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Probabilistic model for the spoilage wine yeast *Dekkera bruxellensis* as a function of pH, ethanol and free SO_2 using time as a dummy variable

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ABSTRACT

The present study uses a probabilistic model to determine the growth/no growth interfaces of the spoilage wine yeast *Dekkera bruxellensis* CH29 as a function of ethanol (10–15%, v/v), pH (3.4–4.0) and free SO_2 (0–50 mg/l) using time (7, 14, 21 and 30 days) as a dummy variable. The model, built with a total of 756 growth/no growth data obtained in a simile wine medium, could have application in the winery industry to determine the wine conditions needed to inhibit the growth of this species. Thereby, at 12.5% of ethanol and pH 3.7 for a growth probability of 0.01, it is necessary to add 30 mg/l of free SO_2 to inhibit yeast growth for 7 days. However, the concentration of free SO_2 should be raised to 48 mg/l to achieve a probability of no growth of 0.99 for 30 days under the same wine conditions. Other combinations of environmental variables can also be determined using the mathematical model depending on the needs of the industry.

1. Introduction

Wine spoilage is a serious problem for the wine industry because it renders the product unacceptable and can lead to large economic losses (Oelofse et al., 2008). *Dekkera bruxellensis*, or its anamorph state *Brettanomyces bruxellensis*, has been described as the main spoilage yeast in red wines (Loureiro and Malfeito-Ferreira, 2003; Querol and Fleet, 2006). Its presence in wines is associated with the detection of phenolic flavours that have a negative impact on the organoleptic characteristics of the final product (Chatonnet et al., 1992). The formation of these volatile phenols by *Dekkera* species has been shown to be the result of the enzymatic transformation of phenolic (hydroxycinnamic) acids naturally present in grape and wine into vinylphenol derivatives through the action of a coumarate decarboxylase activity and then reduced to an ethyl derivative through a vinylphenol reductase enzyme (Godoy et al., 2009; Suárez et al., 2007). These volatile phenols, especially the ethylphenols, have a low sensorial threshold and even small amounts are responsible for off-odours that have been described as 'animal', 'medicinal', 'horse sweat', 'barnyard', 'spicy' and 'phenolic' (Suárez et al., 2007). *Dekkera* species are slow-growing yeasts and their increase in number only occurs when other rapidly fermenting yeasts decrease and nutritionally favourable conditions are still maintained. Wine ageing in wood barrels and the bottling of wines have been recognised as the most critical stages during wine production for *Dekkera* spoilage (Oelofse et al., 2008; Renouf et al., 2006; Suárez et al., 2007).

It seems logical that the most efficient way to prevent wine spoilage by *D. bruxellensis* is to control its development and ethylphenol production through winemaking management using a preventive approach. Therefore, there is a need to improve our knowledge of the factors which control the growth of this microorganism during wine processing. Assuming that sufficient nutrients are available, microbial growth can be controlled primarily by pH, ethanol and temperature; additional factors such as the presence of preservatives like sulphites (SO_2) also contribute. SO_2 is widely recognised in both wine and food industries for its antioxidant and antimicrobial properties. Once dissolved in water, SO_2 exists in equilibrium between molecular SO_2, bisulphite and sulphite forms. This equilibrium is dependent on pH, with the bisulphite anion being the dominant form under wine conditions (pH between 3 and 4). Only molecular SO_2 appears to exert an antimicrobial action and its concentration in wine depends of many factors such as pH, ethanol, temperature, anthocyanin levels and nutrient contents (Fugelsang and Edwards, 2007). Different studies have been carried out for a better understanding of the effect of environmental factors on *Dekkera* growth, evaluating each variable in an independent way (Dias et al., 2003; Oelofse et al., 2008). However, the issues relating

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to the complexity of microbiological spoilage in wine are simply not resolved by individual factors, but rather require a holistic approach (Oelofse et al., 2008).

Probabilistic models have been widely used in predictive microbiology to obtain the growth/no growth interfaces of spoilage and pathogen microorganisms as a function of environmental hurdles (Arroyo-López et al., 2012; Presser et al., 1998; Ratkowsky and Ross, 1995; Valero et al., 2010). The position of the growth/no growth boundaries of non desirable microorganisms are of interest in establishing conditions for product stabilization and also to ensure food safety. The interfaces generated by means of the logistic models have to be chosen for a determined level of probability (p). Setting low levels of p is necessary in order to increase food safety and product stabilization. To our knowledge, no probabilistic model has been developed for the spoilage wine yeast *D. bruxellensis*. The only models available in the literature for this microorganism have been carried out by Garcia Alvarado et al. (2007) to determine the influence of acetic acid and aerobic/anaerobic conditions on *Dekkera* growth, and by Aguilar-Uscanga et al. (2011) to assess the influence of glucose concentration on ethanol production by this spoilage wine yeast.

The main objective of this study was to obtain the growth/no growth boundaries of the spoilage wine yeast *D. bruxellensis* as a function of pH, free SO_2 and ethanol contents, which was accomplished by means of a probabilistic model using time as a dummy variable. The results obtained in this work could be very useful for the industry in order to determine the conditions which inhibit the growth of this species in wines.

2. Material and methods

2.1. Yeast strain and inoculum preparation

The yeast strain used in the present study (CH29) was originally isolated from a highly contaminated commercial red wine. It was molecularly identified as *D. bruxellensis* through the sequencing of the D1/D2 domain of the 26S ribosomal gene and registered with this reference in the Oenological Research Centre Microorganism Collection from INTA, Argentina (GenBank accession number KF002710). This strain was selected among other *D. bruxellensis* strains because of its rapid growth in wine conditions and the presence of both spoilage coumarate descarboxylase and vinylphenol reductase enzymatic activities (data not shown).

