^2 (percentage of variability in the response that can be explained by the model).

2.8. Model validation

For the model validation, a new series of experiments were carried out in synthetic must. Each treatment was a combination of all studied variables. Variable levels were chosen randomly within the range of the values used in the design (interpolation region). Validation treatments were: 1) 21.0 °C, pH 3.00, 225 g/L sugar (coded values, −0.50, −0.66, and 0.50, for temperature, pH and sugar concentration, respectively); 2) 21.0 °C, pH 4.00, 175 g/L sugar (coded values, −0.50, 0.66, −0.50); 3) 27.0 °C, pH 3.00, 225 g/L sugar (coded values, 0.50, −0.66, 0.50); and 4) 27.0 °C, pH 4.00, 175 g/L sugar (coded values 0.50, 0.66, −0.50). Experimental μ_{max} values were compared to those predicted by the RS equations. To give a quantitative measure of the models performance, the accuracy (A) and bias factors (B) were calculated as described by Baranyi et al. (1999). The accuracy factor is based on mean square differences, while the bias factor is based on the arithmetical mean of the differences. The indexes %D ('percent discrepancy' between the predictions and observations), and %B ('percent bias') were also obtained (Baranyi et al., 1999).

3. Results

3.1. Effects of environmental variables on yeast growth parameters

Yeasts had a very short lag period under the conditions included in the experimental design (Table 2). This parameter ranged from 0.89 to 8.39 h in the case of T37, from 0.92 to 10.1 h for the hybrid W27, and from 0.30 to 16.7 h in case of yeast 1802 (excluding run 10 in which no growth was observed after 170 h). Modelling lag phase in the wine model system showed difficulties. The percentages of variability in the response (R^2) that could be explained by the models for λ were low,

Table 2
Treatments included in the central composite circumscribed design and growth parameters (lag phase (λ) and maximum specific growth rate (μ_{max})) obtained for the yeasts *Saccharomyces cerevisiae* T73, *S. kudriavzevii* IFO 1802^T and the hybrid strain *S. cerevisiae* × *S. kudriavzevii* W27.

	Coded values			λ (h)			μ_{max} (h^{-1})		
Run	*T*	pH	*S*	T73	W27	1802	T73	W27	1802
1	−1.00	−1.00	−1.00	4.50 (0.86)	3.14 (1.89)	0.89 (0.94)	0.113 (0.026)	0.157 (0.026)	0.142 (0.010)
2	−1.00	−1.00	1.00	3.12 (1.20)	0.92 (0.15)	0.93 (0.82)	0.096 (0.017)	0.150 (0.010)	0.137 (0.015)
3	−1.00	1.00	−1.00	4.93 (2.48)	8.45 (1.49)	4.04 (2.96)	0.129 (0.019)	0.177 (0.014)	0.161 (0.025)
4	−1.00	1.00	1.00	1.02 (0.44)	3.67 (1.60)	1.92 (1.50)	0.094 (0.012)	0.151 (0.018)	0.161 (0.012)
5	1.00	−1.00	−1.00	2.61 (1.41)	3.08 (0.58)	3.89 (1.17)	0.339 (0.095)	0.459 (0.043)	0.143 (0.023)
6	1.00	−1.00	1.00	2.23 (1.09)	2.47 (0.68)	16.7 (6.08)	0.353 (0.097)	0.415 (0.042)	0.119 (0.044)
7	1.00	1.00	−1.00	2.32 (0.88)	4.34 (0.81)	4.64 (1.03)	0.422 (0.054)	0.644 (0.117)	0.348 (0.054)
8	1.00	1.00	1.00	4.13 (1.23)	3.53 (0.91)	5.71 (0.69)	0.441 (0.088)	0.465 (0.077)	0.275 (0.022)
9	−1.68	0.00	0.00	5.38 (1.87)	5.28 (1.96)	3.95 (1.90)	0.082 (0.007)	0.096 (0.006)	0.117 (0.009)
10	1.68	0.00	0.00	0.89 (1.18)	3.46 (0.78)	170 (0.00)[a]	0.293 (0.040)	0.557 (0.089)	0.000 (0.000)[a]
11	0.00	−1.68	0.00	8.39 (1.95)	10.1 (1.05)	0.30 (1.05)	0.218 (0.034)	0.046 (0.008)	0.143 (0.037)
12	0.00	1.68	0.00	3.09 (1.22)	3.45 (0.67)	6.31 (0.95)	0.317 (0.055)	0.334 (0.032)	0.522 (0.099)
13	0.00	0.00	−1.68	3.84 (1.57)	3.27 (1.49)	2.79 (0.73)	0.480 (0.102)	0.421 (0.098)	0.388 (0.045)
14	0.00	0.00	1.68	2.02 (2.51)	2.39 (1.24)	4.91 (1.39)	0.162 (0.022)	0.272 (0.030)	0.394 (0.087)
15	0.00	0.00	0.00	1.87 (0.82)	4.32 (0.50)	4.49 (0.41)	0.224 (0.037)	0.324 (0.023)	0.354 (0.024)
16	0.00	0.00	0.00	2.57 (1.39)	4.36 (0.85)	4.42 (0.77)	0.241 (0.038)	0.361 (0.051)	0.383 (0.054)
17	0.00	0.00	0.00	1.09 (0.36)	4.07 (0.80)	3.94 (0.54)	0.198 (0.024)	0.319 (0.031)	0.337 (0.028)

