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Ethanol tolerance is decreased by fructose in *Saccharomyces* and non-*Saccharomyces* yeasts

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ABSTRACT

The maximal values of osmotic (fructose) stress and ethanol tolerances dependence on hexose type present in media were quantified for a collection of yeasts isolated from mezcal covering ten different genera, including *Saccharomyces*. The yeasts clustered in five groups where in the least tolerant group yeasts were not able to grow at a fructose concentration above 200 g/l, as compared to yeasts in the most tolerant group that were able to grew at concentration of fructose above 700 g/l. In ethanol agar plates without a carbon source, the maximum tolerance was of 9% v/v of ethanol for all of the yeasts. When ethanol was combined with glucose (20 g/l), a number of *Saccharomyces cerevisiae* strains were able to grow at up to 15% v/v ethanol, whereas the maximum was 10% v/v ethanol for the non-*Saccharomyces* yeasts. However, when fructose was used instead of glucose, none of the yeasts tested was able to grow on plates containing above 9% v/v ethanol, including *S. cerevisiae*. Hence fructose did not improve the tolerance to ethanol as observed for glucose, but rather fructose acted as an inhibitor or increasing the toxicity of ethanol.

1. Introduction

During the fermentation process, yeast cells are subjected to several stress conditions, but osmotic (sugar) and ethanol stresses are the most important in terms of changes throughout the fermentation process (Carrasco, Querol, & Del Olmo, 2001; Tofalo et al., 2009). With regards to the fermentable sugars, glucose and fructose are the main hexoses encountered in almost any natural must, being present in equivalent quantities in grape must, but the proportions may vary depending on the must fermented by each alcoholic beverage industry; nonetheless, the initial concentration of total sugars is approximately 100–250 g/l, with the lower fermentability of fructose providing the first challenge for *Saccharomyces cerevisiae* yeast cells (Arroyo-López, Querol, & Barrio, 2009; Oliva-Hernández, Taillandier, Reséndez–Pérez, Narváez-Zapata, & Larralde-Corona, 2013). The second main stress is the progressive accumulation of ethanol during fermentation. The ethanol is a well-known inhibitor of microorganisms' growth, and it has been reported that the toxic effects of ethanol on yeast cells involve loss of cell viability and inhibition of both yeast growth and inhibition of various transport systems such as the general amino acid permease and the glucose (hexoses) transport system (Lewis, Elkon, McGee, Higbee, & Gasch, 2010; Santos et al., 2008). The combination of nutrient depletion and high ethanol content contributes to fermentative limitations.

Study of stress resistance is usually performed by inducing a stressor shock (ethanol, osmotic, temperature, etc.) and then the capacity of recovery (viability) of the yeast population is verified by counting the number of colonies formed by microorganisms in a solid (nutrient agar) matrix from serial dilutions of the original sample, either by the pour-plate or spread-plate techniques, but also by spotting and verifying the dilution rate at which there is no visible growth. However, to our knowledge this latter methodology has not been applied to quantitatively assess to the tolerances on several genera of yeasts growing under different stress conditions, to be used as a tool to select those strains with a potential high performance in alcoholic beverage's fermentations.

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A high level of ethanol tolerance in a yeast strain is a prerequisite for high efficiency during fermentation and, in turn, for a high yield of ethanol. Accordingly, yeasts isolated from agave fermentations (tequila and mezcal as main examples) are an interesting option since these musts are very rich in fructose and this allows for the isolation of yeasts, *Saccharomyces* (Oliva-Hernández et al., 2013) and non-*Saccharomyces* that are already adapted to using this sugar, making this process a good source of yeasts for fermentation purposes, for example for wine production where a high final concentration of fructose is known to be related to stuck fermentations. The aim of this work was to quantitatively assess the effect of fructose over osmotic and ethanol tolerances of yeasts belonging to ten different genera originally isolated from mezcal fermentations.

2. Materials and methods

2.1. Yeast strains and inocula growth conditions

The 25 yeast strains used belong to the mezcal LBI-CBG yeast collection and are conserved in 60% glycerol at −70 °C, and the commercial fructophilic wine strain *Saccharomyces cerevisiae* Fermichamp (DSM Food Specialities B.V., The Netherlands) was used as control. The strains used are representative of the yeast glucophilic and fructophilic diversity found in the fermentation of mezcal from Tamaulipas (Mexico) and belong to the species *S. cerevisiae* (labelled LCBG-Sc3Y2, -Sc3Y3, -Sc3Y4, -Sc3Y5, -Sc3Y8, -ScMsc3, -Sc4Y3, -Sc3D2, -Sc3D3, -Sc3D4, -Sc3D5 and -Sc3D6), *Kluyveromyces marxianus* (labelled LCBG-Km1D5, -4D3 and -1Y9), *Torulaspora delbrueckii* (labelled LCBG-Td1AN1, -1AN2 and -1AN9), *Pichia* spp. (*P. kluyveri* -LCBG- Pk4D6, *P. guilliermondii* -Pg1Y12 and *P. mexicana* -Pm1AN3), and strains of *Candida parapsilosis* (LCBG-Cp1Y7), *Clavispora lusitaniae* (LCBG-Cl4Y4) *Rhodotorula mucilaginosa* (LCBG-RmP12) and *Zygosaccharomyces bailii* (LCBG-Zb3Y1).

