Susceptibility and resistance to ethanol in *Saccharomyces* strains isolated from wild and fermentative environments

F.N. Arroyo-López ¹, Z. Salvadó ^{2,3}, J. Tronchoni ³, J.M. Guillamón ³, E. Barrio ¹ and
A. Querol ^{3,*}

¹ Institut “Cavanilles” de Biodiversitat i Biologia Evolutiva. Universitat de València.
Edifici d’Instituts, Parc Científic de Paterna. P.O. Box 22085, E-46071 València, Spain.

² Departament de Bioquímica I Biotecnologia. Facultat de Enologia, Universitat Rovira
i Virgili. Tarragona, Spain.

³ Departamento de Biotecnología de Alimentos. Instituto de Agroquímica y Tecnología
de los Alimentos (IATA, CSIC). P.O. Box 73. E-46100 Burjassot, Valencia, Spain.

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***Corresponding author:**

A. Querol, PhD.

Telf. +34 963900022 x 2306

Fax.: +34 963636301

e-mail address: aquerol@iata.csic.es

Abstract

In this work, we apply statistical modeling techniques to study the influence of increasing concentrations of ethanol on the overall growth of 29 yeast strains belonging to different *Saccharomyces* and non-*Saccharomyces* species. A modified Gompertz equation for decay was used to objectively estimate the Non-Inhibitory Concentration (NIC) and Minimum Inhibitory Concentration (MIC) for the assayed strains to ethanol, which is related to the susceptibility and resistance of yeasts to this compound, respectively. A first ANOVA analysis, grouping strains as a function of their respective *Saccharomyces* species, revealed that *S. cerevisiae* was the yeasts with the highest, and statistically significant, ethanol resistance values. Then, a second factorial ANOVA analysis, using the origin of strains (wild or fermentative) and their taxonomic classification (*S. cerevisiae*, *S. paradoxus* or *S. bayanus* var. *uvarum*) as categorical predictor variables, showed that no significant differences for the NIC and MIC parameters were found between both ecological niches within the same species, indicative that these physiological characteristics were not presumably modified throughout the adaptation to human-manipulated fermentative environments. Finally, differences among selected strains with respect to ethanol tolerance were correlated to the initial contents of unsaturated fatty acids, mainly oleic acid.

Keywords: Adaptation; ethanol tolerance; statistical modeling; *Saccharomyces*.

Introduction

Ethanol is well known to inhibit yeast growth and viability, affecting various transport systems such as the general amino acid permease and glucose uptake processes. It can also inhibit the activity of crucial glycolytic enzymes and damage mitochondrial DNA (Casey and Ingledew, 1986; Ibeas and Jiménez, 1997; Ingram and Buttke, 1984; Salmon *et al.*, 1993). The main target of ethanol is the fluidity of the plasmatic membrane, greatly affecting its functions and physical-chemical properties (Alexandre *et al.*, 1994a). Because of the pleiotropic effects produced by this compound, a large number of genes are involved in the response of yeasts to ethanol stress (Alexandre *et al.*, 2001; Fujita *et al.*, 2006; Teixeira *et al.*, 2009). These microorganisms have developed diverse strategies to counteract the different types of damage produced by this alcohol (Ding *et al.*, 2009; Stanley *et al.*, 2010). For instance, there is a well-documented correlation between ethanol resistance and the degree of fatty acid (FA) unsaturation of membrane lipids in *Saccharomyces cerevisiae* (Alexandre *et al.*, 1994b; Ding *et al.*, 2009; Thomas *et al.*, 1978). Genes involved in intracellular pH homeostasis are also crucial for resistance to ethanol and other alcohols (Alexandre *et al.*, 2001; Fujita *et al.*, 2006).

The *Saccharomyces* genus contains species associated only with natural habitats (*S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus* and *S. arboriculus*), and others associated with both human-manipulated fermentative and wild environments (*S. cerevisiae*, *S. bayanus* and, in to some extent, *S. paradoxus*). The ability of this genus, and mainly *S. cerevisiae*, to degrade carbohydrates, usually six-carbon molecules such as glucose or fructose, to two-carbon compounds, in particular ethanol, has been unconsciously used by humans for thousands of years to ferment a broad type of beverages (cider, beer, wines, etc.) (Querol and Fleet, 2006). New selective pressures

were introduced on these species, resulting in highly specialized microorganisms which have evolved to optimally utilize these ecological niches. This process can be described as ‘domestication’ and is responsible for the peculiar genetic characteristics found in industrial yeasts (Barrio *et al.*, 2006; Fay and Benavides, 2005). In this survey, we try to determine if ethanol tolerance is a ‘domesticated’ physiological property, which has been modified in industrial *Saccharomyces* yeasts through many years of unconscious human selection, or conversely, it is an intrinsic characteristic of certain *Saccharomyces* species that appeared before the domestication process allowing them to colonize and predominate in human-manipulated fermentative environments.