Before inoculation, the strain was previously grown during 2 days at 28 °C on 20 ml of YPD (yeast–peptone–dextrose) broth media (glucose 40 g/l; yeast extract 5 g/l; peptone 5 g/l) supplemented with 6% ethanol and adjusted to pH 6.0 with HCl (1 M). In order to obtain a better adaptation to the test conditions, the strain was transferred to 40 ml of YPD broth media (glucose 20 g/l; yeast extract 5 g/l; peptone 5 g/l) adjusted to pH 4.0 with HCl (1 M) and supplemented with 50% of a commercial red wine. This last media was incubated for 3 days at 28 °C without shaking. During this time, growth was followed by measuring the turbidimetry at 600 nm until reaching the highest population (9×10^7 cfu/ml) just at the end of the exponential growth phase. The correlation between OD values and viable cell counts was obtained from a previous work (Sturm et al., 2010).

2.2. Growth media and experimental design

The basal media selected to perform the experiments were a complex medium of fermentation proposed by Aguilar-Uscanga et al. (2000), which was modified to mimic Argentine red wine conditions (glucose 0.6 g/l; fructose 1.2 g/l; trehalose 0.3 g/l; yeast extract 2 g/l; $(NH_4)_2SO_4$ 1 g/l; $MgSO_4{*}7\ H_2O$ 0.4 g/l; KH_2PO_3 2 g/l; biotin 0.45 mg/l; thiamine 25 mg/l; p-coumaric acid 5.28 mg/l; ferulic acid 0.66 mg/l). This simile wine medium was then modified with the variables described below.

A full-factorial experimental design, resulting from the combination of 3 levels of ethanol, 3 levels of pH and 7 levels of free SO_2, was used in the present study (Table 1). To reproduce the wine composition, tartaric acid (85%, w/v) was used for pH adjustment and $K_2S_2O_5$ salt (97% purity) to release free SO_2 in the medium. Both compounds are allowed for wine addition by the International Organization for Vine and Wine (OIV, 1998; Ribéreau-Gayon et al., 2006). Ethanol, pH and free SO_2 were determined after the addition according to the international methods for wine and must analysis (OIV, 2009). The variable levels were adjusted to a range of conditions usually found in Argentine red wines, making a total of 63 different levels, which were run in triplicate. Thus, a set of 189 data were obtained for each specific level of the tested dummy variable (at each time period).

2.3. Optical density measurements

Growth was recorded in a Bioscreen C automated spectrophotometer (Labsystem, Helsinki, Finland) at 28 °C for 30 days with a wideband filter (420–580 nm). Measurements were taken every 4 h after a preshaking of 5 s to avoid cell sedimentation. The wells of the microplate were filled with 0.01 ml of inoculum and 0.35 ml of the simile wine medium (modified according to the experimental design), always reaching an initial optical density (OD) of approximately 0.2 units (initial inoculum level of 2×10^6 cfu/ml). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve (data not shown). Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. For each well, growth (coded as 1) was assumed when an OD increase of 0.1 was observed with respect to the initial OD after subtraction of the noise signal. On the contrary, no growth (coded as 0) was recorded when the initial OD did not increase at the different levels of the dummy variable (7, 14, 21 and 30 days). Thus, intermediate values were not obtained. Responses for each replicate were recorded independently, and the whole matrix was subjected to statistical analysis. When the experiments were finished, randomly selected wells (which included both growth and no growth samples representing a 5% of total cases) were spread on YM agar plates and their counts were estimated to corroborate growth/no growth assumption.

2.4. Logistic model and statistical analysis

Logistic regression describes the log odds of the event, which is the natural log of the probability of the event occurring (p) divided by the probability of the event not occurring ($1 - p$). The logit transformation of $p(x)$ is usually defined as:

$$\mathrm{Logit}(p) = \ln[p(x)/(1-p(x))] = \beta_0 + \sum \beta_i x_i. \quad (1)$$

In the present study, the general model described above took the specific form:

$$\mathrm{Logit}(p) = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3}\sum_{j>i}^{3} \beta_{ij} x_i x_j + \sum_{l=1}^{4-1} \beta_{tl} D_{tl} + \varepsilon \quad (2)$$

Table 1
Quantitative and dummy variables tested in the present study with their respective levels.

Variable	Type	Levels
Ethanol	Quantitative	(%, v/v): 10.0; 12.5; 15.0
pH[a]	Quantitative	3.4; 3.7; 4.0
Free SO_2	Quantitative	(mg/l): 0; 8; 16; 25; 34; 42; 50
Time	Dummy	Coded: 1 (7 days); 2 (14 days); 3 (21 days); 4 (30 days)

[a] Tartaric acid was used for pH adjustment. 1.53 mM, 2.04 mM and 3.57 mM were the final concentrations of tartaric acid in the media to reach pH values of 3.4, 3.7 and 4.0, respectively. The undissociated forms of this acid were 93.4% (pH 3.4), 96.7% (pH 3.7) and 98.3% (pH 4.0).

where β_0, β_i, β_{ij} and β_t stand for the coefficients to be estimated by the logistic regression, x_i are the quantitative environmental variables under study (ethanol, pH and free SO_2, in physical values), dummy (D) is the categorical variable (time, t) and ε is a term for error. Dummy variable had 4 levels (k) corresponding to 7, 14, 21 and 30 days, which were coded as t = 1 (1, 0, 0), t = 2 (0, 1, 0), t = 3 (0, 0, 1) and t = 4 (0, 0, 0), respectively, with the highest value taken as the reference level. The initial model also included quadratic terms, the second and third order interaction of quantitative variables as well as the interactions of the dummy variable with the rest of the terms, but they were not retained in the final equation.

The predicted survival probability (p), at each variable combination, may be estimated as:

$$p = \exp(\text{logit}(p))/(1 + \exp(\text{logit}(p))). \quad (3)$$

In Eq. (3), the growth/no growth boundaries for a selected probability (p) can be obtained as a function of pH, free SO_2, and ethanol concentration at each level of the dummy variable. This task was achieved by plotting the resulting equation as a function of one, two or three variables while maintaining the rest of them fixed at predetermined levels.

