Manuscript Details

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Title	FERMENTATIVE BEHAVIOUR AND COMPETITION CAPACITY OF CRYOTOLERANT Saccharomyces SPECIES IN DIFFERENT NITROGEN CONDITIONS
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Abstract

The selection of yeasts with low nitrogen requirement is a current need in winemaking. In this work, we analysed nitrogen requirements of strains belonging to the cryotolerant species S. uvarum, S. eubayanus and S. kudriavzevii, in order to evaluate their potential for conducting the fermentation of low nitrogen content grape musts. Our result demonstrated that S. eubayanus is the species less influenced by the increasing nitrogen concentrations in both growth and fermentation conditions. Strains showing the best behaviours, S. eubayanus NPCC 1285 and S. uvarum NPCC 1317, were selected to be tested in mixed cultures with S. cerevisiae T73 at different temperatures (12ºC, 20ºC and 28ºC) in synthetic grape must with different nitrogen concentrations (60, 140 and 300 mg/L YAN). The cryotolerant strains dominated the fermentations carried out at 12ºC while S. cerevisiae prevailed at 28ºC independently from the nitrogen concentration. At intermediate temperature, 20ºC, S. eubayanus mono and mixed cultures showed the best fermentative behaviour especially with low and intermediate nitrogen concentration. In summary, cryotolerant Saccharomyces species, particularly S. eubayanus, could be interesting tools to avoid fermentations stucks caused by low nitrogen content in grape musts.

Keywords	Saccharomyces eubayanus; Saccharomyces uvarum; Saccharomyces kudriavzevii; nitrogen; wine.
Taxonomy	Yeasts in Food Fermentation, Saccharomyces Sensu Stricto, Beverage Fermentation

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Dear editor,

Herewith we send you a new revised version of our manuscript entitled “Fermentative Behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions” (FOOD_2018_89).

In this new version, we have introduced most of the changes suggested by the reviewers and we have rewritten and edited some sections of the article to improve clarity. For clarity, these changes have been highlighted in yellow in the text. We want to thank the detailed comments provided by the reviewers. These were very helpful for the improvement of our manuscript. We also enclose with this revised version a rebuttal letter answering the concerns raised by the reviewers.

I am looking forward to receiving your comments about the manuscript and, hopefully, this new version is acceptable for the journal.

Sincerely,

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RESPONES TO THE REVIEWERS

-Reviewer 1

In the paper FOOD_2018_89, titled "Fermentative Behaviour And Competition Capacity Of Cryotolerant *Saccharomyces* Species In Different Nitrogen Conditions" the authors evaluated the growth of Saccharomyces uvarum, S. eubayanus and S. kudriavzevii at different nitrogen concentrations and performed mixed fermentations with S. cerevisiae to assess the effect of nitrogen and temperature on fermentative performances and yeast dominance.

The topic is of particular interest as the possibility to carry out industrial fermentation at very low temperature with minimal nitrogen supplementation is indeed a common request by winemakers. I found the paper interesting, however some issues raised regarding some experiments and results obtained:

In the material and methods section the authors used a quite unconventional system to allow escape of CO2 bubbles from the test tubes : "The lids of the tubes were half open in order to allow CO2 release" (lines 145-146). However, the authors are giving appropriate controls (lines 150-151: "Three tubes filled with 10 mL SM without inoculation are used as triplicate controls for the evaporation weight loss (EWL)"). Did the authors observe any growth form contamination in these control tubes?

Sorry for the misleading phrasing. Actually, we used regular lab tubes with screw caps, and these caps were not completely tightened in order to let CO_2 release. The tubes were still fully covered by the cap, therefore, there won't be any contamination problem. The controls were used to estimate the evaporation of the fermentation system in order to compare with the weight loss caused by CO_2 release. The phrase has been modified in the manuscript (lines 163-164).

The author should, in my opinion, report the maximum cell population (should be D in the revised Gompertz equation) along with the duration of the lag phase (lambda) and the specific growth rate. Indeed the maximum cell population parameter is necessary to better evaluate the ability of the strain to effectively grow on the medium (and to utilize nitrogen). Indeed growth rate estimates the time required to cell to duplicate, not how many times the cells replicate. Thus, in the selection of strains for the subsequent analyses, the maximum cell population should be included.

This is a very good point. We have also considered this, however, as reviewer 2 mentioned, the plate screening method is limited by OD saturation. OD value can only be measured accurately up to around 0.5. Since dilution is not applicable with the plate, OD values beyond 0.5 is only estimation. Therefore, the maximum cell population, although is also an important factor for strain evaluation, is not applied in this stage. Since all the strains reached OD saturation, maximum growth rate, which represent the speed to reach OD saturation, is an efficient parameter to evaluate the growth capacity.

However, we agree with the reviewer that lag phase duration is a good parameter to complement the maximum growth rate data. We have introduced a new supplementary table (Supplementary Material Table S2) with all the data of the lag phase time (λ) for all the strains, following the recommendation of the reviewer.