To answer this question, it is necessary to have a quantitative methodology that allows an objective and reliable comparison among yeasts. Lambert and Pearson (2000) developed a simple and valuable method for the estimation of the Minimum Inhibitory Concentration (MIC) and Non-Inhibitory concentration (NIC) of a compound using Optical Density (OD) measurements. MIC is related to the resistance or tolerance of the microorganism to the compound, and is the lowest concentration which results in maintenance or reduction of an inoculum’s viability (marks the concentration above which no growth is observed). On the contrary, NIC is related with the susceptibility of the microorganism to the compound, and it is the concentration above which the inhibitor begins to have a progressive and negative effect on growth (Bautista-Gallego *et al.*, 2008; Lambert and Pearson, 2000).

In the present work, we use statistical modeling techniques to perform a comparative study of the susceptibility and resistance to ethanol, as well as of the lipid cell composition, of a considerable number of *Saccharomyces* strains isolated from diverse ecological niches. Our final goal is to understand the role played by this

compound in the adaptation of some species of this genus to human-manipulated fermentative environments.

Materials and Methods

Yeast strains and inocula preparation

A total of 29 yeast strains, belonging to different *Saccharomyces* and non-*Saccharomyces* species, were used in the present study. Yeasts were selected to obtain representative isolates from natural (15 strains) and fermentative (14 strains) habitats, where possible. Their origins and references are listed in Table 1.

Inocula were prepared by introducing one single colony from pure cultures of each strain into 5 ml of Yeast-Malt-Peptone-Glucose broth medium (YM, Difco™, Becton and Dickinson Company, Sparks, USA). After 48 h of incubation at 27°C for *S. bayanus* var. *uvarum*, *S. arboriculus* and *S. kudriavzevii* strains, which are considered more cryophilic (Querol and Fleet, 2006), or 30°C for the rest of mesophilic yeast species, 1 ml of each tube was centrifuged at 9,000 x g for 10 min, the pellets washed with sterile saline solution (9 g/l), centrifuged and re-suspended in 0.5 ml of sterile saline solution to obtain a concentration of about 7.3 log₁₀ CFU/ml.

Growth medium

The basal growth medium selected for all experiments was Yeast Nitrogen Base (YNB, Difco™) supplemented with 20 g/l of glucose as carbon source (pH~5.5). Medium was sterilized by filtration (0.2 µm) and modified with sterile pure ethanol (99.8%) (Scharlau Chemie S.A., Spain) to obtain the following concentrations: 0, 7.8, 15.6, 23.4,

39.1, 54.7, 62.5, 78.2, 93.8, 117.3, 156.4 and 194.5 g/l. To convert these concentrations to percentages only is necessary to divide by 7.818, which is obtained according to the density of ethanol in the range of temperature 25-30°C. Therefore, the ethanol percentages assayed in this work were included in the interval 0 to 25% (v/v).

Optical density measurements

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 27°C (for *S. bayanus* var. *uvarum*, *S. kudriavzevii* and *S. arboriculus* strains) or 30°C (for the rest of isolates). Optimal growth temperatures for each species were chosen to reduce a possible inhibitory effect of this factor. Measurements were taken every hour over 3 days after a pre-shaking of 20 s. Therefore, all the experiments were carried out under aerobic conditions. The wells of the microplate were filled with 0.01 ml of inoculum and 0.25 ml of YNB medium (modified with ethanol), reaching an initial OD of approximately 0.2 (corresponding to a starting cell number of $\sim 10^6$ cells/ml). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate. This way, a total of 1044 growth curves (12 levels of ethanol x 29 strains x 3 replicates) were obtained and analysed.

Estimation of the NIC and MIC parameters

The basis of the technique used for estimating the NIC and MIC parameters was the comparison of the area under the OD/time curve of a positive control (absence of ethanol, optimal conditions) with the areas of the tests (presence of ethanol, increasing

inhibitory conditions). The use of the area under OD/time curve as a measure of overall yeast growth was recently proved by Arroyo-López *et al.* (2009) due to its relation to biological growth parameters. This parameter resulted to be inversely related to the lag phase, but linearly related to both the maximum population level and maximum specific growth rate of yeasts (Bautista-Gallego *et al.*, 2008; Arroyo-López *et al.*, 2009). As the amount of inhibitor in the well increases, the effect on the growth of the organism also increases. This effect on the growth is manifested by a reduction in the area under the OD/time curve relative to the positive control at any specified time. The areas under the OD/time curves were calculated by integration using OriginPro 7.5 software (OriginLab Corporation, Northampton, USA). The relative amount of growth for each ethanol concentration, denoted as the fractional area (fa), was obtained using the ratios of the test area ($area_{test}$) to that of the positive control of the yeast ($area_{cont}$), according to the following formula:

$$fa = (area_{test})/(area_{cont}) \quad (1)$$

The plot of the fa versus $\log_{10}$ ethanol concentration produced a sigmoid-shape curve that could be well-fitted with the modified Gompertz function for decay (Lambert and Pearson, 2000), which has the following expression:

$$fa = A + C \cdot \exp[-\exp(B(x-M))] \quad (2)$$

where, A is the lowest asymptote of fa (approximately zero), B is a slope parameter, C is the distance between the upper and lower asymptote (approximately 1) and M is the $\log_{10}$ ethanol concentration of the inflexion point. These parameters were obtained by a non-linear regression procedure, minimizing the sum of squares of the difference between the 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