The logistic regression model described above was fitted to the growth/no growth data obtained in this work. The model fit was performed using SYSTAT 12 software package (Systat Software Inc., Washington, USA). The automatic variable stepwise selection (maximum number of allowable runs set to 50) with backward option was used to choose the significant coefficients ($p \leq 0.05$). The retained coefficients were selected with the option likelihood of the ratio tests (chi-squared).

The log–likelihood ratio statistic was used to assess the importance of each of the explanatory variables on the response (yeast growth/no growth data). This statistic (which follows a chi-square distribution) indicates whether the coefficients of the model are significantly different from zero, taking into account the number of exploratory variables (degree of freedom) retained in the model (Hosmer and Lemeshow, 2000). The goodness of the fit was also evaluated using other criteria such as: i) the MacFadden's rho-squared (a transformation of the likelihood statistic, $\rho^2 = 1 - (LL(b) / LL(0))$), with values between 0.2 and 0.4 considered highly satisfactory; ii) the Nagelkerke's R^2 statistics (a modification of the Cox and Snell R^2, $R^2_{cs} = 1 - \exp[-2 / n[LL(b) - LL(0)]]$) adjusted to range from 0 to 1 (by dividing it by the maximum value ($R^2_N = R^2_{cs} / R^2_{max}$ with $R^2_{max} = 1 - \exp[2(n^{-1})LL(0)]$)), with values closer to one indicating better fits of the model. The meaning of these indexes are not similar to R^2 but can be interpreted as an approximate variance in the outcome accounted for the model (Gordon, 2012). Overall hit rate (number of global correct prediction divided by sample size), sensitivity (percent of correct predictions in the reference category) and sensibility (percent of correct prediction in the given category) were also estimated.

The coefficients of the logit model represent the changes in the log of the odds (natural log of the probability that an event would happen (p), growth in this case, divided by the probability, $1 - p$, that it would not happen, no growth) due to one unit change while in categorical variables they mean the changes due to the shift from one level with respect to the reference level. The interpretation is similar in the other cases. A more convenient way of interpreting these coefficients is through the odds ratio = exp(b), derived from the multiplicative form of logit (p). Then, exp(b) means the change in the odds due to one unit change in the variable under study when no change in the others is introduced. In this case, a value of 1 ($e^0 = 1$) indicates that the variable under study does not cause any effect on the odds. Values below 1 indicate a decrease while values higher than 1 mean an increase.

The predicted 3D or 2D growth/no growth interfaces were produced with STATISTICA 7.0 software package (StatSoft Inc., Tulsa, USA). The limits were estimated for a growth probability p value of 0.01, considered appropriate for the product assayed (wine).

3. Results

The total number of cases analyzed in the present work was 756 (189 at each level of the dummy variable), with a total distribution between growth/no growth data of 321/435, respectively. For all randomly selected wells (38), the growth/no growth assumption was satisfactorily confirmed on YM agar medium. This task was carried out by comparing counts at the different sampling times with respect to the initial inoculum level. No false positives were obtained. The global dummy model was chosen instead of several models for each specific time value because of the similar inhibition patterns observed in the growth/no growth interfaces of the different time values, which only showed a parallel displacement (data not shown). In this circumstance, a model with time as the dummy variable turned out to be simpler than several models for each value of time. The units used for the model development were intentionally adapted to the terminology used by the wine producers.

3.1. Global logistic model

The global probabilistic model fitted the data satisfactorily as suggested by the following statistical tests: McFadden's rho-squared, 0.629; Nagelkerke's R^2 0.774; likelihood ratio test $2 * (LL(N) - LL(0) = 648.6$, $p < 0.0001$ (8 df); Hosmer and Lemeshow test chi-squared = 10.21, $p = 0.251$ (8 df). In addition, the overall percentage of hits was 88.4%, with a sensitivity of 86.3% and a specificity of 89.9%. The false positive and negative proportions obtained from the equation were 15.2% and 11.2%, respectively.

The significant coefficients ($p \leq 0.05$) retained in the model, after the backward procedure selection, are shown in Table 2. Thereby, the final obtained equation was:

$$\text{Logit}(p) = \ln(p/(1-p)) = 67.18 - 12.76 * [\text{pH}] - 5.112 * [\text{Et}] - 1.484 * [\text{free } SO_2] + 1.047 * [\text{Et} * \text{pH}] + 0.348 * [\text{free } SO_2 * \text{pH}] + \text{dummy}. \quad (4)$$

With values of the dummy variable of −3.098 for the first level (7 days), −1.050 for the second level (14 days) and −0.532 for the third level (21 days), while the fourth level (30 days) was taken as the reference value. The dummy 1 value (−3.098) means a decrease in logit (p) from the time of reference (30 days) when moving from this to the first sampling period (7 days). Similar interpretations can be given for the rest of the dummy values. On the other hand, the odds ratio for dummy 1, dummy 2, and dummy 3 levels (Table 2) indicate that the odds, which represent the probability of an event ocurring/probability of an event not ocurring, decrease by a factor (exp(dummy)) of 0.045, 0.350 or 0.588 when the growth is observed at 7, 14 or 21 days with respect to the reference level. In other words, this means that the odds of growth decreased as time was shorter, although there were no significant differences between the fourth and the third levels of

Table 2
Coefficients of the global logistic model (standard errors in brackets) retained after the backward procedure selection ($p \leq 0.05$) with their respective odds ratios and their confidence limits (CL) for $p = 0.95$.

Parameter	Estimated coefficient	Odds ratio	Lower CL	Upper CL
Constant	67.18 (20.30)	–	–	–
pH	−12.76 (5.26)	0.000 (0.00)	0.000	0.087
Ethanol	−5.112 (1.426)	0.006 (0.009)	0.000	0.099
Free SO_2	−1.484 (0.227)	0.227 (0.051)	0.145	0.354
Dummy 1	−3.098 (0.427)	0.045 (0.019)	0.020	0.104
Dummy 2	−1.050 (0.370)	0.350 (0.130)	0.169	0.723
Dummy 3	−0.532 (0.367)	0.588 (0.215)	0.286	1.206
Ethanol * pH	1.047 (0.369)	2.850 (1.053)	1.382	5.879
Free SO_2 * pH	0.348 (0.058)	1.416 (0.082)	1.264	1.586

Note: Dummy 4 (30 days) was used as reference value.

the dummy variable (30 and 21 days) (data not shown). The interpretation of the other coefficients is not so straightforward because their two-way interactions were also significant. Then, the effects of the variables and their interactions would need to be explored graphically.