-Reviewer 2

Manuscript FOOD-2018-89 reports on the fermentation behaviour of single and mixed cultures of Saccharomyces species under winemaking conditions in response to a combination of various nitrogen concentrations and different fermentation temperatures. The impact of these parameters has been studied previously, but in isolation and not in the combination investigated in this study. Although most of the results were intuitively expectable, it is assumed that the novelty of this study lies in the combination of the different parameters.

The manuscript is rather well written, expect for a few issues mentioned in the minor comments below. I just have three general comments/requests:

(1) In the introduction, please clarify the connection between cryotolerance and nitrogen requirement.

Sentences describing the connection between low temperature and nitrogen requirement have be introduced in line 65-73.

(2) In all the graphs (not only in the supplementary tables), please indicate the statistical significance more clearly.

We now show the statistical significance among the different strains and conditions in figures 4, 6 and S1 by letters on top of the bars. We also show the statistical differences of figures 1 and 2 in supplementary tables 1 and 3 because the representation of this significance by letters in the figures lead to confusion among the different conditions (too many letters). Finally, we consider that statistical analysis has little meaning for figures 3 and 5, representing density reduction and percentages of *S. cerevisiae* in mixed culture fermentations.

(3) Which range of OD was considered by the authors during the microtitre plate assay? Since dilutions were not feasible, only OD values up to 0.5 are theoretically accurate. Anything beyond this value is at best an estimation, with the error increasing pregressively as the yeasts grew. In this context, only the lag phase duration and the early growth rate (beginning of exponential phase) could be measured accurately. How did the authors calculate the maximum specific growth rate accurately? Could this issue not explain some unexpected results such as the absence of correlation between growth performance and fermentation performance for some strains?

We considered all the measurements during the exponential growth phase. We agree with the reviewer that, in the microtiter plate assay, the non-linear response of OD measurement at high cell concentration is an issue. Thus, we do not consider the maximum OD as an accurate parameter. Moreover, not in this study, but in previous works, we also compared growth data obtained from microtiter plates and from falcon tubes (50 ml), in which dilutions can be done, and the calculated growth parameters did not differ too much in both systems. Thus, we trust that the data obtained are quite accurate.

It is not the first time that we (Gutiérrez et al. 2012) and other authors (Kemsawasd et al. 2015) have observed a lack of positive correlation between growth and fermentation performance (more explained now at the beginning of Discussion). As mentioned above, we have confidence in the accuracy of the data and we do not think that the lack of correlation is related with poor quality of the data obtained by the microtiter plate assay.

Minor comments:

Line 52: add "the" before "wine industry"

Modified

Line 55: "grapes are often overripe": the overall problem is a bit more complex than that. If climate change only resulted in grapes being overripe, it would be easy to solve the problem by harvesting earlier. Please rephrase.

We agree. This statement has been replace by a more detailed description of the climate change problems (lines 61-65)

Line 61: "have" instead of "has"; "often causes the main fermentative problems": please clarify

All this paragraph have been rephrased to highlight the connection between low temperature and nitrogen shortage stresses (lines 65-73)

Line 63: "have been done": colloquial, please rephrase

Modified

Line 100 and throughout the manuscript: "for comparative purposes" instead of "with comparative purpose"

Modified

Line 101: "medium" instead of "media"

Modified

Line 113: "as in the natural grape must": this statement implies that the nitrogen composition of grape must is always the same. Please rephrase.

It was badly expressed. We meant that the average percentage of inorganic (ammonium) and organic (amino acids) nitrogen in natural grape musts is approximately 40:60 respectively. We have now rephrased to make it clearer .

Line 124: Please specify the number of repeats in this paragraph.

The experiment was carried out in triplicate and it is now mentioned in line 152.

Line 128: litre is abbreviated as both l and L in the manuscript. Please standardise throughout.

Modified

Line 129: how was the cell count determined?

We indirectly determined the number of cells by OD measurement. Previously, we checked the correspondence between number of cells and OD value, by using a Neubauer chamber for cell counting. For all the species used in our study, OD_{600nm} = 0.1 approximately equals to 10^6 cells/ml. This has been stated in the manuscript (Line 134-137).

Line 130: "incubated" instead of "cultivated"?

Modified

Line 135: "carried out" instead of "done"

Modified

Line 139: I do not understand how this equation was used in the manuscript. Please clarify.

The equation is applied by using R code. It has been now clarified in the text (lines 160-161).

Line 142: unit of the lag phase duration?

Modified (h)

Line 146: how was sterility ensured if the flasks were half opened?

The sentence was definitely not very suitable because it provoked misunderstanding in R1 and R2. What we were trying to say was that the screw caps of the fermentation tubes were not completely tightened in order to let CO_2 release. Therefore, the tubes were actually fully covered by the caps. It has been rephrased in the text (lines 164-165).

Line 146: "synthetic must be modified": this sentence makes not sense, please rephrase

Modified

Line 210: Were the results obtained in the microtiter plate assay confirmed at a larger scale? At least some of them to confirm accuracy?