Note: Standard deviation calculated from duplicate experiments in parenthesis.
T, temperature; *S* (sugar concentration).
[a] No growth was observed in this treatment for yeast 1802 and μ_{max} and λ were set up at 0.000 h^{-1} and 170 h for analysis, respectively.

Table 3
Regression coefficients estimated by means of the ANOVA analysis for the response factor *Y* (maximum specific growth rate, h^{-1}) in function of temperature (*T*), pH and sugar concentration (*S*) for the yeasts *Saccharomyces cerevisiae* T73, *S. kudriavzevii* IFO 1802^T and the hybrid *S. cerevisiae* × *S. kudriavzevii* W27.

Regression coefficient	Value	Standard deviation	*p*-value
Model for yeast T73	$R^2=0.812$		
β_0 (Mean/Inter)	0.220	0.013	[a]0.003
β_1 (linear *T*)	0.108	0.006	[a]0.003
β_{11} (quadratic *T*)	−0.014	0.006	0.175
β_2 (linear pH)	0.025	0.006	0.054
β_{22} (quadratic pH)	0.013	0.007	0.180
β_3 (linear *S*)	−0.040	0.006	[a]0.023
β_{33} (quadratic *S*)	0.032	0.007	[a]0.041
β_{12} (interaction *T**pH)	0.019	0.008	0.139
β_{13} (interaction *T***S*)	0.010	0.008	0.325
β_{23} (interaction pH**S*)	−0.001	0.008	0.861
Model for yeast W27	$R^2=0.952$		
β_0 (Mean/Inter)	0.331	0.013	[a]0.001
β_1 (linear *T*)	0.155	0.006	[a]0.001
β_{11} (quadratic *T*)	0.006	0.006	0.439
β_2 (linear pH)	0.054	0.006	[a]0.012
β_{22} (quadratic pH)	−0.041	0.006	[a]0.025
β_3 (linear *S*)	−0.037	0.006	[a]0.026
β_{33} (quadratic *S*)	0.013	0.007	0.184
β_{12} (interaction *T**pH)	0.026	0.008	0.080
β_{13} (interaction *T***S*)	−0.023	0.008	0.099
β_{23} (interaction pH**S*)	−0.019	0.008	0.140
Model for yeast 1802	$R^2=0.854$		
β_0 (Mean/Inter)	0.362	0.013	[a]0.001
β_1 (linear *T*)	0.006	0.006	0.416
β_{11} (quadratic *T*)	−0.121	0.006	[a]0.003
β_2 (linear pH)	0.076	0.006	[a]0.006
β_{22} (quadratic pH)	−0.024	0.007	0.072
β_3 (linear *S*)	−0.006	0.006	0.396
β_{33} (quadratic *S*)	−0.003	0.006	0.650
β_{12} (interaction *T**pH)	0.039	0.008	[a]0.040
β_{13} (interaction *T***S*)	−0.011	0.008	0.296
β_{23} (interaction pH**S*)	−0.005	0.008	0.572

[a] Significant coefficients (*p*-value ≤ 0.050).

with 67.9% for T73, 62.7% for yeast 1802, and 45.3% for the hybrid W27. In addition, the lack of fit was significant ($p<0.05$) for the ANOVA of the hybrid W27 and yeast 1802, and only the model for T73 had a non significant lack of fit ($p>0.143$). Diverse transformations for λ (1/λ, $\log_{10}\lambda$, and $1/\sqrt{\lambda}$) were also fitted by RSM, but with similar results. In any case, the experimental values recorded for λ showed that these yeasts can start the fermentation in few hours after inoculation even at low values of temperature and pH and high sugar concentration (Table 2). Only run 10 for yeast 1802 (34.1 °C, pH 3.50, 200 g/L) showed no growth at the end of the experimental period, indicating that such conditions were inhibitory for this microorganism.