7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to experimental data. The NIC and MIC parameters were later estimated as (Lambert and Pearson, 2000):

$$NIC = 10^{(M - \frac{1.718}{B})} \quad MIC = 10^{(M + \frac{1}{B})} \quad (3)$$

ANOVA analyses

To check for significant differences among yeast species for NIC and MIC parameters, an analysis of variance was performed by means of the one-way ANOVA module of Statistica 7.0 software. Strains were previously grouped as a function of their respective *Saccharomyces* species, including a total of 5, 1, 1, 1, 10, 4, and 4 isolates for *S. kudriavzevii*, *S. mikatae*, *S. arboriculus*, *S. cariocanus*, *S. cerevisiae*, *S. bayanus* var. *uvvarum* and *S. paradoxus*, respectively. For those yeast species including fermentative and wild isolates, a second factorial ANOVA analysis was carried out to determine if there were significant differences for the NIC and MIC values according to the origin of the strains. The analysis was done using ‘yeast species’ (3 levels: *S. cerevisiae*, *S. bayanus* var. *uvvarum* and *S. paradoxus*) and ‘yeast origin’ (2 levels: fermentative and wild) as categorical predictor variables. In this way, a total of 6 groups were obtained including 18 yeast strains and 56 cases (3 determinations by strain). In both ANOVA analyses, the Scheffé test was applied, which is considered to be one of the most conservative post-hoc comparison tests (Winer, 1962).

Determination of the fatty acid and sterol cell composition

Three strains with marked differences in ethanol tolerance (*Sc* T73, *Sk* CR85 and *Sb*

BM58) belonging to the species *S. cerevisiae*, *S. kudriavzevii* and *S. bayanus* var. *uvarum*, were selected to determine their FA and sterol profiles. Previously, yeasts were grown over 24 h at 28°C in YNB medium under aerobic conditions without ethanol. In this way, we obtained information on the cell composition in the absence of this compound. Experiments were carried out in triplicate.

FAs and sterol cell composition were determined by gas chromatography (GC) and one-dimensional thin-layer chromatography (TLC), respectively, according to the methodology described by Redón *et al.* (2009). Analytical GC was performed on a Hewlett-Packard 6850 apparatus (Agilent Technologies Inc, Santa Clara, USA). Relative amounts of given FAs were quantified from their respective chromatographic peak areas, and the total FAs was obtained as their sum. These values were related to the cell dry weight and later expressed as the percentage of the total FAs extracted. For sterols, the compounds lanosterol, ergosterol and squalene (Sigma-Aldrich, St. Louis, USA) were applied to every plate as internal standards. Then, cell lipids were charred with 10% CuSO₄ in 8% H₃PO₄ and heated at 180 °C for 4 min on a TLC Plate Heater (CAMAG). An image of the plate was acquired with Image Scanner (Amersham Biosciences, Sweden). Each spot of the image was quantified as integrated OD with Quantity One software (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK). Calibration curves were constructed by plotting the integrated OD of the sterol standard over the amount of sterol loaded. Finally, to check for significant differences among yeasts respect to their FA and lipid composition profiles, the one-way ANOVA module of Statistica 7.0 and the Scheffé post-hoc comparison test were also used.

Results

Quantifying ethanol effects

In this work, the response of 29 yeast strains against the stress caused by increasing concentrations of ethanol (from 0 to 194 g/l, equivalent to 0 – 25%) was quantified in laboratory media using OD measurements. In all cases, the plot of the fa versus ethanol concentration (expressed as $\log_{10}$) gave a typical sigmoidal decay function. Consequently, yeast susceptibility to ethanol was nonlinearly dose related. Figure 1 shows as an example the curve fitting for yeast *Sc T73*. Clearly, the whole sigmoid-shaped curve can be divided into three sections: i) points corresponding to concentrations from zero up to the NIC (concentrations at which no effect of the inhibitor was observed and fa was around 1), ii) concentrations between NIC and MIC (within which growth inhibition progressively occurred and the fa decreases), and iii) a third section above MIC (where no growth relative to the control was recorded and fa was around 0). These regions are denoted in Figure 1 as NIR, PIR and NGR, respectively. The curve fitting was good in the 87 cases analyzed (29 yeast strains x triplicate), with an R^2 ranging from 0.967 to 0.999 (data not shown).

Figure 2 shows the concentration range at which the increasing inhibitory effect of ethanol was noticed for the different strains assayed in this work. In the case of NIC, this parameter ranged from 19.7 g/l (calculated for the non-*Saccharomyces* yeast *Hu*), to 73.9 g/l (obtained for the wild *S. cerevisiae* isolate *Sc 9*). Focusing on each yeast species, the *S. cerevisiae* isolates showed NIC values included in the interval between 36.7 and 73.9 g/l (the widest dispersion), the *S. paradoxus* strains between 51.3 and 73.5 g/l, the *S. bayanus* var. *uvareum* strains between 27.3 and 56.2 g/l, and finally the *S. kudriavzevii* isolates between 27.4 and 44.3 g/l. Overall, the non-*Saccharomyces* and *S.*

kudriavzevii strains exhibited the lowest NIC values (< 46 g/l), indicating that these strains are more sensitive to low ethanol concentrations than the rest of yeasts, which can tolerate, in most cases, up to 60 g/l of ethanol (~8%) without any effect on their growth (Figure 2).