We did not confirm in this work but we did previously it in other studies of the group (Gutierrez et al. 2013; 2016). Growth in microtiter plates was compared with falcon tubes of 50 ml and, in general, the correlation was quite good, with an average measurement error (coefficient of variation) lower than 10 %.

Line 219 and throughout manuscript: “after fitting growth curves”: I do not understand what the authors mean. Please rephrase.

This sentence has been replaced by “after calculating by Gompertz equation”

Lines 233-236: Was the deviations from the average value calculated per nitrogen concentration? Otherwise it is surprising not to see a clearer link between nitrogen supply and maximum biomass. Please clarify.

Yes, the average value was obtained for each nitrogen concentration. It was stated in line 246-247.

Line 257: I do not understand this sentence, please rephrase

We have now changed to the next sentence “Interestingly, contrary to the expected, the strains selected for their high or low nitrogen requirements showed similar fermentative behaviour in each nitrogen concentration” (lines 282-283.

Line 269: Unclear, please rephrase

We agree it was confusing. We have rephrased all the paragraph, trying to make it clearer.

Line 270: “uncorrelated”?

Modified. Line 293-298.

Lines 281-285: Why selecting 2 strains from the same group (Figure 1) and not 1 strain from each of the 2 groups for better comparison of distinct behaviours?

Our work is considering a future application in the industry. Therefore, we have selected strains with favourable characters for wine fermentation, which are the strains with relatively low nitrogen requirements in the same group. It has been clarified in the manuscript (line 302.

Line 306 and throughout manuscript: “imposition” cannot be used in this context. “dominance” rather?

Modified

Line 324: “cerevisiae” instead of “cerevsiae”

Modified

Line 325: “under this condition” instead of “with this condition”; “were” instead of “are”

Modified

Line 336: “had” instead of “have”

Modified

Line 438: “their”

Modified

-Reviewer 3

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In the paper entitled "Fermentative behavior and competition capacity of cryotolerant Saccharomyces species in different nitrogen conditions" (FOOD-2018-89), the authors investigated the growth and fermentative performances during wine fermentation of non-*cerevisiae* yeast strains from various habitats, as well as their capacity to compete with S. cerevisiae in mixed cultures. This study that addresses an issue of great interest for the winemaking sector, has been well conducted scientifically, with an appropriate experimental design and adequate methodologies. However, some issues regarding the setting-up and the presentation of the data must be addressed before this manuscript deserves to be published in International Journal of Food Microbiology.

Major comments:

My major concern relates to the coherence and linkage between the heat-map and the values of the maximum growth rate reported in Supp. Mat. Table1 for the strains T73, CR85 and CR90. Taking into account the values reported in the table, the boxes corresponding to these strains should be colored in red (higher than the average for each nitrogen concentration) while they appeared in green in the heat map. The authors must also confirm the accuracy of and, if necessary, change all the comments related to these data (for example lines 389-395).

Thanks a lot for pointing out the mistake. We have carefully checked the result, and it was caused by a mistake during normalization. We have changed the data and modified the heat map and related comments (lines 246-262).

Another point that, to my mind, is important to mention regarding the scientific issues addressed in this paper, concerns the relationships between the genetic background and the phylogeny of the species and strains and their phenotypes. This point should be discussed in the first paragraph of the discussion.

The relationship between the genetic background, the phylogeny of S. uvarum strains and their phenotype has been briefly discussed in line 420-429.

Minor comments:

- Line 43-44. Yeasts can also metabolise small peptides. This information should be included in the text.

We have deeply modified this paragraph for a better comprehension, mentioning other nitrogen sources that can be metabolised by yeasts (lines 45-51).

- Line 89 -91. The major aim of the study (combined analysis of the incidence of temperature and nitrogen availability on the fermentation performances of cryotolerant Saccharomyces species) should be more clearly specified in this paragraph.

We have now tried to clearly highlight the aim of the study, following the recommendation of the reviewer (lines 103-109). We have also explained the connection between both stresses during wine fermentation (lines 67-73).

- Line 147-151. The frequency of the weights should be precised.

We have now mentioned in Mat & Met (line 171). The frequency of the weights was approximately every 12 hours

- Line 212-241. Please, include the temperature used to carry out the experiments.

Done. They were performed at 25 ºC.

- Line 306 and further. change 'imposition' by 'implantation'.

Modified

- Line 363-364. More details should be added on the previous work : yeast species used, how many strains included in the studies, …

We have given more details about these previous works, which are somehow related with the present study (lines 384-395)

- Line 414-418. This sentence is not clear. Please, reformulate.

The sentence was clearly confusing. It now is as follows: "The competition ability of several S. cerevisiae strains under different nitrogen concentration has been evaluated (García-Ríos et al., 2014; Lemos Junior et al., 2017; Vendramini et al., 2017); however, little is known about the competition ability between S. cerevisiae versus other species from the Saccharomyces genus" (lines 451-455).

Highlights

Increasing nitrogen concentration has little influence on *S. eubayanus* growth.