An initial pre-culture of the tested yeast was grown on YPD agar plates containing 1% yeast extract, 2% peptone, 2% w/w D-glucose, plus 2% bacteriological agar (Difco Laboratories, France), all on a w/w basis and incubated at 30 °C for 48 h. A loop of this pre-culture was used as inoculum for liquid YPD medium incubated 24 h at 30 °C with shaking at 200 rpm. The optical density of the cultures was determined at 600 nm and the initial inoculum concentration was adjusted using sterile Ringer solution to an absorbance of 0.5 for all tolerance experiments.

2.2. Setup of the drop CFU counting technique for stress tolerance assessment

In order to quantitatively verify the level of stress tolerance of the yeast collection, the first step was to set up a technique for plate counting, based on the classic method of Miles and Misra as revised by Hedges (2002) but modifying the volume used. To set up the counting conditions, cells were counted both in standard plate count technique as in Neubauer chamber using an 18 h culture of the control strain Fermichamp growing on YPD medium. Several volumes (5–20 μl) and dilution factors (10^0 to 10^{-6}) were assayed. The experiment was performed on 9 cm petri dishes containing 15 ml of YPD agar for every yeast inocula (control plate) and the different stress conditions tested (osmotic/carbon source, and ethanol). Triplicates were assayed for each strain under every experimental condition.

2.3. Fructose osmotic tolerance assessment

As fructose is consumed slower than glucose during fermentation, the highest concentration at which the yeasts were able to grow in fructose was assessed across an ample range, and the base agar medium (YP, 1% yeast extract, 2% peptone, 2% bacteriological agar, all on w/w basis) was supplemented with 2% fructose (w/w) (as in YPF medium), 5%, 10% and then in increments of 5% up to 90% fructose. Serial dilutions were spotted on the media, the plates were incubated at 30 °C until colonies were countable (from 1 to a maximum of 9 days for the most stressful conditions) and all the Petri dishes were sealed with Parafilm M™ (Brand, Germany) to minimize water loss. The experiments were conducted at least three times, and as controls, YPF, YPD and YPDF (equimolar glucose and fructose) agar media were used, the three of them rendering the same colony counts.

2.4. Ethanol tolerance assessment with and without hexoses added

Ethanol stress analysis was conducted on agar media with and without hexoses (either glucose or fructose) and in an equimolar glucose and fructose mixture to assess the effect of the simultaneous presence of the two hexoses. Base YP medium plus 2% w/w of either D-glucose (YPD agar) or D-fructose (YPF agar) or equimolar glucose and fructose (YPDF agar) was used with specific quantities (2, 5, 8, 9, 10, 11, 12, 13, 14, 15 or 16% v/v) of ethanol added to the temperate but still melted medium after autoclaving to complete the required volume of medium just prior to being poured into Petri dishes. Then, the drop colony counting technique described above was used. On the other hand, to assess the tolerance and utilisation of ethanol as the sole carbon source, inocula were spotted on base YP agar medium containing 2, 5, 7, 8, 9, 10, 11 or 12% v/v ethanol. The plates were incubated at 30 °C from 2 up to a maximum of 9 days, and all the dishes were sealed with Parafilm M™ (Brand, Germany) to minimize ethanol and water loss. The experiments were conducted at least three times and as a control the inoculum dilutions were spotted on regular YPD agar medium without ethanol. The average values presented always had a standard deviation of less than 10%.

2.5. Statistical analyses

The raw colony count (CFU/ml) data were converted to their corresponding logarithmic values to facilitate statistical analysis, which was performed using the Analyse-it software for Microsoft Excel (version 2.20) and the JMP routine of the SAS software for ANOVA analysis. For the global tolerance analysis we made use of five values (obtained with normal YPD plus four ethanol and fructose tolerances) per strain in a box plot type description (Krzywinski & Altman, 2014) as the most straightforward statistical method to classify the tolerance range of the *S. cerevisiae* strains. It is worth noting that when no growth was observed, an arbitrary value of 1 was used to allow logarithmic calculations, and the value of zero was reported for this condition accordingly.