Table 2 also shows the μ_{max} obtained for the different yeasts according to the experimental design. This parameter ranged from 0.082 to 0.480 h^{-1} in the case of T73, from 0.046 to 0.644 h^{-1} for the hybrid W27, and from 0.000 to 0.522 h^{-1} for yeast 1802. The regression coefficients estimated for the three yeasts and their significances, deduced from the ANOVA analysis of regression, are shown in Table 3. All models had a non-significant lack of fit ($p>0.05$). In addition, the variance in the response that was explained by these models was always high: 81.2% for T73, 85.4% for yeast 1802 and 95.2% for the hybrid W27. This way, the models can be considered appropriated to describe the effects of environmental variables on μ_{max}.

A regression coefficient that was significant (*p*-value ≤ 0.05) for the three models was β_0, which represents the mean/intercept term. In other words, it is the value of μ_{max} at the reference point 24.0 °C, pH 3.50, 200 g/L (0,0,0 in coded values). Other significant coefficients for yeast T73 were also the linear effect of temperature, and the linear and quadratic effects of sugar concentration (Table 3). The effect of the environmental variables on μ_{max} of T73 can be studied by means of their respective coefficients. In the case of temperature, μ_{max} increased its value when this environmental variable ranged from 13.9 to 34.1 °C. One unit increase in temperature (in other words, the factor is moved from 0 to +1, change of 6 °C) may, theoretically, increase μ_{max} in 0.108 h^{-1}. The value of the coefficient for the sugar content can be interpreted in a similar way: an increase of +1 in sugar concentration (or 50 g/L in physical level) originates a change of −0.040 (h^{-1}) in the linear term and +0.032 (h^{-1}) in the square term on the response, showing a net decrease. No significant coefficients were found for pH (Table 3). However, these effects can be better studied by means of the profiles obtained by its respective polynomial equation, plotted in Fig. 1. These graphs depict the changes in the parameter modelled as