A relationship between origin and nitrogen requirement is observed for *S. uvarum* strains.

Nitrogen content in must is involved in the competition capacity of *S. eubayanus* and *S. uvarum*.

Cryotolerant *Saccharomyces* species could be used in nitrogen-limited grape must fermentations.

FERMENTATIVE BEHAVIOUR AND COMPETITION CAPACITY OF CRYOTOLERANT *Saccharomyces* SPECIES IN DIFFERENT NITROGEN CONDITIONS

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ABSTRACT

The selection of yeasts with low nitrogen requirement is a current need in winemaking. In this work, we analysed nitrogen requirements of strains belonging to the cryotolerant species *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*, in order to evaluate their potential for conducting the fermentation of low nitrogen content grape musts. Our result demonstrated that *S. eubayanus* is the species less influenced by the increasing nitrogen concentrations in both growth and fermentation conditions. Strains showing the best behaviours, *S. eubayanus* NPCC 1285 and *S. uvarum* NPCC 1317, were selected to be tested in mixed cultures with *S. cerevisiae* T73 at different temperatures (12ºC, 20ºC and 28ºC) in synthetic grape must with different nitrogen concentrations (60, 140 and 300 mg/L YAN). The cryotolerant strains dominated the fermentations carried out at 12ºC while *S. cerevisiae* prevailed at 28ºC independently from the nitrogen concentration. At intermediate temperature, 20ºC, *S. eubayanus* mono and mixed cultures showed the best fermentative behaviour especially with low and intermediate nitrogen concentration. In summary, cryotolerant *Saccharomyces* species, particularly *S. eubayanus*, could be interesting tools to avoid fermentations stucks caused by low nitrogen content in grape musts.

Keywords: *Saccharomyces eubayanus*, *Saccharomyces uvarum*, *Saccharomyces kudriavzevii*, nitrogen, wine.

1. Introduction

Nitrogen is a key nutrient during wine fermentation, affecting both fermentation kinetics and the formation of wine aroma. It is the major limiting nutrient for growth under oenological conditions. Although alternative nitrogen sources, such as oligopeptides, amides, biogenic amines and nucleic acids, can be found and might constitute a substantial nitrogen resource in grape juice (Ough et al., 1991; Henschke and Jiranek, 1993; Perry et al., 1994; Marsit et al., 2015), yeast assimilable nitrogen (YAN) is mainly composed of ammonium and amino acids (Henschke and Jiranek, 1993). Different factors including grape variety, geographical origin, climate conditions and some technological processes affect the YAN content in musts and thus the fermentation kinetics (Butzke, 1998; Dubois et al., 1996; Nicolini et al., 2004). Previous works have determined that, in general, a minimum of 140 mg/L of YAN is required for yeast to complete alcoholic fermentation (Bell and Henschke, 2005; Bely et al., 1990; Butzke, 1998). Nevertheless, it strongly depends on the yeast species developing during the fermentation process.

Saccharomyces cerevisiae is the main yeast species used in the wine industry. Its favoured characteristics such as high fermentation efficiency, high ethanol tolerance and consistency of wine quality help *S. cerevisiae* to maintain its dominant position (Mas et al., 2016). However, the context of the worldwide Oenology has altered with the climate change, which affects the grape composition and ends up with grape musts with low nitrogen and high sugar concentrations (van Leeuwen et al., 2016). This situation gives the wine industry a big challenge to meet consumers' preference for wines with lower alcohol and fruitier aromas. One of the oenological practices applied by winemakers is to use lower fermentation temperatures, as far as 10–12°C to preserve aroma compounds in wines

(Beltran et al., 2006; Alonso del Real et al., 2017). However, low-temperature fermentation produced similar metabolic and transcriptional effects to those obtained in nitrogen-limited fermentations (Beltran et al., 2006; Pizarro et al., 2008). Both conditions decreased biomass yield and fermentation rate during wine fermentation. This is not unexpected, because low temperature produces a rigidification of the plasma membrane that impairs the activity of some permeases, reducing the transport of nutrients (Beltran et al., 2006). Thus, low-temperature influences the quantity and the quality of yeast nitrogen requirements. In summary, the lack of nitrogen, the high sugar concentration, and the low fermentation temperature have made a very complicated harsh condition for yeasts and often causes the main fermentative problems.