For MIC, this parameter ranged from 44.6 g/l (again for *Hu* strain) to 141.4 g/l (in the case of the fermentative strain *Sc PE35M*). The *S. cerevisiae* isolates always showed the highest MIC values (between 95.6 and 141.4 g/l), followed by the *S. paradoxus* strains (between 82.7 and 93.4 g/l) and then, with similar values, by the *S. bayanus* var. *uvarum* isolates (between 77.3 and 84.9 g/l). Lower MIC values were obtained for all the *S. kudriavzevii* strains (between 45.6 and 71.9 g/l) (Figure 2). The wild type strains of *S. mikatae* (81.3 g/l) and *S. arboriculus* (81.9 g/l) had very similar MIC values compared to the *S. paradoxus* and *S. bayanus* var. *uvarum* strains, while *S. cariocanus* (71.3 g/l) was closer to *S. kudriavzevii* (especially to the strains *Sk CR90* and *CA111*). The non-*Saccharomyces* strains studied in this work (corresponding to species *Hanseniaspora uvarum*, *Torulaspora delbrueckii* and *Kluyveromyces marxianus*), had MIC values below 80 g/l, always lower than the *S. cerevisiae* and *S. paradoxus* strains. Table S1 (see supporting information) shows the numerical NIC and MIC values obtained for all the strains assayed in this work.

Figure 3 shows the results of the one-way ANOVA analysis carried out for the NIC and MIC parameters after grouping strains as a function of their respective *Saccharomyces* species. Because different strains were considered for each species, the obtained values represent a better approximation to overall yeast response against ethanol than a single strain. *S. kudriavzevii* was the yeast with the lowest NIC values (average 35.3 g/l, Figure 3 upper panel), indicative of a higher susceptibility to ethanol,

showing significant differences with respect to *S. cerevisiae* (57.8 g/l), *S. mikatae* (59.4 g/l) and *S. paradoxus* (59.8 g/l). For *S. bayanus* var. *uvarum*, *S. cariocanus* and *S. arboriculus*, the NIC values were 45.8, 48.7 and 55.1 g/l, respectively. In the case of the MIC parameter (Figure 3 lower panel), *S. cerevisiae* was clearly the yeast with the highest ethanol resistance value (average 112.5 g/l), and statistically different compared to the rest of *Saccharomyces* species (59.3, 71.3, 80.5, 81.3, 81.9 and 88.7 g/l for *S. kudriavzevii*, *S. cariocanus*, *S. bayanus* var. *uvarum*, *S. mikatae*, *S. arboriculus* and *S. paradoxus*, respectively). As can be also observed in Figure 3, the differences between *S. kudriavzevii* (a species exclusively isolated from wild environments) and *S. paradoxus* and *S. bayanus* var. *uvarum* (which are yeasts better adapted to human-manipulated fermentative environments) were also statistically significant.

In those species including fermentative and wild isolates (*S. cerevisiae*, *S. paradoxus* and *S. bayanus* var. *uvarum*), a second ANOVA analysis was carried out to determine if there were significant differences according to the origin of the strains. Table 2 shows the results obtained for this analysis using the Scheffé post hoc comparison test. Non significant differences were found for the NIC or MIC average values between natural and fermentative habitats within the same yeast species. Significant differences were obtained for NIC parameter between the group formed by the *S. cerevisiae* wild isolates (average value of 65.9 g/l) and the group of the *S. bayanus* var. *uvarum* fermentative isolates (39.3 g/l). In the case of the MIC parameter, the natural and fermentative *S. cerevisiae* groups showed again a significant higher resistance to ethanol (~112 g/l) than the groups formed by the *S. paradoxus* (~90 g/l) and *S. bayanus* var. *uvarum* (~80 g/l) strains (see Table 2). Figure S1 (see supporting information) shows the graphical representation of this analysis. It is worth noting that similar results were also obtained when the ANOVA analysis was performed by

considering the four *S. cerevisiae* wine isolates as an independent group (data not shown).

Fatty acid and sterol cell composition

Three selected strains belonging to species with clear differences in ethanol tolerance were analyzed for their sterol and FA composition profiles, to determine if there was a possible cause-effect relationship. We chose *Sc T73* and *Sb BM58* because both strains were initially isolated from wine fermentations characterized by the presence of high ethanol levels, while *Sk CR85* was isolated from Spanish oak tree samples.

Table 3 shows the distribution of the different types of FAs and sterols in these strains. The medium-chain FAs (MCFA, C6 to C14) represented approximately 3% of the total FAs, being the only FA group with no significant differences among yeasts. However, the long-chain saturated FA contents (SFA) were significantly different among strains, showing saturation levels between 17% and 34%. In yeast *Sc T73*, these FAs represented around a half of the percentage of FA present than in the other two species. We also found significant differences among yeasts with respect to the long-chain unsaturated FAs (UFA), which divided each strain into a separate class. For strain *Sk CR85*, the UFA represented ~63% of the total FAs, followed by *Sb BM58* (~70%) and, finally, by *Sc T73* with the highest value (~80%). Differences among strains were especially evident when the ratio UFA/SFA was obtained, with *Sc T73* almost doubling the value of the other two strains. The mean of FA chain length was also significantly longer in yeast *Sc T73* than in strains *Sb BM58* or *Sk CR85*.