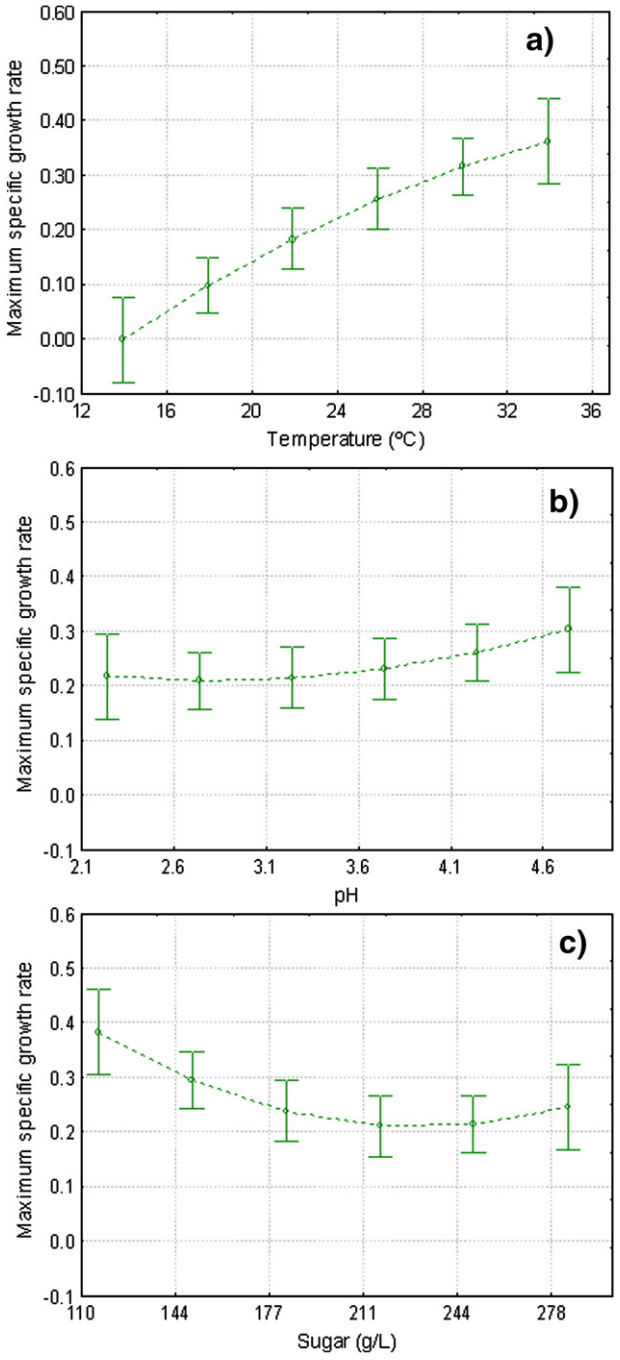

Fig. 1. Changes in μ_{max} (h^{-1}) obtained by the polynomial equation of yeast *Saccharomyces cerevisiae* T73 as a function of a) temperature, b) pH, and c) sugar concentration, with all other factors held constant at the central point (24.0 °C for temperature, 3.50 for pH and 200 g/L for sugar).

the factor moves along its axis, with all other factors held constant at the central point (0,0). Within the interval assayed, μ_{max} always increased with temperature (Fig. 1a). On the contrary, μ_{max} decreased progressively as the sugar concentration increases and reaches a minimum around 220 g/L (Fig. 1c). From Fig. 1b, it is also clear that the initial pH did not affect growth of this species, although a slight increase of μ_{max} was noticed when pH rose.

In the case of hybrid W27, the significant coefficients were: the linear effect of temperature, pH and sugar concentration, as well as the quadratic term of pH (Table 3). This means that μ_{max} increased linearly when temperature ranged from 13.9 to 34.1 °C due to its positive term (0.155). The negative value of the linear effect of sugar is indicative of an inverse relationship: an increase of 1 unit in sugar decreases μ_{max} by 0.037 h^{-1}. Interpretation of the effect of pH is more complex due to the combined effect of linear and quadratic terms. In fact, the response may have an increase because of its positive linear term (0.054) and a decrease due to the negative value of its quadratic effect (−0.041). As previously, these effects are better showed by the respective profiles obtained for the factors (Fig. 2). The effects of temperature and sugars are fairly clear (Fig. 2a and c) and the overall effect of pH is now more evident. μ_{max} increased progressively from pH 2.24 up to ≈3.80, where reaches a maximum (Fig. 2b).

The significant coefficients for strain 1802 were: the linear effect of pH, the quadratic effect of temperature, and the interaction temperature*pH (Table 3). Interpretation of coefficients is similar to the previous yeasts. The presence of significant quadratic coefficient for temperature is indicative of a curvature on this parameter. The positive value of the interactive term between temperature and pH (0.039) may indicate a potential synergistic effect between both factors although no physiological interpretation can be deduced, given de empirical character of the model. For estimation purposes, an increase of 1 unit in the interaction temperature*pH increases μ_{max} in 0.039 h^{-1}. The obtained profiles clarify the effects. The effect of temperature showed a convex shape and an optimum in μ_{max} was reached around 24 °C (Fig. 3a). According to Fig. 3b, μ_{max} increased as pH moved from 2.24 up to 4.76 (positive sign for its coefficient). Finally, μ_{max} did not change as the sugar concentration increased (Fig. 3c).

Overall, the temperature was, apparently, the variable with the greater effect on yeast growth as may be deduced from the comparative study of Figs. 1–3, in which the changes in temperature always produced the higher variations on μ_{max}. Within the experimental region, the effect of temperature was never related to the level of sugar but showed a clear relationship with pH (significant interaction pH*temperature) only in the case of yeast 1802, which may indicate that it was strain dependent.

The RS equations obtained for the three yeasts as a function of temperature, pH and sugar concentration can be deduced from Table 3, by replacing the appropriate terms in Eq. (3). To reduce the complexity of the equations, only significant coefficients can be used. These polynomial equations, in coded terms, can be used by the industry to estimate the response (parameter modelled) of the three strains as a function of diverse combinations of environmental variables within the experimental range (interpolation region). Thus, the model may be useful for winemakers. For this purpose, it could be advisable to transform physical values into coded terms according to Eq. (1), and work with the coded polynomial equations to make predictions.

3.2. Validation results

Predictions of the models for the three yeasts were good. The best indexes for μ_{max} were obtained for yeasts T73 ($A = 1.04$, $B = 0.98$) and the hybrid W27 ($A = 1.06$, $B = 0.98$) with a pert cent discrepancies between predictions and observations of 4.80% and 6.30%, respectively. The bias factor (B) for both yeasts showed values very close to 1, with a slight tendency in the model to predict slower growth than the observations (%$B = -0.78$ for T73 and -0.63 for hybrid W27). In the case of yeast 1802, the indexes were slightly worse ($A = 1.17$, $B = 1.14$), with a percent discrepancy of 17.5% and a bias factor > 1, indicative that the models predict faster growth than the observations (%$B = +5.82$).