3. Results

The reproducibility of CFU counting technique was first validated using an 18 h culture of the control strain Fermichamp growing in YPD broth. The volume of 10 μl was chosen as the most convenient in terms of handling, visualisation and integrity of the drop on the agar surface (Fig. 1A). A working dilution, typically 10^{-3} was used considering that the initial sample had an absorbance ($OD_{600\ nm}$) of 0.5, equivalent to approximately 3×10^6 CFU/ml, which renders a count around 20–50 colonies per drop deposited, and using the direct sample (no dilution) for the most stressful conditions. We used this technique to evaluate the effects of increasing ethanol and fructose concentrations on yeast growth, as the ones shown in Fig. 1B obtained for selected *S. cerevisiae* and non-*Saccharomyces* strains. At such a small volume used, it was

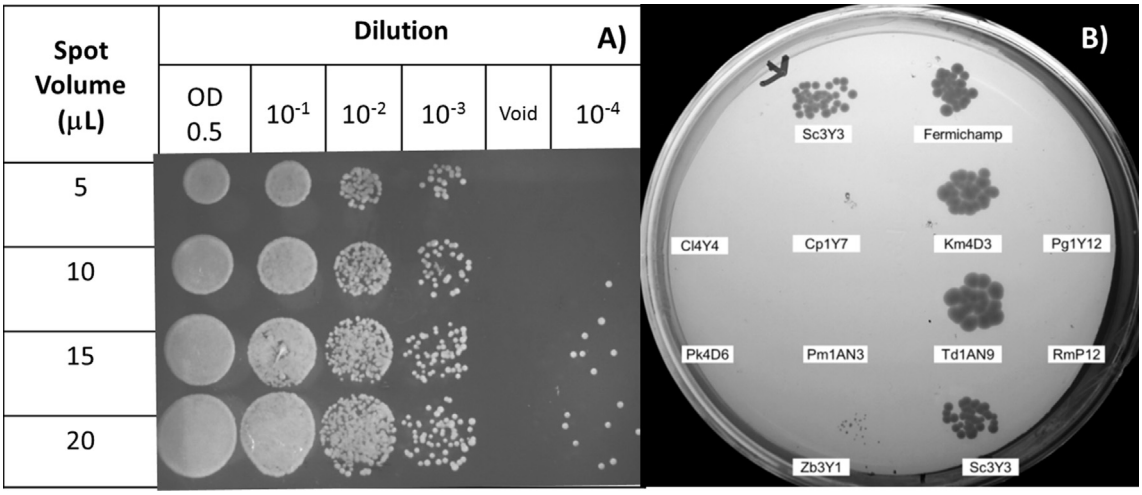

Fig. 1. Setup of the drop-plate colonies count technique for assessing: A) volume and dilution rate needed to accurately quantify the number of colony forming units in a known sample of control strain Fermichamp growing in YPD medium at 18 h; B) example of the growth of colonies at 72 h of incubation by some representatives of the 10 different yeast species isolated from mezcal, growing in YPD plus 8% v/v ethanol at a 10^{-3} dilution.

possible to quantify the viability of up to 12 strains per plate and also to eliminate the need for spreading the sample over the whole plate, which speeded up the analysis.

As expected we found a positive correlation between the cell counts assessed in spread-plates and that in both drop-plates and Neubauer chamber counts (Fig. 2), which corresponds to the interval from 0.1 to 1 of absorbance at 600 nm ($OD_{600\ nm}$) of the cultures. It is worth mentioning that for the less stressful conditions, the colonies could be reliably counted from 10 h of incubation by using a 2 to 4× magnification objective.

3.1. Tolerance of mezcal yeasts to osmotic stress caused by fructose

The growth of the yeasts on increasing fructose concentrations spanned the entire range tested (Fig. 3A and 3B); but in general terms, the yeasts grow could be grouped into five visually distinguishable fructose tolerance groups: 1) low tolerance (up to 200 g/l of fructose) was observed only for *S. cerevisiae* 3D4; 2) moderate tolerance (up to 350 g/l of fructose) included only *S. cerevisiae* strains 3D2 and 3D3; 3) high tolerance (up to 500 g/l of fructose) included *S. cerevisiae* 3D5, 3D6, Msc3 and 3Y5 as well as the non-*Saccharomyces* strain Pk4D6; 4) very high tolerance (up to 650 g/l of fructose) included the control fructophilic strain Fermichamp and the non-*Saccharomyces* strains Cp1Y7, Km4D3, and RmP12; and 5) extreme tolerance (above 700 g/l of fructose) included *Saccharomyces* strains 3Y2, 3Y3, 3Y4, 4Y3, and 3Y8 plus the non-*Saccharomyces* strains Km1D5, Cl4Y4, Km1Y9, Td1AN1, Td1AN2, Td1AN9, Pg1Y12, Pm1AN3 and Zb3Y1. As can be observed (Fig. 3), the mezcal strains showed a wide range of tolerance to increasing fructose concentrations.