We also analyzed the FA cell composition profile compound to compound (data not shown). We found that differences in the UFA/SFA ratio were mainly due to the palmitic and stearic SFAs, and the palmitoleic and oleic UFAs. Table 3 also shows the

values of these FAs expressed as $\mu\text{g}/\text{mg}$ of dry weight. The only FA that did not show significant differences was the stearic acid. On the contrary, the three strains showed significant differences for the palmitic SFA and oleic UFA, being *Sc T73* the yeast with lowest amount of palmitic acid and highest of oleic acid. *Sb BM58* was the yeast with highest values of the sixteen-carbon palmitoleic UFA, more than twice compared to the other two species.

The main recognized membrane sterol in yeasts is ergosterol, which did not show significant differences among the three species with values ranging from 5.7 to 8.0 $\mu\text{g}/\text{mg}$ (see Table 3). However, the squalene differentiated yeasts into 2 class, one formed by *Sk CR85* and *Sb BM58* ($\sim 6.7 \mu\text{g}/\text{mg}$) and another only formed by the strain *Sc T73* (with a lower production of this sterol, less of 1 $\mu\text{g}/\text{mg}$). The lanosterol also showed significant differences among strains, but the production of this sterol was very low compared to the other compounds.

Discussion

Of the seven *Saccharomyces* species studied in this work, including a total of 26 strains isolated from different ecological niches, *S. cerevisiae* significantly was the most resistant yeast to high ethanol levels, followed by *S. paradoxus*, whilst *S. kudriavzevii* was the least resistant. Similar results were also reported by Belloch *et al.* (2008) with other strains of these species, showing that whilst *S. cerevisiae* strains were able to grow up to ethanol levels of approximately 117 g/l, the type strain of *S. kudriavzevii* grew poorly at 40 g/l and it was unable to grow at 80 g/l. However, must fermentations carried out in our laboratory with this strain showed that ethanol levels of 85 g/l ($\sim 11\%$) can be produced by this microorganism (personal data). A possible explanation for this difference is that through fermentation yeast cells can gradually respond to the ethanol

stress, increasing their tolerance. Several authors have reported that the composition of the medium (amino acids, lipids, etc) may also influence yeast ethanol resistance (Ding *et al.*, 2009).

Among all the *S. cerevisiae* isolates, *Sc GB* (a ‘flor’ yeast from Sherry wine) and *Sc PE35M* (isolated from Masato fermentations) were the most resistant yeasts to ethanol, while the non-*Saccharomyces* wine strains *Hu* and *Td* resulted to be very sensitive. Aguilera *et al.* (2006) also reported that a *S. cerevisiae* ‘flor’ wine strain was less affected by the presence of high ethanol levels (among other *S. cerevisiae* and non-*Saccharomyces* strains), but a *T. delbrueckii* strain showed the highest sensitivity to this compound. During velum formation the ‘flor’ yeasts are able to aerobically consume ethanol as a main carbon source by an oxidation process. In fact, it was reported that the ethanol resistance of yeasts greatly depended on mitochondria, and that the ethanol tolerance of a *S. cerevisiae* laboratory strain could be enhanced by introducing the mitochondria from a ‘flor’ wine yeast (Ibeas and Jiménez, 1997; Jiménez and Benítez, 1988). For this reason, we thought at first that the higher ethanol tolerance exhibited by certain strains could be due to a greater capability to consume ethanol in the presence of oxygen. However, when we carefully revised the growth curves for all strains, we did not find any evidence of diauxic growth, which would be indicative of ethanol consumption.

Susceptibility and resistance to ethanol were not statistically different between wild and fermentative isolates of *S. cerevisiae*. Similar results were also obtained for the species *S. paradoxus* and *S. bayanus* var. *uvarum*. This indicates that, presumably, both physiological characteristics were not modified through yeast adaptation to fermentative environments provided by human activity, but on the contrary, are natural properties

intrinsic to the species which have undergone little changes. These results are consistent with previous evidences obtained by Thomson *et al.* (2005). These authors dated that the duplication of the ancestral alcohol dehydrogenase (ADH) gene occurred ~80 million years ago, rather before than the much more recent origin of human-controlled alcohol production (~9,000 years ago). The ancestral ADH enzyme was able to ferment sugars into ethanol, but could not consume ethanol as efficiently as modern *Saccharomyces* yeasts do (Thomson *et al.*, 2005). With the acquisition of a new and modified ADH enzyme (ADH2), which had a higher affinity for ethanol consumption, *Saccharomyces* yeasts took advantage over its competitors, by first producing high ethanol levels to be subsequently respired (Piskur *et al.*, 2006; Woolfit and Wolfe, 2005). The ability to produce, accumulate and consume ethanol acquired by *S. cerevisiae* imposed a selective pressure to become ethanol tolerant, and preliminary data obtained in this work support this hypothesis. Susceptibility and resistance to ethanol seem to be properties previous to the adaptation to human-manipulated fermentative processes because it is also present in wild *Saccharomyces* yeasts. However, other phenotypes, as for example sulfite resistance, have been clearly proved to be domesticated characters present in wine yeasts but absent in wild strains (Barrio *et al.*, 2006; Pérez-Ortín *et al.*, 2002).