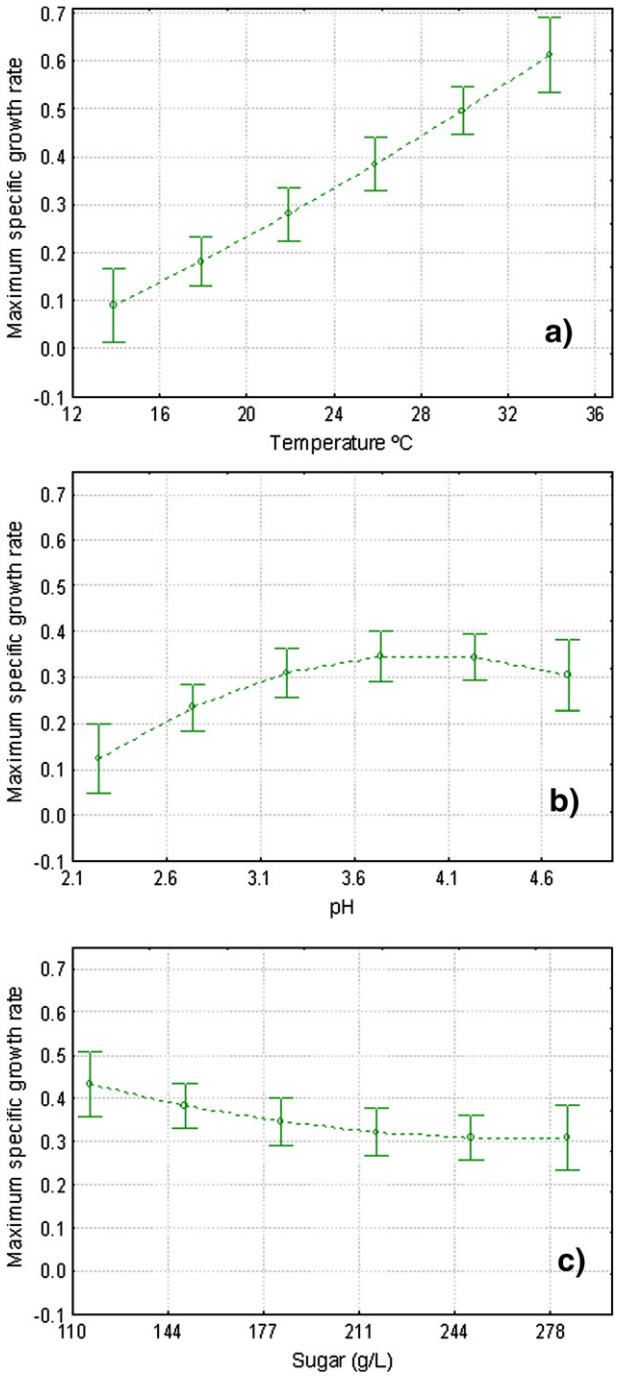

Fig. 2. Changes in μ_{max} (h^{-1}) obtained by the polynomial equation of the hybrid *S. cerevisiae* × *S. kudriavzevii* W27 as a function of a) temperature, b) pH, and c) sugar concentration, with all other factor held constant at the central point (24.0 °C for temperature, 3.50 for pH and 200 g/L for sugar).

3.3. Assessing the response of the hybrid strain respect to its parental species

The range checked for the three environmental variables (temperature 13.9–34.1 °C, pH 2.24–4.76, and sugar concentration 116–284 g/L) is usually found in wine fermentations. Thus, from the results

obtained in this study, we can assess and compare the response of the hybrid strain W27 with respect to its parental species under a simulated wine environment.

As mentioned above, λ values were very low, showing that all yeasts had a short adaptation time to wine environmental conditions even when the pre-incubation of the inoculums were carried out in a medium with a higher pH (5.5) and lower sugar concentration (10 g/L).