3.2. 3D growth/no growth surfaces

The growth/no growth surfaces as a function of the three quantitative variables can be obtained at each sampling time by fixing a probability level in Eq. (4) and plotting the resulting expression on 3D axes. This presentation is convenient for simultaneously studying the effect of the variables and their interactions. Fig. 1 shows the graphs for the diverse levels of the dummy variable (time) for a selected growth probability of 0.01. As it can been deduced, the surfaces always had an overall good fit at this probability value, and the circles (conditions where yeast grew) were always below the surface, clearly delimiting the inhibition region (above the surface). There were significant differences among the results obtained at t = 1 (7 days) from those obtained at any other time period. The results obtained at t = 2 (14 days) were also significantly different from those obtained at dummy 3 (21 days) and dummy 4 (30 days), but the results at t = 3 and t = 4 were similar (Table 2). This is indicative of the fact that the growth response of *D. bruxellensis* was different and the inhibitory interface moved with time, showing a considerably higher number of growth circles at t = 3 and t = 4 than at t = 1 and t = 2. The 3D surfaces obtained at the different sampling times (Fig. 1) can be used to determine different combinations of the quantitative variables which could adequately preserve ($p = 0.01$) the wine from 7 to 30 days. However, it is difficult to calculate these values directly from Fig. 1, as well as to determine the importance of each individual variable, thus another graphical representation could be more explanatory.

3.3. Individual inhibitory profiles

The profiles (changes in the growing probabilities) as a function of each inhibitor factor can be easily derived from Eq. (4), using the following general equation:

$$p = \frac{1}{1 + e^{-(61.18 - 12.76 \cdot pH - 5.112 \cdot Et - 1.484 \cdot SO_2 + 1.047 \cdot Et \cdot pH + 0.348 \cdot SO_2 \cdot pH + dummy)}}. \quad (5)$$

From this equation, the probability of growth for each specific variable can be determined, fixing the levels for the other two variables. These graphs are of interest for studying the inhibitory profiles of each quantitative variable as a function of its concentration.

The changes in growth probability as a function of pH for t = 1 and t = 4 at the different ethanol concentrations tested is shown in Fig. 2. Concentrations of 50 mg/l of free SO_2 were always inhibitory, regardless of the pH level assayed. For the other concentrations of free SO_2 evaluated, the growth probability increased as the pH increased, with the probability profile being strongly affected by the presence of ethanol. Therefore, in the absence of free SO_2, there was no practical inhibition when the ethanol content was 10.0 or 12.5%, but the probability considerably decreased when the ethanol level increased up to 15%. At this ethanol concentration, the profiles at time period t = 1 and t = 4 were markedly different; while at 10.0 and 12.5% they were quite similar (Fig. 2). The addition of free SO_2 markedly decreased the growth probability, regardless of the pH or the level of the dummy variable. It was also observed that, except for the highest free SO_2 level, for the same levels of the quantitative variables, the probability of growth also increased with time (growth probability for t = 4 was higher than for t = 1).

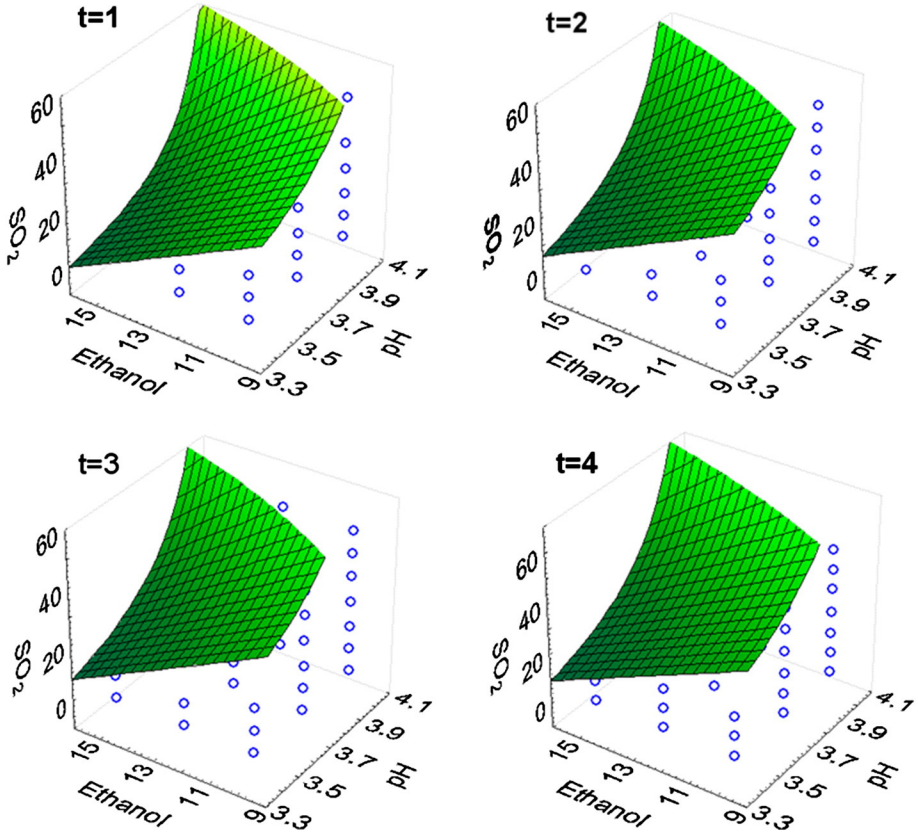

Fig. 1. 3-D growth/no growth interfaces for a growth probability level of 0.01 as a function of pH, ethanol (%, v/v) and free SO_2 (mg/l) for the four levels of time assayed (t = 1, 7 days; t = 2, 14 days, t = 3, 21 days; t = 4, 30 days). Opened circles show conditions where the yeast was able to grow.