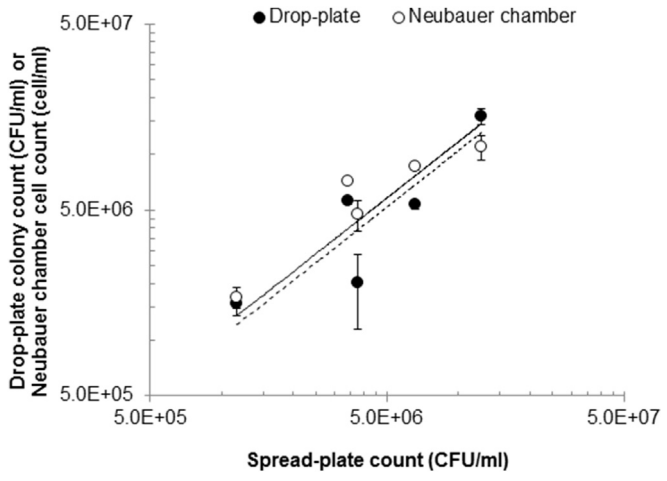

Fig. 2. Linear correlation of the cell count of control strain *S. cerevisiae* Fermichamp obtained on the spread-plate count and the drop-plate count (full symbol, continuous line, $R^2 = 0.945$) and the Neubauer chamber count (void symbol, dotted line, $R^2 = 0.899$). Standard deviations were calculated from three independent experiments.

3.2. Tolerance to ethanol according to hexose present

The tolerance to increasing concentrations of ethanol, was assessed in more detail for all the *Saccharomyces cerevisiae* yeasts in the presence of either glucose or fructose at a concentration of 20 g/l, which resulted in practically the same amount of growth (G symbolizing here growth in CFU/ml on a logarithmic scale) from 0 up to 8% of ethanol. Specifically, growth on glucose was $G_{glc} = 6.41 \pm 0.24$ log CFU/ml, while with fructose, growth was $G_{fru} = 6.35 \pm 0.38$ log CFU/ml, thus a ratio of $G_{fru}/G_{glc} = 1.00 \pm 0.03$ log CFU/ml for all the *S. cerevisiae* strains, including Fermichamp. Hence the diminishing growth in this range of increasing ethanol (0–8% v/v of ethanol) concentrations can be solely attributed to the inhibitory effect of the ethanol and not to the hexose used. When only ethanol was used with no hexose (YP medium), the growth ratio of the strains under this condition was: $G_{ethanol}/G_{hexose} = 0.8 \pm 0.06$ log CFU/ml, except for those yeasts that were not able to growth under this medium (*S. cerevisiae* Sc4Y3, 3D2, and 3D3, corresponding to tolerance group 3 in Fig. 5), therefore these were not considered for the calculation.

Noteworthy, when ethanol concentration was higher than 8% in the presence of fructose (at 20 g/l), which could be a typical case in the late stage of almost any alcoholic beverage fermentation, for all the yeast species tested in this work including *S. cerevisiae*, a drastic drop in maximal tolerance to ethanol was observed (Fig. 4A and B), which is graphically emphasized with an arrow towards the left. This unique behaviour observed only when fructose was present instead of glucose, is equivalent to a drop in tolerance from 129 to 77 g/l of ethanol. This general behaviour, not reported before as far as we know in yeasts, evidenced an inhibitory effect of the fructose