However, the response was different when the parameter analysed was μ_{max}. A comparison for this parameter with respect to temperature for yeasts T73, W27 and 1802 can be carried out by means of the profiles obtained for μ_{max} using their respective RS equations, fixing the pH and sugar concentration at 3.50 and 200 g/L respectively (see Figs. 1a–3a). Strain T73 and the hybrid W27 had a linear response in the range studied (13.9–34.1 °C) with the maximum value for both yeasts at 34.1 °C, while 1802 showed a quadratic response with a maximum around 24 °C. In the case of pH, the profiles obtained for μ_{max}, fixing the temperature and sugar concentration at 24.0 °C and 200 g/L, showed that the response for yeasts 1802 and the hybrid W27 was more similar between them than with respect to T73 (Figs. 1b–3b). All yeasts increased μ_{max} when pH increased, but the hybrid W27 showed an evident quadratic effect for this variable with an optimum around 3.5–4.0 (see Table 3 and Fig. 2b). Yeasts 1802 and T73 also showed a slight curvature for the pH, but their quadratic effects were not significant according to the corresponding ANOVAs (Table 3). The profiles obtained for μ_{max} as a function of sugar concentration fixing the temperature and pH at 24.0 °C and 3.50 (Figs. 1c–3c), showed that μ_{max} of 1802 was practically not affected by the sugar concentration in the range studied (116–284 g/L). However, for yeasts W27 and T73 the response was similar, and μ_{max} decreased when sugar concentration increased, with a marked quadratic effect in the case of T73 (see Table 3).

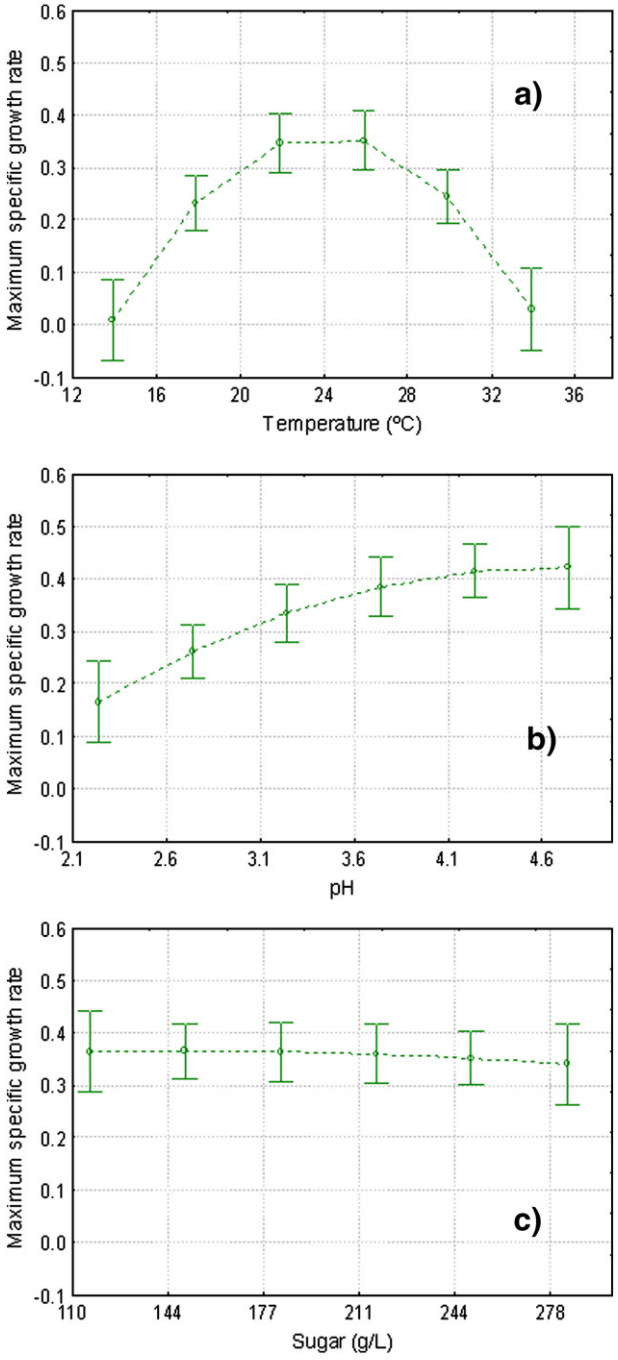

Fig. 3. Changes in μ_{max} (h^{-1}) obtained by the polynomial equation of yeast *S. kudriavzevii* IFO 1802^T as a function of a) temperature, b) pH, and c) sugar concentration, with all other factor held constant at the central point (24.0 °C for temperature, 3.50 for pH and 200 g/L for sugar).