Yeast species are known to differ in their ability to produce and tolerate ethanol, and although this phenomenon has been studied over the last few decades, the reason why some strains are more tolerant to ethanol than others remains unclear. *S. cerevisiae* is recognized as the most predominant yeast in alcoholic beverages, and especially in wines, where, in the later stages of fermentation, high ethanol levels are reached (Pretorius, 2000). It is known that *S. cerevisiae* possesses diverse strategies to counteract the stress produced by high ethanol concentrations, as: i) to change the membrane

composition to antagonize membrane fluidization (by increasing levels of UFA and ergosterol), ii) the expression of factors that stabilize and/or repair denatured proteins, iii) the synthesis of proteins involved in the expression of stress-related genes, and iv) an increase in plasma membrane ATPase activity which counteracts the ethanol-induced proton influx (Ding *et al.*, 2009). However, until our knowledge, no detailed studies have been carried out with other related *Saccharomyces* species and, thus, it is difficult to compare and determine the specific causes why they support lower ethanol levels, especially *S. kudriavzevii*.

For this reason, we have analyzed the FA and sterol cell composition of three strains belonging to different *Saccharomyces* species. The most marked difference was noticed in the UFA/SFA ratio, with *Sc T73* > *Sb BM58* > *Sk CR85*. This ratio was clearly related with the susceptibility and resistance of yeasts to ethanol, which was 67.6, 47.4 and 33.4 g/l for NIC, and 110.6, 80.9 and 54.5 g/l for MIC (for strains *Sc T73*, *Sb BM58* and *Sk CR85*, respectively). The initial higher UFA/SFA ratio of the *S. cerevisiae* strain could provide the possibility to compensate better the stress originated by the sudden immersion in high ethanol levels. It is worth noting that this capability was a natural predisposition, because cells were not pre-adapted in a medium enriched with ethanol. It has been reported that under semi-anaerobic conditions, as presumably occur during fermentation, cell UFA decreased and SFA increased for the lack of oxygen (Torija *et al.*, 2003). However, in the present study, there was a lower natural predisposition to synthesize UFA by the strains *Sc BM58* and *Sk CR85*, even in the presence of oxygen. Torija *et al.* (2003) also mentioned an initial lower UFA/SFA ratio in *S. bayanus* dry cells (2.8) compared to *S. cerevisiae* cells (3.9). It is proved that the increasing proportion of UFA (C18:1) is also accompanied by a decrease in the proportions of the FA C16:0 and C16:1 in response to increasing ethanol concentrations

(You *et al.*, 2003). These authors demonstrated that oleic acid was the most efficient UFA in overcoming the toxic effects of ethanol in growing yeast cells, whilst palmitoleic acid did not confer any ethanol tolerance. We have obtained similar results, and the yeast with the highest oleic levels and the lowest palmitoleic levels (*Sc T73*) was also the most ethanol resistant. Finally, an increase of sterols in membrane would compensate the fluidity effect occasioned by ethanol (Ding *et al.*, 2009), but in this study there were no significant differences among yeasts in the main membrane sterol, ergosterol. However, we found differences in squalene, with *Sc T73* presented almost 6 times less than the other two species.

Further and complementary researches should be conducted with additional strains to understand the adaptive role played by membrane composition differences on ethanol tolerance, but it seems obvious that the higher natural predisposition exhibited by *S. cerevisiae* to counteract ethanol stress resulted very useful to colonize and predominate in many fermentative habitats supplied by human activity.

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Table 1. Origin and designation of the 29 yeast strains used in this study