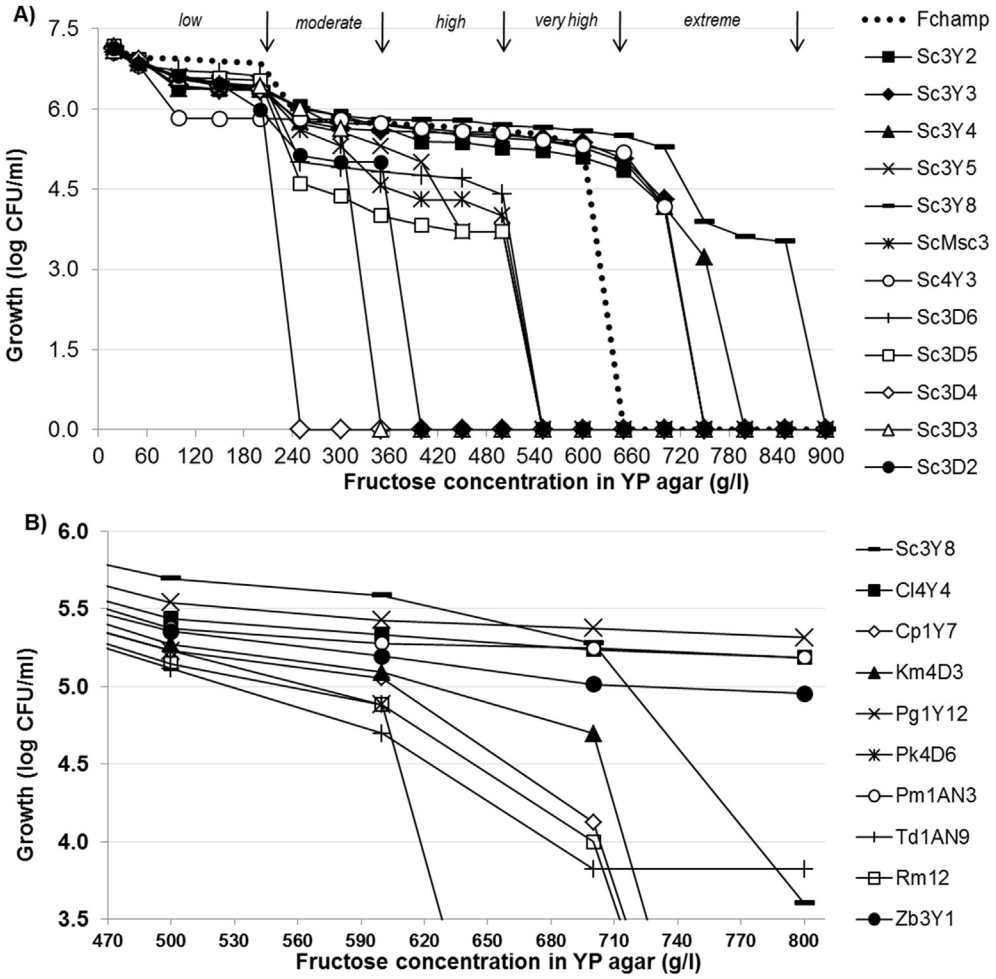

Fig. 3. Fructose tolerance as evidenced by growth capacity on solid YP agar medium supplemented with increasing fructose concentrations for A) *Saccharomyces cerevisiae* (plus Fermichamp as a control), and B) selected representative non-*Saccharomyces* mezcal strains, showing only a detail of the upper part of the graphic for clarity, and including *S. cerevisiae* 3Y8 data for comparison. In all cases the colony counts had standard deviations of less than 5%. The arrows (↓) show the upper limit of each range of tolerance.

(or a general effect of potentiation of the toxic effect of ethanol) rather than a surplus of carbon source for all the yeasts, including the fructophilic control strain Fermichamp.

As a consequence, those strains initially classified as highly resistant to ethanol in YPD, especially some *S. cerevisiae* (Sc) strains that were able to grow at up to 15% v/v of ethanol (3Y2, 3Y3, 3Y4, 3Y5, 3Y8, 3D2, 3D4, 3D5 and Fermichamp), had a maximal tolerance of only 9% ethanol when fructose was the carbon source. This was also the limit for the non-*Saccharomyces* strains Cl4Y4, RmP12 and Zb3Y1 (Fig. 4B) in this hexose. For the rest of the strains, the maximum resistance to ethanol was 8%, both in glucose and fructose.

When an equimolar mixture of glucose and fructose was used instead (YPDF medium), we observed that the presence of glucose partially counteracted the negative effect of fructose for the tested *S. cerevisiae* strains as well as for the non-*Saccharomyces* yeasts *K. marxianus*, *P. guilliermondii*, *T. delbrueckii* and *Z. bailii*, allowing them to increase their tolerance to at least 2% v/v more ethanol with respect to the value obtained with only fructose. This means that *S. cerevisiae* (3Y3 and Fermichamp) and *K. marxianus* 4D3 increased their tolerance from 9 to 12% ethanol in the equimolar medium, whilst *T. delbrueckii* 1AN9 and *Z. bailii* 3Y1 increased their tolerance from 8 to 10% ethanol in this medium, with the latter growing an order of magnitude further in this equimolar mixture than in only glucose at the same ethanol concentration. For the rest of the species there was no positive effect of using the equimolar glucose/fructose medium.

3.3. Analysis of the stress tolerances of S. cerevisiae mezcal strains

The end of almost any alcoholic fermentation is characterized by both high fructose and ethanol concentrations, and also by the almost absolute predominance of *S. cerevisiae* species, hence we used a combination of the unstressed growth and that obtained at four stressful conditions (high ethanol levels with and without hexoses present, and growth at high osmotic pressure caused by fructose) as a means of classifying the phenotypes observed for this species.