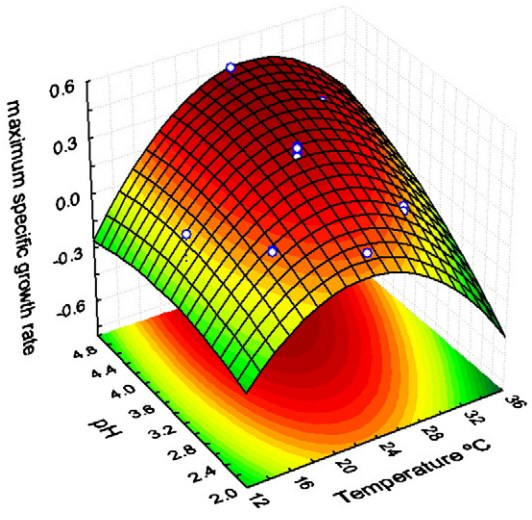

Fig. 4. Response surface for μ_{max} (h^{-1}) of *S. kudriavzevii* IFO 1802^T as a function of temperature and pH for a sugar concentration of 116 g/L (50% glucose + 50% fructose).

Results above commented were obtained fixing two variables at the centre of the experimental design (0, 0) and changing the other variable along its range (−1.68 to +1.68). They must be interpreted as the different response of the yeasts only under these levels. But it is also very interesting to find the optimal growth conditions inside the experimental region for the combinations of the three environmental variables. These values can be obtained by optimizing mathematically the profiles for predicted values of μ_{max} using their respective equations. They were attained at: a) 34.1 °C, pH 4.76 and 284 g/L (50% glucose + 50% fructose) in the case of yeast T73, with a predicted μ_{max} of 0.551 ± 0.132 h^{-1}; b) 34.1 °C, pH 4.76 and 200 g/L for the hybrid W27 with a predicted μ_{max} of 0.660 ± 0.151 h^{-1}; and c) 24.0 °C, pH 4.76 and 116 g/L for the yeast 1802 with a predicted μ_{max} of 0.438 ± 0.152 h^{-1}. Fig. 4 shows as an example the RS for μ_{max} of yeast 1802 as a function of temperature and pH for a sugar concentration of 116 g/L. It shows a clear optimum around 24.0 °C and pH 4.76 in agreement with the data deduced from the previous profiles for this yeast. The quadratic effect along the temperature axis is also clear.

4. Discussion

In this work, RSM based in a central composite design proved to be a very useful tool to assess the response of different yeasts under

varying conditions of temperature, pH and sugar concentrations usually found during wine fermentations. The methodology was performed following a complete procedure of predictive microbiology (experimental design, primary model, secondary model and validation) and was applied to wine yeast strains. Validation indexes obtained from independent experiments showed that RS equations can be used by industry as a guide to predict yeast growth in wine fermentations. To the best of our knowledge, this is the first time that this type of predictive model is used to compare the response of a hybrid strain with respect to representatives of its parental species, obtaining at the same time valuable information about the possible effect of climatic change on yeast growth.

The effect of the environmental variables on λ was complex to model due to the short lag period and the narrow interval in which their maximum and minimum values were reported (7.49 h for yeast T73, 9.21 h for hybrid W27, and 16.4 h for yeast 1802). Difficulties in estimating the bacterial lag time were also recognized by numerous authors and some of them have reviewed the diverse modelling possibilities (McMeekin et al., 1993; Swinnen et al., 2004). In the case of *B. intermedius* i100, a yeast also isolated from grape must, was reported a very short lag phase (7.80 h) in a medium with low pH (3.50) (Medawar et al., 2003). These authors also found a high standard deviation during the estimation of the λ parameter (±3.40 h). Similar results were found in this work for *Saccharomyces* strains.

Models for μ_{max} always had a non-significant lack of fit. The temperature was the variable with the higher effects for the three yeasts. This fact was also reported by Arroyo-López et al. (2006) for *P. anomala,* by Sorensen and Jakobsen (1997) for *D. hansenii*, and by D'Amato et al. (2006) for *S. cerevisiae*. Serra et al. (2005) studied the influence of temperature and pH on the μ_{max} of *S. bayanus* var. *uvarum* P3 and *S. cerevisiae* VL3c, both yeasts related with wine fermentations. They showed that the temperature was also the factor with the main influence on the response whereas the pH was less important. The optimal temperature for *S. bayanus* var. *uvarum* growth was 30 °C when the pH was kept constant at 4.0, and the optimal temperature for *S. cerevisiae* VL3c at pH 5.0 was 35 °C. In this work, the highest μ_{max} value for yeast T73 was reported at 34.1 °C and pH 4.76 (upper limits established by the experimental region), which suggest that the optimum value of temperature for this strain could be even higher. Torija et al. (2003) studied the effects of temperature (15–35 °C) on the growth of different strains of *S. cerevisiae* in white must. Overall, the fermentation was faster at high temperatures (30–35 °C). Charoenchai et al. (1998) also mentioned that growth rates for *S. cerevisiae*, *P. anomala*, *K. apiculata*, and *T. delbrueckii* increased with temperature, although their study only reached 25 °C. Charoenchai et al. (1998) reported that variation of medium pH between 3.0 and 4.0 did not significantly affect the growth rate and cell biomass of wine yeasts. However, Fleet and Heard (1993) observed that growth rate and must fermentation by *S. cerevisiae* were decreased as the pH was decreased from 3.5 to 3.0. In this work, an increase in the pH from 2.24 to 4.76 increased μ_{max} for yeasts T73, W27 and 1802. On this respect, if acidity decreases in grapes due to the climatic change, growth rate may increase for *Saccharomyces* wine strains. In the present study the pH of the synthetic must was adjusted with tartaric acid ($pK_a = 2.98$). Therefore, the undissociated form of this organic acid could also have an additional effect on yeast growth in the lower ranges of pH (for example, at pH 2.24 a great proportion of the acid is still undissociated).