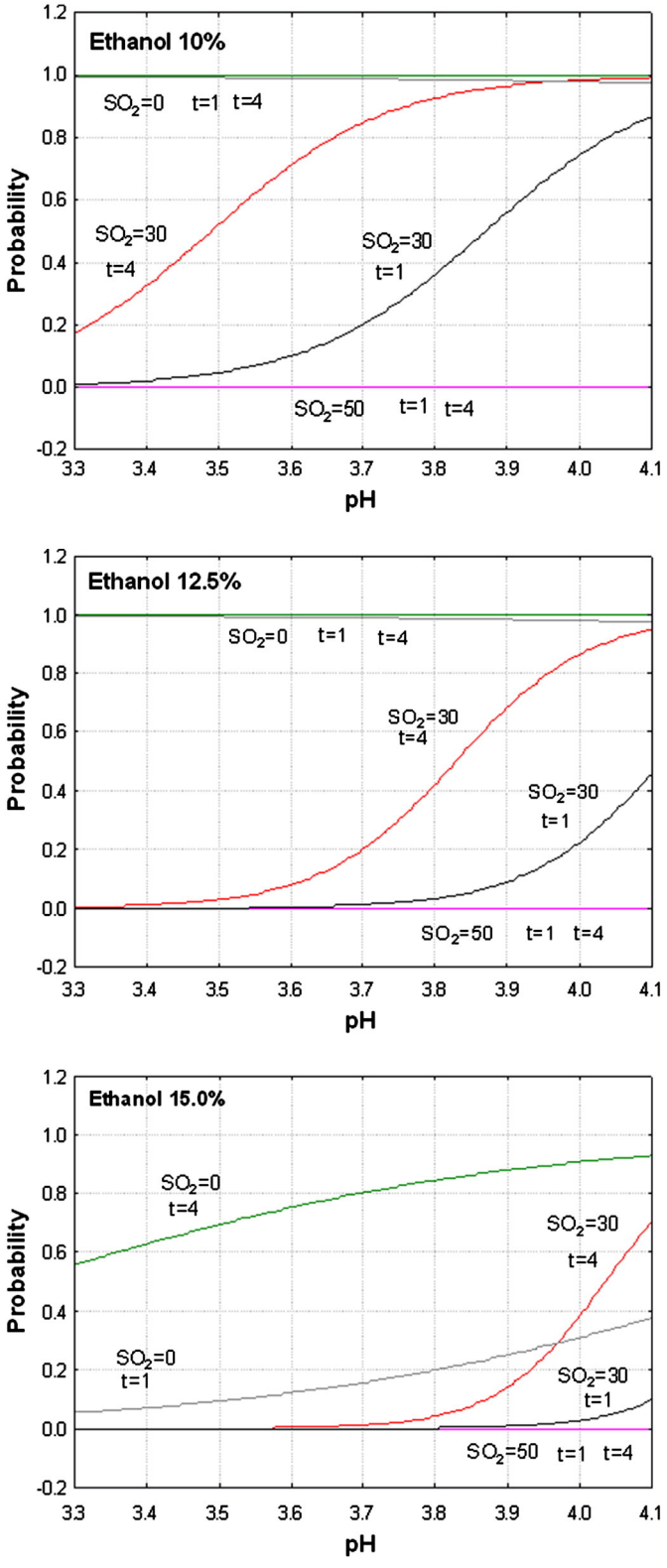

Fig. 2. Growth probability profiles as a function of pH for t = 1 (7 days) and t = 4 (30 days), fixing the levels of ethanol at 10.0, 12.5 and 15.0% (v/v), and the levels of free SO_2 at 0, 30 and 50 mg/l.