As can be observed (Fig. 5), the tolerance to the studied stresses for the *S. cerevisiae* mezcal strains, expressed as the pooled growth attained under the conditions tested, can be classified into 3 groups and one unique behaviour as evidenced by their box plot distributions as follows: yeasts in group 1 (strains Sc3Y2, 3Y3, 3Y4, 3Y8, Msc3 and control strain Fermichamp) performed well under the four stresses tested and had the lowest interquartile range (IQR) and highest minimal values, corresponding to the more robust (fitter) growth of these strains; yeasts in group 2 (including yeasts Sc3Y5, 3D5 and 3D6) were similar to the former but had a lower

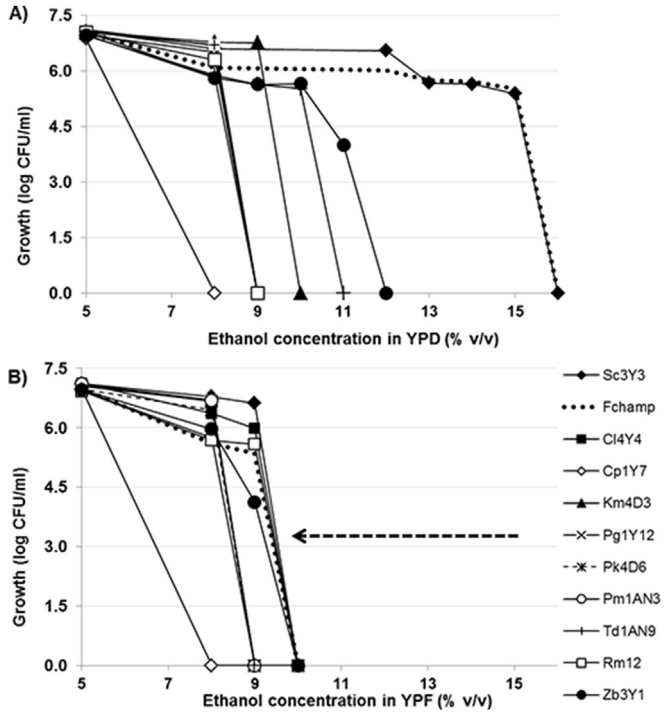

Fig. 4. Tolerance to increasing ethanol concentrations displayed by representative strains of each of the species found in mezcal growing in the presence of A) glucose, or B) fructose, both at a concentration of 20 g/l. There was a drop in resistance (emphasized by the dotted arrow) for all species when ethanol was above 8% v/v and fructose was the carbon source.

tolerance to high concentration fructose; group 3 yeasts (Sc3D3, 3D2 and 4Y3) were tolerant to ethanol in the presence of glucose or fructose but not to ethanol without any hexose, and they had a low tolerance to high concentration fructose; and finally, as an individual case, strain Sc3D4 was tolerant to ethanol with or without the presence of any hexose, showing a high median growth value of 6.36, similar to group 1, but was very sensitive to high concentration fructose. All this indicated a high phenotypic diversity of the *S. cerevisiae* mezcal strains in terms of tolerance to ethanol and fructose.

4. Discussion

Determination of the levels of tolerance to stress are usually performed under liquid culture, which requires the use of agitation devices, transfer of volume for absorbance quantification and control of water loss in the case of microplate readers. As all this is avoided in agar plates, it constitutes an invaluable tool for screening of yeasts and determination of their limit tolerances to stress as far as the media utilised can be contained in the agar matrix.

4.1. Suitability of the drop plate method for stress tolerance analysis

With respect to the drop-plate technique for quantifying the growth of a yeast culture, Supanwong and Pichai (1995) in a short report showed their use for S. *cerevisiae* TISTR 5168 and the reliability of spotting several dilutions on the same plate, although no details were given concerning the volume of the cell solutions used for the drops, nor different media were tested. In this aspect, the method presented here and experimental setup proposed allowed us the quantitative analysis of our strains with capability to be performed in both, a high number of replicates and/or of strains in a short time (overnight for the less stressful conditions), and it can be easily adapted in any lab with a minimum of microbiological equipment.