Another factor that should be considered with detail is the sugar concentration because yeast can delay its growth at high concentrations of glucose and fructose, which was clear for strains T73 and the hybrid W27 (Table 2). Sugar concentrations from 200 g/L to 300 g/L decreased *S. cerevisiae* growth rate, as reported by Charoenchai et al. (1998) and D'Amato et al. (2006). These authors found the lowest growth rate at the higher glucose concentrations. Belloch et al. (2008) studied the response of *Saccharomyces* hybrids in laboratory media under low pH (2.8) and 250 g/L of glucose. The majority of yeasts tested were able to grow at 30 °C, but with the methodology used, they could not detect small changes in their responses. Therefore, the grape sugar concentration increase produced by the climatic change could negatively affect the kinetics of the wine fermentations.

The response of the yeasts was different depending on the variable under study. In the case of temperature, the response of the hybrid W27 was similar to T73 showing a positive and linear effect when temperature moved from 13.9 to 34.1 °C, while yeast 1802 showed a negative and quadratic effect with an optimum of temperature around 24.0 °C. D'Amato et al. (2006) also reported a positive and linear effect for a *S. cerevisiae* wine strain in the interval of temperature 15–35 °C. These authors did not find an optimum of temperature in the range studied. However, when the studied factor was pH, the hybrid W27 had a similar response to yeast 1802 and different to T73. The response of the hybrid W27 as a function of the sugar concentration had a similar behaviour to T73 and different to 1802. Serra et al. (2005) reported that hybrid strains between *S. bayanus* var. *uvarum* and *S. cerevisiae* globally had a response as a function of the temperature similar to the parental *S. bayanus* var. *uvarum*, although not completely identical. In the present work, the hybrid W27 response to temperature was also similar to that of the parental *S. cerevisiae* representative (strain T73), but showing a higher μ_{max} (Table 2). The yeasts T73 and 1802 were chosen as representative strains of the parental species involved in the origin of hybrids, such as W27, because the real parental strains of natural hybrids are unknown. This fact can explain the small differences in the response of the hybrid with respect to the parental representative. González et al. (2007) showed that the fermentation patterns of hybrid W27 and yeast T73 in Tempranillo musts at different temperatures were also very similar, especially in the range of temperature between 18 and 32 °C. González (2005) performed a detailed molecular characterization of the hybrid W27 using flow cytometry, comparative genome hybridization with DNA macroarrays and RFLP analysis of 37 genes located in the different chromosomal arms. Her results showed that hybrid W27 is an aneuploid strain, with three types of chromosomes, a complete set of the 16 chromosomes of *S. cerevisiae*, most chromosomes of the *S. kudriavzevii* parental, and several chimerical chromosomes resulting from recombination between "homeologous" chromosomes from *S. cerevisiae* and *S. kudriavzevii*. This complex genome structure could explain the response of the hybrid with respect to the representative strains of the parental species depending on the environmental variables under study.

5. Conclusions

Results obtained in this work show that changes in values of fermentation temperature, pH and sugar concentration of the must originated by the climatic change may affect to wine yeast growth. RS equations built for T73 and the hybrid W27 can be used by the industry to predict the growth of these yeasts under different combinations of the environmental variables. *S. kudriavzevii,* which has never been isolated from wine environments, showed optimal growth under low pH, high sugar concentration and temperatures around 24 °C, not unusual during winemaking. This observation raises the question about why this species in not found in wine environments, whilst its hybrids with *S. cerevisiae* are found in Central European wine fermentations. Further studies on the interaction between these species during fermentation are necessary to answer such a question.

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