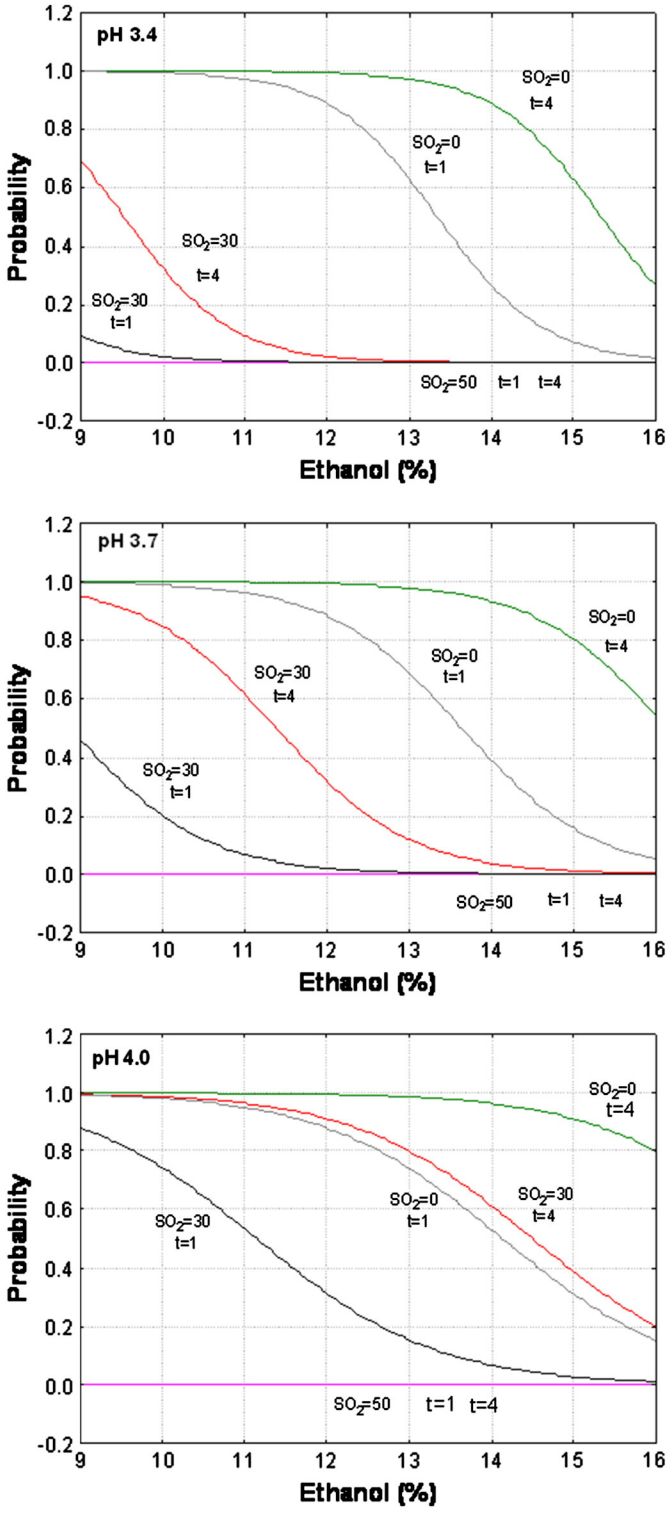

Fig. 3. Growth probability profiles as a function of ethanol concentration (%, v/v) for t = 1 (7 days) and t = 4 (30 days), fixing the levels of pH at 3.4, 3.7 and 4.0 units, and the levels of free SO_2 at 0, 30 and 50 mg/l.

The probability profile as a function of the ethanol concentration is shown in Fig. 3 for times t = 1 and t = 4 and diverse levels of pH. At 50 mg/l of free SO_2, there was no growth, regardless of the levels of the other variables studied. With respect to the concentration of ethanol, the growth probability decreased as the presence of this compound increased. Once again, when the time period increased (from t = 1 to t = 4), the growth probability also increased.

Fig. 4 shows the growth probability as a function of the free SO_2 concentration, for t = 1 and t = 4 and diverse levels of pH and ethanol. As can be observed, the growth probability considerably decreased when the free SO_2 concentration increased, with a more marked effect at the lowest pH level. At pH 3.4 and above 50 mg/l of free SO_2, there was

no growth regardless of the ethanol concentration or the time period. However, as the pH increased, the inhibitory effect of free SO_2 decreased. Therefore, at pH = 3.7, the required concentration of free SO_2 to achieve a growth probability of 0.01 was slightly outside the experimental region used in this study (50 mg/l) at 10% of ethanol, while at the same concentration of ethanol and at pH 4.0 there was a growth probability ranging from 0.3 to 0.9 for 50 mg/l of free SO_2.

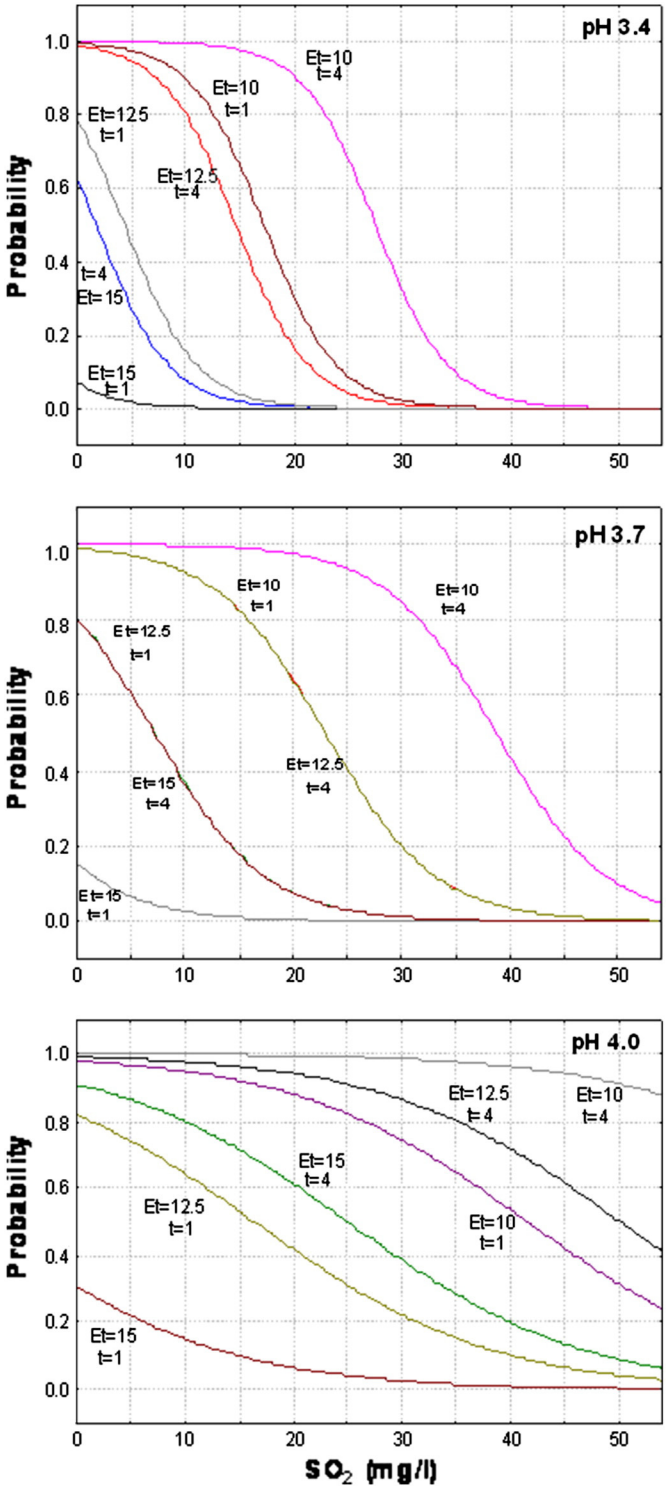

Fig. 4. Growth probability profiles as a function of free SO_2 concentration (mg/l) for t = 1 (7 days) and t = 4 (30 days), fixing the levels of pH at 3.4, 3.7 and 4.0 units, and the levels of ethanol at 10, 12.5 and 15% (v/v).