4.2. Tolerance of yeasts to high concentration fructose

The upper limit of growth at fructose concentrations above 750 g/l observed in this work for two of the *S. cerevisiae* yeasts (3Y4

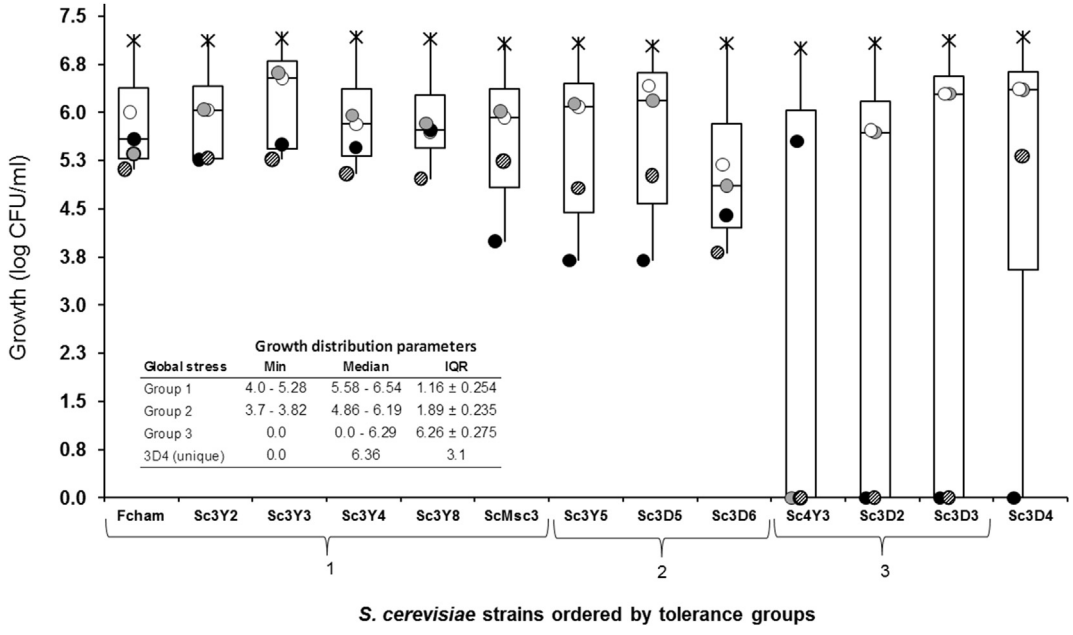

Fig. 5. Global box plot type analysis of the tolerance of *Saccharomyces cerevisiae* mezcal strains comparing the average growth attained on solid media under the following conditions: normal YPD (black stars), YPD plus 12% ethanol (white dots), YPF plus 9% ethanol (grey dots), YP plus 8% ethanol (striped dots) and YP plus 500 g/l fructose without ethanol (black dots). Values describing the distribution of each tolerance group and the unique case are presented in the upper right side of the figure. All the experiments were performed at least three times, average values are presented and the standard deviation was always less than 10%.

and 3Y8) is higher than the tolerance to fructose in liquid medium reported by Arroyo-Lopez et al. (2009) who observed a maximum of 640 g/l for *S. cerevisiae*, which could be explained by the different types of media used by these authors and in the present work. For the non-*Saccharomyces* yeasts, specifically for *P. guilliermondii* and *Zygosaccharomyces baillii* isolated from Spanish candied fruit factories, Martorell, Stratford, Steels, Fernandez-Espinar, and Querol (2007) found that the maximum glucose tolerance, also tested in a YPD-based medium, was 576 g/l (3.2 M) for the former and around 727 g/l (4.04 M) for the latter. These values are lower than the ones obtained in this work using fructose instead of glucose, with tolerance to fructose of 800 g/l (4.44 M) for the two strains of these species tested in this work (Pg1Y12 and Zb3Y1) as well as for the two non-*Saccharomyces* yeasts Cl4Y4 and Pm1AN3 belonging to the genera *Clavispora* and *Pichia*, respectively.

It is worth noting that information concerning yeast responses to osmotic stress has been obtained by using potassium chloride (Carrasco et al., 2001; Zuzuarregui & del Olmo, 2004), sorbitol (Wimalasena et al., 2014) and glucose (Martorell et al., 2007; Tofalo et al., 2009), but the effect of the stress caused by fructose itself, which can indeed drastically decrease the ethanol tolerance not only for *S. cerevisiae* but for all of the species studied here, is seldom analysed, except for the work of Arroyo-Lopez et al. (2009), and the modelling work of Zinnai, Venturi, Sanmartin, Quartacci, and Andrich (2013). The latter researchers found no difference in the rate of consumption of these hexoses below 50 g/l ethanol, but above this concentration glucose consumption increases, and by means of a mathematical model the authors suggest that the limiting step may occur either at the active transport of fructose through the membrane or at the isomerization of fructose to glucose.