In this context, strains belonging to cryotolerant *Saccharomyces* species, showing good adaptation at low temperature, lower nitrogen requirements and lower ethanol yields, may play an important role in wine fermentations. *Saccharomyces uvarum*, *Saccharomyces kudriavzevii* and *Saccharomyces eubayanus* have been identified as natural cryotolerant species in the genus. In particular, *S. uvarum* has been isolated from both natural habitats (Almeida et al., 2014; Bing et al., 2014; Gayevskiy and Goddard, 2016; Rodríguez et al., 2014) and fermentation environments including wines, ciders and apple chichas (for a review see Rodríguez et al., 2016). The fermentation profile of *S. uvarum* is different from that shown by *S. cerevisiae*. This species has a shorter lag phase than *S. cerevisiae* at low fermentation temperature (around 13°C). Comparing with *S. cerevisiae*, it also produces higher amounts of glycerol and lower amounts of ethanol (Castellari et al., 1994; Masneuf et at., 2010), and it generates a differential aromatic profile, particularly characterised by a higher production of 2-phenylethanol which gives a very pleasant rose-like floral odour

(Bertolini et al., 1996; Masneuf et al., 2010; Origone et al., 2018). These different traits support the great potential of *S. uvarum* being widely used in wine industry at low temperature fermentation. The other two cryotolerant species, *S. kudriavzevii* and *S. eubayanus*, have only been found in natural habitats, although it has been demonstrated that they contribute to ferment beverages through their presence as part of the genome of chimeric strains with other *Saccharomyces* species, predominantly *S. cerevisiae* (González et al., 2006; González et al., 2007; Libkind et al., 2011).

Many studies have been carried out to explore the nitrogen demanding character of *S. cerevisiae* both phenotypically and genotypically (García-Ríos et al., 2014; Gutiérrez et al., 2012, 2013, 2016). However, there are few studies about the nitrogen requirement of the cryotolerant species of the genus. It has only been described that *S. uvarum* strains have lower nitrogen requirement than *S. cerevisiae* (Masneuf et al., 2010), which suggests the putative competitiveness of this species during specific wine fermentations.

In our study, we phenotypically analysed the combined effect of temperature and nitrogen availability on the fermentation behaviour of cryotolerant yeast strains. Firstly, Nitrogen requirements of a set of *S. uvarum* and *S. eubayanus* strains recently isolated from Patagonia as well as *S. kudriavzevii* strains from natural habitats in Spain were studied. Secondly, Competition assays have also been evaluated by selected cryotolerant strains against *S. cerevisiae* in different conditions of temperature and nitrogen content in order to find out the optimal condition for the better implantation of these cryotolerant species.

2. Materials and methods

2.1. Strains and media used in the study

112 Thirty-two strains belonging to *S. uvarum*, *S. eubayanus* and *S. kudriavzevii* species,
113 isolated from natural (Lopes et al., 2010; Rodríguez et al., 2014) and fermentative
114 (Rodríguez et al., 2017) environments were used in this work. *S. uvarum* BMV58 and
115 CECT12600 and *S. cerevisiae* T73 were also used for comparative purpose.
116 Yeast extract peptone dextrose (YPD) medium (2% glucose, 2% peptone, 1% yeast extract)
117 was used for yeast propagation.
118 A synthetic grape must (SM), similar to natural grape must, but with a defined composition,
119 was used in our study to determine yeast growth and fermentation characteristics. The SM
120 was prepared as described by Riou et al. (1997), but with some modifications. The SM
121 contains 200 g/L reducing sugar (100 g/L glucose and 100 g/L fructose), malic acid 5 g/L,
122 citric acid 0.5 g/L and tartaric acid 3 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4$ 0.25
123 g/L, $CaCl_2$ 0.16 g/L, NaCl 0.2 g/L, trace elements ($MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$
124 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH_4)_6Mo_7O_{24}$ 1 mg/L), vitamins
125 (myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid 2 mg/L, thiamine
126 hydrochloride 0.25 mg/L, pyridoxine hydrochloride 0.25 mg/L and biotine 0.003 mg/L).
127 The composition of nitrogen source in the SM is 40% ammonium and 60% amino acids, as
128 described in Beltran et al. (2004). The composition of amino acids in 1 liter amino acid
129 stock is L-tyrosine 1.5 g, L-tryptophan 13.4 g, L-isoleucine 2.5 g, aspartic acid 3.4 g,
130 glutamic acid 9.2 g, L-arginine 28.3 g, L-leucine 3.7 g, L-threonine 5.8 g, glycine 1.4 g, L-
131 glutamine 38.4 g, L-alanine 11.2 g, L-valine 3.4 g, L-methionine 2.4 g, L-phenylalanine 2.9
132 g, L-serine 6 g, L-histidine 2.6 g, L-lysine 1.3 g, L-cysteine 1.5 g and L-proline 46.1 g,
133 which corresponded to 13.75 g/L assimilable nitrogen. The final pH of the SM was

adjusted to 3.3 with sodium hydroxide. The SM was sterilised by filtration through 0.22 µm pore size membrane filter (Thermo scientific).

Fermentation medium was inoculated with a yeast overnight preculture to reach an OD_{600nm} of approximately 0.1. Preliminary experiments have been carried out to relate cell counts, by using Neubauer chamber (Brand GMBH, Germany), with OD value. The result showed that, for the different species used in our study, OD_{600nm} = 0.1 approximately equals to $1x10^6$ cells/mL (data not shown).