3.4. Growth/no growth interfaces as a function of two variables

Fig. 5 shows the 2D growth/no growth interfaces of *D. bruxellensis* for a growth probability of 0.01 as a function of pH and free SO_2 for diverse levels of ethanol. The advantage of using these interfaces is that ethanol is not a variable that could be easily manipulated in wines because its levels are given by the fermentative process. Thus, the required concentration of free SO_2 or modifications in pH through the addition of tartaric acid by the industry to achieve yeast inhibition could be easily deduced from such graphs. In any case, other growth/no growth interfaces are also possible to calculate from the general equation for any given growth probability.

It can be concluded from Fig. 5 that when the concentration of ethanol increased, the interfaces were displaced to the left, indicating a clear inhibitory effect of this compound on yeast growth. The generated 2D interfaces showed that the inhibition for a short period of time (t = 1) was achieved with lower pH and higher free SO_2 concentrations for the same ethanol level than when time increased (interfaces moved towards the right). Thus, the required concentration of free SO_2 at 10% of ethanol to preserve the wine for a period of time of 7 days at pH 3.4 was approximately 33 mg/l, but increased up to 43 mg/l to achieve inhibition for 30 days. However, when the proportion of ethanol increased up to 15%, the preservation of wine for 7 days was achieved with only 7 mg/l of free SO_2, and with 17 mg/l for 30 days. In the same way, at 12.5% of ethanol and pH 3.7 for a growth probability of 0.01, the addition of 30 mg/l of free SO_2 to inhibit yeast growth for 7 days was required. However, the concentration of free SO_2 should be raised up to 48 mg/l to obtain a no growth probability of 0.99 for 30 days under the same wine conditions. Other combinations of the environmental variables can be determined by the mathematical model depending on the needs of the industry. As deduced from Fig. 5, preservation for longer periods of time needs higher concentrations of free SO_2 and ethanol and lower pH levels. According to these interfaces, as pH values increased, the inhibitory effect of the other variables decreased. At pH = 3.8–3.9, inhibition for a period of time of 30 days can only be achieved with concentrations of ethanol and free SO_2 at the maximum levels used in this study.

4. Discussion

In this work, the combined effects of the main factors that limit *D. bruxellensis* growth have been evaluated using a logistic/probabilistic model with time as a categorical/dummy variable. The variable levels were adjusted to a range of conditions usually found in Argentine red wines, and they are of easy management for winemakers. Because of the number of replicates used by condition (only 3), this model gives a first indication of the growth probability of this spoilage yeast as a function of pH, ethanol and free SO_2. However, for more accurate determination, a model built with more replicates would be suitable.

The results show that all environmental variables assayed had a considerable inhibitory effect on yeast growth, as well as the interactions among ethanol * pH and free SO_2 * pH. SO_2 proved to be a considerable inhibitor of the yeast growth in wines (Stratford, 2006). Regarding SO_2 and its effect on *D. bruxellensis* yeast, studies have yielded different results, and this microorganism is regarded as either sensitive or resistant. Resistance of yeasts to sulphites is not always found in all strains of an individual species. Some authors showed that a low concentration of molecular SO_2 (0.1 mg/l) did not affect the viability or culturability of the *D. bruxellensis* cells, but at higher molecular SO_2 concentrations (0.8 mg/l) no cells grew on the agar plates (Du Toit et al., 2005). Similarly, Agnolucci et al. (2010) reported that very low concentrations of free molecular SO_2 (≤1 mg/l) induced a viable but not culturable state in *D. bruxellensis* acting as a powerful chemical stressor, although the production of the spoilage metabolism (volatile phenols) was not arrested. The viable but not culturable state in the *D. bruxellensis* yeast was also observed by Du Toit et al. (2005). On the contrary, Barata et al. (2008),