Species	Strain	Origin	Designation [†]
<i>S. cerevisiae</i>	9	Forest soil (Hungary)	<i>Sc</i> 9
	96.2	Oak tree bark (Spain)	<i>Sc</i> 96.2
	CECT10131	<i>Centaurea alba</i> flower (Spain)	<i>Sc</i> 10131
	Lalvin T73 ^C	Wine fermentation (Spain)	<i>Sc</i> T73
	EC1118 ^C	Champagne (France)	<i>Sc</i> EC1118
	RVA ^C	Wine fermentation (Spain)	<i>Sc</i> RVA
	GBFLOR-C	Wine fermentation (Spain)	<i>Sc</i> GB
	PE35M	Masato fermentation (Peru)	<i>Sc</i> PE35M
	CPE7	Sugarcane fermentation (Brazil)	<i>Sc</i> PE7
	TEMOHAYA-MI26	Agave fermentation (Mexico)	<i>Sc</i> TEMO
<i>S. paradoxus</i>	CECT1939 ^T = CBS432 ^T	Tree bark (Russia)	<i>Sp</i> 1939
	116.1	Oak tree bark (Spain)	<i>Sp</i> 116.1
	120M	Pulque fermentation (Mexico)	<i>Sp</i> 120M
	K54	Wine fermentation (Croatia)	<i>Sp</i> K54
<i>S. bayanus</i> var. <i>uvarum</i>	NCAIM789	Oak tree bark (Hungary)	<i>Sb</i> NCAIM
	CECT1969 ^T	Black currant (The Netherlands)	<i>Sb</i> 1969
	BM58 ^C	Wine fermentation (Spain)	<i>Sb</i> BM58
	CECT 12627	Wine fermentation (Spain)	<i>Sb</i> 12627
<i>S. kudriavzevii</i>	NBRC 1802 ^T = IFO 1802 ^T	Decayed leaf (Japan)	<i>Sk</i> IFO
	CA111	Oak tree (Spain)	<i>Sk</i> CA111
	CR85	Oak tree (Spain)	<i>Sk</i> CR85
	CR89	Oak tree (Spain)	<i>Sk</i> CR89
	CR90	Oak tree (Spain)	<i>Sk</i> CR90
<i>S. mikatae</i>	NBRC 1815 ^T = IFO 1815 ^T	Tree bark (Japan)	<i>Smik</i>
<i>S. arboriculus</i>	CBS10644 ^T	Tree bark (China)	<i>Sarb</i>
<i>S. cariocanus</i>	CBS8841 ^T	Insects (Brazil)	<i>Scar</i>
Non-Saccharomyces	<i>Hanseniaspora uvarum</i> NS1	Wine fermentation (Spain)	<i>Hu</i>
	<i>Torulaspora delbrueckii</i> NS2	Wine fermentation (Spain)	<i>Td</i>
	<i>Kluyveromyces marxianus</i> NS3	Wine fermentation (Spain)	<i>Km</i>

[†] Yeast designation used in the present work, ^T Type strain, ^C Commercial strain.

Table 2. NIC and MIC average values obtained by means of a factorial ANOVA analysis using ‘yeast origin’ (2 levels: fermentative, wild) and ‘yeast species’ (3 levels: *S. cerevisiae*, *S. bayanus* var. *uvarum*, *S. paradoxus*) as categorical predictor variables to group strains.

Number of strains in the group/cases	Categorical predictor variables		NIC (g/l)	MIC (g/l)
	Yeast species	Origin		
3 / 9	<i>S. cerevisiae</i>	Wild	65.86 (9.51) ^b	110.78 (9.05) ^b
7 / 21	<i>S. cerevisiae</i>	Fermentative	54.38 (14.32) ^{a,b}	113.16 (19.23) ^b
2 / 6	<i>S. paradoxus</i>	Wild	60.15 (12.58) ^{a,b}	89.00 (4.37) ^a
2 / 6	<i>S. paradoxus</i>	Fermentative	59.43 (3.25) ^{a,b}	88.32 (5.83) ^a
2 / 6	<i>S. bayanus</i> var. <i>uvarum</i>	Wild	51.26 (5.89) ^{a,b}	78.80 (8.16) ^a
2 / 6	<i>S. bayanus</i> var. <i>uvarum</i>	Fermentative	39.33 (11.58) ^a	82.55 (3.52) ^a

[†]Values followed by different superscript letters, within the same column, are significantly different according to a Scheffé post hoc comparison test. Standard deviations between parentheses.

Table 3. Fatty acid and sterol cell composition of yeasts *Saccharomyces cerevisiae* T73, *S. bayanus* var. *uvvarum* BM58 and *S. kudriavzevii* CR85 in laboratory medium (YNB) at 28°C.

	Yeast strains		
	<i>Sk</i> CR85	<i>Sb</i> BM58	<i>Sc</i> T73
[†] Fatty acids			
SFA	33.16 ± 1.24 ^b	28.12 ± 0.07 ^b	17.20 ± 3.61 ^a
UFA	63.51 ± 1.32 ^a	69.58 ± 0.07 ^b	80.20 ± 3.14 ^c
MCFA	3.33 ± 0.83 ^a	2.30 ± 0.04 ^a	2.60 ± 0.87 ^a
Ratio UFA/SFA	1.91 ± 0.11 ^a	2.47 ± 0.01 ^b	4.66 ± 1.03 ^c
CL	16.33 ± 0.09 ^a	16.34 ± 0.03 ^a	16.89 ± 0.27 ^b
^{††} Fatty acid / sterol			
Palmitic acid (C16:0)	30.88 ± 0.24 ^b	52.3 ± 0.4 ^c	10.95 ± 6.99 ^a
Palmitoleic acid (C16:1)	45.68 ± 1.69 ^a	110.02 ± 0.98 ^b	43.85 ± 22.46 ^a
Stearic acid (C18:0)	3.36 ± 2.91 ^a	7.09 ± 0.04 ^a	8.43 ± 2.56 ^a
Oleic acid (C18:1)	20.22 ± 2.05 ^a	39.04 ± 0.2 ^b	44.11 ± 0.99 ^c
Squalene	6.67 ± 0.31 ^b	6.84 ± 3.27 ^b	0.83 ± 0.11 ^a
Lanosterol	0.04 ± 0.01 ^a	0.53 ± 0.27 ^{ab}	0.66 ± 0.27 ^b
Ergosterol	8.02 ± 4.94 ^a	7.19 ± 4.45 ^a	5.73 ± 3.4 ^a

[†] Values expressed as percentages of the total fatty acids. SFA, long-chain saturated fatty acids (C14:0, C16:0 and C18:0); UFA, unsaturated fatty acids (C14:1, C16:1 and C18:1); MCFA, medium-chain fatty acids (C8:0, C10:0 and C12:0); CL, mean fatty acid chain length.