2.2. Strain screening based on nitrogen requirement

2.2.1. Growth character screening

S. uvarum, *S. eubayanus* and *S. kudriavzevii* strains were screened by both growth and fermentation character under different nitrogen concentrations. The growth character was determined by microtiter plate screening method. 96 well microtiter plates were used. Each well was filled with 250 µL of synthetic must of different nitrogen concentrations and with 10^6 yeast cells/mL. Growth curves were monitored by recording the increase of optical density (OD) at wavelength 600 nm. The microtiter plates were incubated in SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany) at 25°C with 500 rpm orbital shaking. The optical density of each well was measured every 30 minutes until the growth reached the stationary phase. The growth characters of 32 strains were screened under 12 nitrogen concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 300 mg/L YAN). All the nitrogen conditions were assayed by triplicate. The growth character of each strain under different nitrogen condition can be calculated by directly fitting OD measurements versus time to the Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

157 $$y = D * exp\ \{-exp[((\mu_{max} * e)/D) * (\lambda - t) + 1]\}$$

158 Where y= ln (OD_t/OD_0), OD_0 is the initial OD and OD_t is the OD at time t; D = ln

159 (OD_∞/OD_0) is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the

160 maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). R code (statistical

161 software R, v.3.0) was used to facilitate calculating μ_{max} and λ with Gompertz equation.

162 ***2.2.2. Fermentation character screening***

163 The fermentation character was screened by microscale fermentation method. The

164 fermentations were carried out in 15 mL tubes with 10 mL synthetic must. The screw caps

165 of the tubes were not completely tightened in order to allow CO_2 release. Synthetic must

166 was modified with three different nitrogen concentrations which were 60, 140 and 300

167 mg/L YAN. These values correspond to nitrogen limited, standard and excessive conditions

168 (Bely et al., 1990). The fermentations were followed by CO_2 production which can be

169 represented by the weight loss of the fermentation tubes. Three tubes filled with 10 mL SM

170 without inoculation are used as triplicate controls for the evaporation weight loss (EWL).

171 The fermentation tubes were weighted around every 12 hours. The weight loss (WL) can be

172 simply calculated as: $WL = W_0 - W_t - EWL$. The fermentations were considered as finished

173 when WL stops to increase. Lag phase time (λ) and maximum fermentation rate (V_{max})

174 were calculated by applying the weight loss data into Gompertz model as described in

175 2.2.1.

176 **2.3. Competition fermentation**

177 In order to determine the influence of nitrogen concentration and fermentation temperature

178 on the yeast population in a mixed culture, competition fermentation was carried out

179 between *S. cerevisiae* strain T73 and one of the non-*cerevisiae* strains. The strains used for

fermentation were first propagated in liquid YPD media overnight and then transferred to SD media (2% glucose, 0.017% yeast nitrogen base) with 230.8 mg/L NH_4Cl (corresponding to 60 mg/L YAN) in order to eliminate the influence of YPD nitrogen-rich media.

Fermentations were carried out in 100 mL bottles with 80 mL synthetic must of three nitrogen concentrations (60, 140 and 300 mg/L YAN). All the fermentations were inoculated with 2 $x10^6$ cells/mL. In the case of mix-culture fermentations, $1x10^6$ cells/mL of each strain was inoculated. In order to observe the influence of temperature on competition capacity of each strain, the fermentations were carried out at three temperatures: 12, 20 and 28ºC, representing low, medium and high temperature respectively. The fermentation process is monitored by measuring the density of the media (g/L), after a slight centrifugation at 5.000 rpm for 5 min for cell removal and using a portable densitometer (Mettler Toledo, USA). The fermentation was considered as finished when the density reaches 0.998 g/L (Gutiérrez et al., 2012). Mono-culture fermentations were also carried out as references.

2.4. Strain population dynamics in competition fermentation

As *S. uvarum* and *S. eubayanus* are less heat tolerant than *S. cerevisiae*, we confirmed the incapability of *S. uvarum* and *S. eubayanus* to grow on at 37°C, whereas they perfectly grow at 30°C in YPD (data not shown). On the other hand, the colony forming ability of *S. cerevisiae* strains is the same at 30°C and 37°C. The percentage of strains belonging to *S. cerevisiae* or to non-*cerevisiae* can be simply determined by plating the sample on two YPD plates and incubated at 30ºC and 37ºC. CFUs were counted after 2 days of incubation. The CFU number on the 37°C plate represents the population of *S. cerevisiae* in the mixed