4.3. Tolerance of yeasts to high concentration of ethanol

Concerning ethanol, the level of tolerance on YPD of half of the *S. cerevisiae* strains coincides with that reported by Carrasco et al. (2001) and also by Páez et al. (2011) for some of their *S. cerevisiae* strains isolated from agave musts, with 15% v/v ethanol being the upper limit in this work. The response to ethanol in the absence of any sugar was determined (YP−EtOH), where a maximum tolerance of 9% v/v ethanol was obtained, which is in line with a report on other strains with low, moderate and high ethanol tolerances ranging between 5 and 14% (Ding et al. 2009; Tofalo et al., 2009). All the non-*Saccharomyces* strains tested in this work were unable to grow on YPD plates containing more than 10% ethanol except for *Z. bailii* 3Y1, as also was observed in the work of Santos et al. (2008), who suggested that the more ethanol affects the hexose transport system, the more residual fructose was present in the stuck-wine (fructose) medium, but this was also dependant on the specific strain and its growth stage.

None of the yeasts tested here were able to grow on either YPF−EtOH plates or YP−EtOH plates containing above 9% ethanol, even when the sample was spotted directly without dilution, which indicated that the presence of fructose did not improve the survival rate of any of the yeasts in the presence of ethanol but rather had an inhibitory or disruptive effect, as evidenced by the abrupt shift in tolerance from 15 to 9% v/v ethanol (Fig. 4B). This effect of decreasing viability and increasing mortality rate caused solely by fructose has only been reported as far as we know by Semchyshyn, Lozinska, Miedzobrodzki, and Lushchak (2011) for *S. cerevisiae* at a much lower concentration (4%), and it could at least partially explain the shift in viability observed in this work. Interestingly, in experiments using equimolar concentrations of glucose and fructose we observed that the presence of glucose partially counteracted the negative effect of fructose and provided to some of the strains (*Saccharomyces* and some non-*Saccharomyces*) a further increase in ethanol tolerance of at least 2% v/v ethanol more in comparison to growth values attained using only fructose, thus evidencing some competition-type behaviour of these sugars, probably at their initial uptake in the membrane, as suggested by Zinnai et al. (2013), or a conformational change of ethanol in the hexoses mixture, which could be more toxic and/or less prone to be transported. This however requires further experimental confirmation. From the genetic expression point of view, Piper et al. (1994) have long demonstrated that at the membrane level both temperature and ethanol stress responses have similar patterns of Hsps (Heat-shock proteins), which are induced (Hsp104, Hsp70 and Hsp26) at ethanol levels above 4% and up to 10%. Notably, Hsp140 and Hsp70 induction is equally strong when either heat-shock (39 °C for 40 min) or 8% ethanol exposure is tested, as is the rapid loss of plasma-membrane ATPase probably in a cellular attempt to maintain homoeostasis by extruding the protons that passively enter the yeast when the membrane is affected by such stresses.

We globally analyse the performance of the *S. cerevisiae* mezcal strains. It was observed that they could be classified into three main groups, wherein the first one includes the commercial strain Fermichamp, which is currently used to reactivate stuck fermentations given its fructophilic nature. We therefore anticipated that the strains in this group would be the ones with the highest ethanol productivity; we found as a general trend that the maximal ethanol yield of the strains in a synthetic agave-like medium where fructose/glucose ratio is 9:1, (Oliva-Hernández et al., 2013) was the highest for strains in group 1 ($Y_{etOH/S} = 0.43 \pm 0.05$), except for strain 3Y2, which had a low ethanol yield ($Y_{etOH/S} = 0.28$) but was included in the high stress tolerance group 1 (Fig. 5). Overall, the global analysis revealed the high tolerance of this species to osmotic and ethanol stresses, and the robustness of its ethanol production, as observed by Mendes et al. (2013) using a polyphasic approach to classifying *S. cerevisiae*, which included and their resistance to ethanol (10 and 14% v/v) in liquid medium. These authors concluded that phenotypic groups were formed according to the technological use of the strains (domestication), and this was independent of their geographic origin. Our results are in agreement with this observation and we were also able to assess the high phenotypic (stress tolerance) yeast variability of this fermentation system.

5. Conclusions

We found that fructose causes a dramatic decrease in the tolerance level and survival rate of all the yeasts tested, *Saccharomyces* and non-*Saccharomyces*, a finding that to our knowledge has not been reported before, as usually tolerance/resistance determinations in the literature are carried out using solely glucose as the carbon source. Accordingly, there is a high risk of overestimation of ethanol tolerance of a given strain when tested in the laboratory using only glucose, when it is intended for performing fermentation in fructose-rich substrates. The methodology and analysis procedure proposed here for stress tolerance quantification applied to several genera of yeasts is proposed for screening and selection of those strains with a high performance on fermentation processes, but also could be used as a tool for isolation of those variants/segregants of a yeast/bacteria population with a more resistant phenotype, allowing a direct calculation of the ratio of this subpopulation from the original sample.

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