^{††} Values expressed as µg/mg of dry weight.

Note: Values followed by different superscript letters, within the same row, are significantly different according to a Scheffé post hoc comparison test. They are the mean (±standard deviations) of three independent experiments.

Figure legends

Figure 1. Non-inhibitory region (NIR), progressive inhibitory region (PIR) and no-growth region (NGR) of the yeast *Saccharomyces cerevisiae* T73 as a function of the decimal logarithm of ethanol concentration (g/l). Curve fitting was achieved with a modified Gompertz function for decay (Lambert and Pearson, 2000).

Figure 2. Ethanol concentration range (g/l) where an increasing inhibitory effect was observed for the 29 yeast strains. Values are averages from triplicate experiments. Dashed lines represent the standard deviations for the different strains.

Figure 3. One-way ANOVA for the NIC and MIC parameters (dependent variables) grouping strains as a function of their respective *Saccharomyces* species (*Skud*, *S. kudriavzevii*; *Scar*, *S. cariocanus*; *Sbay*, *S. bayanus* var. *uvarum*; *Scer*, *S. cerevisiae*; *Smik*, *S. mikatae*; *Sarb*, *S. arboriculus*; *Spar*, *S. paradoxus*). 26 yeast strains, including a total of 78 cases, were introduced in the analysis.

NIC

MIC

Supporting information to: **Susceptibility and resistance to ethanol in *Saccharomyces* strains isolated from wild and fermentative environments**

Table S1. NIC and MIC ethanol average values (standard deviations from triplicate experiments in parentheses) obtained for the 29 yeast strains assayed in this work.

Strain designation	NIC (g/l)	MIC (g/l)
<i>Sc 9</i>	73.9 (8.3)	115.8 (0.6)
<i>Sc 96.2</i>	66.4 (6.2)	104.5 (1.8)
<i>Sc 10131</i>	57.2 (6.8)	111.9 (14.0)
<i>Sc T73</i>	67.6 (7.3)	110.6 (6.4)
<i>Sc EC1118</i>	60.3 (2.2)	112.2 (3.5)
<i>Sc RVA</i>	64.9(5.2)	97.7 (3.2)
<i>Sc GB</i>	48.4 (17.4)	135.0 (17.5)
<i>Sc PE35M</i>	36.7 (11.1)	141.4 (12.1)
<i>Sc PE7</i>	54.3 (4.8)	99.6 (4.3)
<i>Sc TEMO</i>	48.6 (3.2)	95.6 (11.8)
<i>Sp 1939</i>	51.3 (4.1)	86.1 (2.0)
<i>Sp 116.1</i>	73.5 (2.4)	93.4 (2.0)
<i>Sp 120M</i>	61.7 (1.2)	92.1 (3.8)
<i>Sp K54</i>	56.0 (0.5)	82.3 (0.1)
<i>Sb NCAIM</i>	46.3 (2.9)	77.3 (6.8)
<i>Sb 1969</i>	56.2 (2.1)	80.3 (10.6)
<i>Sb BM58</i>	47.4 (4.3)	80.9 (2.0)
<i>Sb 12627</i>	27.2 (3.4)	84.9 (4.7)
<i>Sk IFO</i>	33.9 (1.0)	52.6 (1.0)
<i>Sk CA111</i>	44.3 (6.5)	67.2 (4.6)
<i>Sk CR85</i>	33.4 (1.4)	54.5 (1.2)
<i>Sk CR89</i>	27.4 (0.5)	45.6 (0.7)
<i>Sk CR90</i>	34.8 (8.2)	71.9 (5.0)
<i>Smik</i>	59.4 (0.7)	81.3 (1.8)
<i>Sarb</i>	55.1 (4.9)	81.9 (5.0)
<i>Scar</i>	48.7 (5.6)	71.3 (5.6)
<i>Hu</i>	19.7 (2.0)	44.6 (0.8)
<i>Td</i>	20.8 (0.3)	62.0 (1.9)
<i>Km</i>	45.6 (0.2)	79.6 (4.8)

Supporting information to: **Susceptibility and resistance to ethanol in *Saccharomyces* strains isolated from wild and fermentative environments**

Figure S1. Graphical representation of the factorial ANOVA analysis carried out for the NIC and MIC parameters (dependent variables) with ‘yeast origin’ (natural or fermentative) and ‘yeast species’ (*S. cerevisiae*, *S. paradoxus* or *S. bayanus* var. *uvorum*) as categorical predictive variables. 18 yeast strains including a total of 54 cases were introduced in the analysis.