203 culture and the CFU difference between the two plates represents the population of non-
204 *cerevisiae* strain in the mixed culture.
205 At the end of the competition experiment, fermentation samples were taken and analysed
206 for the main wine chemical parameters including glucose, fructose, ethanol, glycerol and
207 main organic acids. The samples were first centrifuged and diluted 3 times with deionized
208 water, then filtrated through 0.22 mm pore size nylon filters (Micron Analitica, Spain). The
209 analysis was carried out on a UHPLC (Thermo Scientific, ultimate 3000) equipped with a
210 refraction index detector and a UV-visible detector (Thermo Scientific). The mobile phase
211 used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 45°C. The
212 metabolites were separated by a HyperREZ XP Carbohydrate H+ 8 mm column (Thermo
213 Scientific) and their concentration was calculated by using external standards.
214 **2.5. Statistical analysis**
215 All the experiments were carried out at least in triplicate. Physiological data were analysed
216 by the Sigma Plot 13.0 software and the results are expressed as mean and standard
217 deviation. Significance was determined by analysis of variance (ANOVA) using the
218 Statistica, version 7.0, software package. The statistical level of significance was set at a P
219 value of ≤ 0.05 with a Tukey HSD test. Multi-factorial ANOVA based on the percentage of
220 *S. cerevisiae* in the mixed culture fermentation was also carried out (Statistica, version 7.0)
221 in order to analyse the significance of the influences of temperature (12, 20 and 28°C),
222 nitrogen concentration (60, 300 mg/L YAN), and the type of mixed culture (*S. cerevisiae* +
223 *S. eubayanus*, *S. cerevisiae* + *S. uvarum*). The data group of fermentation carried out at
224 20°C with 140 mg/L YAN was not included in the multi-factorial ANOVA, since it is the
225 unique group with 140 mg/L YAN. Phenotypic data were fitted to the modified Gompertz

model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the function in the R statistical software, v.3.0. Hierarchical clustering used in heatmap plot was performed by MeV MultiExperiment Viewer with Euclidean distance metrics and group clustering was based on group averages (average linkage).

3. Results

3.1. Nitrogen requirements of *S. eubayanus*, *S. uvarum* and *S. kudriavzevii* in pure cultures

Nitrogen requirements of a set of 32 non-conventional cryotolerant *Saccharomyces* yeast strains including *S. uvarum* (17 different strains), *S. eubayanus* (13 different strains) and *S. kudriavzevii* (2 different strains) were evaluated in synthetic grape must at 25°C. During the first stage, yeast growth was evaluated by OD increases in microtiter plates containing synthetic must with different total nitrogen concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 300 mg/L YAN). Growth curves were fitted to modified Gompertz equations for all yeast strains and conditions (a total of 1260 growth curves, including the three reference strains). After calculating by Gompertz equation, μ_{max} (maximum growth rate) and λ (lag phase time) values were successfully obtained for all analysed strains and conditions and their absolute values are exhibited in Supplementary Material Tables 1 & 2. Lag phase time was mainly strain-dependent and increasing nitrogen concentration did not show significant differences on λ value of each strain. In order to easily detecting different nitrogen requirement among analysed species and strains, a heatmap analysis was carried out with μ_{max} data (Figure 1). For this analysis, the μ_{max} value obtained for each strain was normalised by mean value of all strains at each particular nitrogen concentration. Values lower and higher than the average are indicated in green and red respectively in the

heatmap (Figure 1). This heatmap is hierarchically clustered into four clusters. The first cluster is made up of the strains with the highest μ_{max}, which mostly belong to *S. eubayanus*, with the exception of two *S. uvarum* strains (NPCC1317 and NPCC1323), which were isolated from apple chicha. The second cluster is formed by a group of strains with higher or similar growth rate than the average value of all strains (red to black colour). These strains were mostly isolated from *A. araucana* tree (Wild), which is a gymnosperm endemic of the lower slopes of the Chilean and Argentinian south-central Andes, typically above 1000 m of altitude (Rodríguez et al, 2014). The third group was represented by strains mostly with lower μ_{max} than the average (black to green colour). This cluster is mainly made up of *S. uvarum* isolated from wild and fermentative environments (chicha and cider) and, curiously, the three wine strains, two *S. uvarum* references BMV58 and CECT12600, and the control *S. cerevisiae* T73, are included in this third cluster, showing similar interspecific growth features. The fourth group includes two strains of *S. kudriavzevii*, showing the lowest growth rate of all the tested strains.

In order to evaluate if the behaviour evidenced in yeast growth is also observed in the fermentation performance of the yeasts, we selected strains showing high, low and intermediate nitrogen requirements to be evaluated in fermentations with low (60 mg/L YAN), high (300 mg/L YAN) and intermediate (140 mg/L YAN) nitrogen concentrations. The strains with high nitrogen requirements were selected among those showing low μ_{max} values at low nitrogen concentrations and high μ_{max} values at high nitrogen concentrations according to the normalised data from Figure 1 (*S. eubayanus* NPCC 1296, 1301 and *S. uvarum* NPCC 1321, 1418). Conversely, the strains with low nitrogen requirements were selected among those showing high μ_{max} values at low nitrogen concentrations and also

high (or average) μ_{max} values at high nitrogen concentrations (*S. eubayanus* NPCC 1282, 1283, 1285 and *S. uvarum* NPCC 1288, 1314, 1317). *S. eubayanus* strain NPCC 1292 and *S. uvarum* strain NPCC 1290, showing intermediate nitrogen requirements, were also evaluated. For comparative purposes, we additionally included the two *S. kudriavzevii* strains CR85 and CR90, the *S. uvarum* reference strains BMV58, CBS 7001 and CECT 12600 and the *S. cerevisiae* reference strain T73.