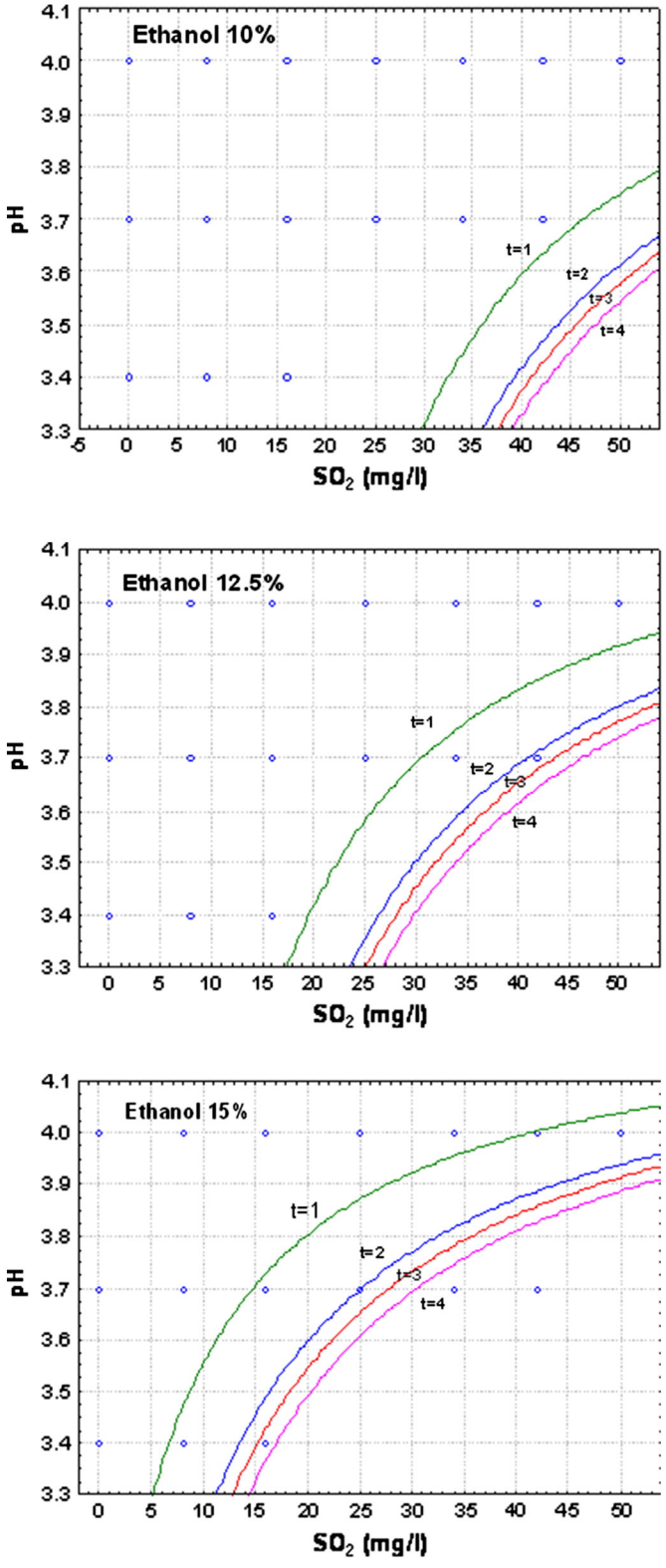

Fig. 5. 2D growth/no growth interfaces as a function of pH and free SO_2 for diverse levels of ethanol (10, 12.5 and 15%, v/v) and dummy variable (t = 1, 7 days; t = 2, 14 days; t = 3, 21 days; t = 4, 30 days). Probability of growth fixed at $p = 0.01$. Opened circles show conditions where the yeast was able to grow.

analysing a wide range of strains, observed the existence of an active but non-culturable population after the addition of SO_2 to wine. In line with this, the effect of SO_2 on the enzymatic activities has been proven. Benito et al. (2009), found that in the absence of other limiting factors the spoilage enzymatic activities of several strains of *D. bruxellensis* stop with a free SO_2 concentration of 20 mg/l at pH 3.5. Finally, the use of 0.5 to 0.8 mg/l molecular SO_2 has been recommended to control *Dekkera* spp. (Oelofse et al, 2008). However, achieving this level could be difficult in wines with a high pH given that the maximum allowable limits for the addition of SO_2 by the OIV is 150 to 300 mg/l of total SO_2 (OIV, 1998).

Ethanol has been largely used as a preservative in foods. The degree of protection from spoilage conferred by ethanol depends on the concentration used and the species of yeast involved, but overall spoilage is minimized by ethanol in an excess of 15% (v/v) and it is eliminated at concentrations greater than 22% (Stratford, 2006). Yeasts do not have the same sensitivity or resistance to ethanol. The most resistant yeast species to ethanol are not surprisingly those fermentative species that produce ethanol at their highest concentrations. These include *Dekkera anomala* and *D. bruxellensis*, *Saccharomyces cerevisiae* and *Saccharomyces bayanus*, *Saccharomycodes ludwigii* and *Zygosaccharomyces bailii* (Arroyo-López et al., 2010; Stratford, 2006). Some strains of *D. bruxellensis* have even demonstrated to have higher tolerance to ethanol when compared with *S. cerevisiae* (Phowchinda et al. 1997). Dias et al. (2003) reported that *D. bruxellensis* growth and 4-ethylphenol production were inhibited by increasing the concentration of ethanol, and fully prevented at 13% (v/v) in synthetic grape must media. In this work, the strain *D. bruxellensis* CH29 was able to grow at 15% of ethanol in the absence of free SO_2 at the highest pH level assayed (4.0). However, when the other factors became more restrictive, ethanol tolerance decreased. Thereby, at pH 3.4 and 30 mg/l of free SO_2, the growth probability of *D. bruxellensis* was drastically reduced at the lowest ethanol concentration assayed (10% v/v). This fact indicates a possible synergistic effect between both factors to limit yeast growth.

Acidic beverages are particularly associated with yeast spoilage. Low pH alone does not contribute directly to protecting foods from yeasts or moulds (Stratford, 2006). Furthermore, examination of the growth of yeasts shows that only when the pH falls below 3.0 are yeasts low-pH-stressed, as indicated by a slower growth. However, low pH has a dramatic effect on food preservation in the presence of other preservatives, such as SO_2. As mentioned before, the antimicrobial action of SO_2 was strongly influenced by pH. It was shown that the *Dekkera* species is able to grow under acidic conditions, in a pH ranging from 3.0 to 4.5, however growth was weaker at pH 2.5 in grape juice (Gilis, 1999).

The results of this work highlight time as a factor to be considered for setting the values of growth limiting conditions. It was shown that the growth response of *D. bruxellensis* CH29 was different and the inhibitory interfaces moved with time. More restrictive initial conditions were necessary to inhibit *Dekkera* growth at 30 days than at 7 days. This could be related to both the adaptation of the *D. bruxellensis* strains to stressful conditions and to the decreased levels of ethanol and free SO_2 with time. Thus, these factors should be periodically monitored, especially in wine aged in wooden barrels to ensure that protection against *Dekkera* is maintained (Benito et al., 2009). Moreover, the use in this work of microtitre plates could introduce a major bias when comparing with growth in real wine conditions because the small volume of each well does not prevent oxygen dissolution, which stimulates growth and decreases the free SO_2 concentrations in a short period of time. This fact, together with ethanol evaporation, could explain why the SO_2 concentrations required to inhibit yeast growth are higher than expected and growth probability increased as time progressed.

5. Conclusions

In summary, the logistic regression model obtained in this work shows that all the quantitative (ethanol, pH, free SO_2) and dummy (time period) variables studied had a considerable effect on *D. bruxellensis* growth, providing a good fit of experimental data. Time was especially relevant, requiring more drastic conditions to ensure wine stability for a

longer period of time. Thereby, the model gives us a first indication of the growth probability of this spoilage yeast under wine conditions and could be a valuable tool for the wineries because it allows, as a function of the needs of the industry, estimating diverse combinations of the environmental variables which inhibit the growth of this species from 7 to 30 days.

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