Weight loss data due to CO_2 release were calculated by Gompertz equation and parameters V_{max} (representing the maximum rate of CO_2 production) and λ_{CO2} (representing the time required to start the fermentation) were obtained for each strain at three nitrogen conditions: 60, 140 and 300 mg/L (Figure 2 and Supplementary Material Table 3). Interestingly, contrary to the expected, the strains selected for their high or low nitrogen requirements showed similar fermentative behaviour in each nitrogen concentration. Generally, the increasing nitrogen concentration enhanced the V_{max}, but with different intensity in the various group of strains. *S. eubayanus* strains were less affected by the increasing nitrogen concentration, showing similar values in V_{max} obtained at both 140 mg/L and 300 mg/L. The V_{max} of *S. uvarum* Patagonian strains (NPCC strains) improved greatly along with increasing nitrogen concentration, especially high value was observed at 300 mg/L. The greatest influence of nitrogen concentration on V_{max} was observed for the wine *S. uvarum* and *S. kudriavzevii* strains, which showed around twice V_{max} values at high nitrogen concentrations compared with that observed at 60 mg/L YAN (Figure 2A). Regarding the time needed to start fermentation (Figure 2B; λ_{CO2}), *S. eubayanus* and *S. uvarum* mostly shortened this lag period as nitrogen concentration increased. Conversely, the two *S. kudriavzevii* strains showed a negative correlation between nitrogen

295 concentration and the time needed for starting fermentation, which showed higher λ_{CO2}
296 values at intermediate and high nitrogen concentrations. Finally, the shortest λ_{CO2} was
297 observed in the wine strains *S. uvarum* BMV58, CECT12600 and *S. cerevisiae* T73,
298 regardless the nitrogen concentration. This is of great significance for the reason that, it
299 evidences that regardless of the species or the nitrogen concentration, the isolating origin of
300 the strains is the most influencing factor for a good adaptation to the growth medium.

301 **3.2. Competition tests *S. cerevisiae* vs. *S. eubayanus*/*S. uvarum***

302 For their industrial application potential, one *S. eubayanus* strain (NPCC 1285) and one *S.*
303 *uvarum* strain (NPCC 1317), showing the best performance at low nitrogen concentrations
304 (especially evident in growth assays) were selected to perform mixed culture fermentation
305 with *S. cerevisiae* reference strain T73, in order to evaluate their competition capacity in
306 synthetic grape musts with different nitrogen concentrations. We evaluated, in the first
307 stage, the competition at both low and high nitrogen content (60 mg/L and 300 mg/L
308 YAN). Moreover, due to the well-known differential temperature preference of the yeasts
309 involved in the competitions, two different fermentation temperatures (28ºC and 12ºC)
310 were evaluated for the mixed cultures. For comparative purposes, monoculture
311 fermentations were also carried out with the three strains involved in the competitions.
312 Our result showed that both fermentation temperature and nitrogen concentrations have
313 significant influences on the fermentation kinetics and on the competitiveness of the strains.
314 At 28°C, most of the fermentations were completed with both nitrogen concentrations (only
315 *S. eubayanus* monoculture was unable to end up fermentation). However, the low YAN
316 concentration yielded sluggish fermentations (Figure 3A-B). Conversely, at 12ºC, the
317 combination low temperature and low YAN resulted in unfinished fermentations for all the

318 tested monocultures or mixed cultures (Figure 3C). At low temperature but high YAN
319 concentration, *S. uvarum* and *S. eubayanus* monocultures showed the highest fermentation
320 activity and the shortest fermentation times (Figure 3D). The higher fermentation activity
321 for *S. cerevisiae* at 28 ºC and for *S. eubayanus*/*S. uvarum* at 12 ºC generally correlated with
322 higher CFU counts (Figure 4). Regardless YAN concentration, the cryotolerant species
323 showed greater final population size at 12 ºC. Conversely, only *S. eubayanus* produced
324 more cells than *S. cerevisiae* at 28ºC and low YAN.
325 In terms of strain composition in mixed culture fermentations, temperature of fermentation
326 determined the competitiveness of the different strains and nitrogen did not have any
327 impact on their implantation. At 28ºC, *S. cerevisiae* completely dominated the two mixed-
328 culture fermentations, with implantation percentages higher than 60% in all samples only
329 after 6 h of fermentation (Figure 5A-B). Conversely, at 12 º C, the cryotolerant yeasts *S.*
330 *eubayanus* and *S. uvarum* dominated the mixed fermentations. Nevertheless, this
331 implantation was not absolute, with percentages of *S. cerevisiae* around 10-30% at the end
332 of the fermentations (Figure 5C-D).
333 As 12ºC is an extreme low fermentation temperature to conduct fermentation in the
334 wineries, we decided to test the selective effect of a milder temperature for yeast
335 development. Thus, we subsequently evaluated the same mono and mixed cultures at 20ºC.
336 In this case, we also considered to include, additionally, an intermediate nitrogen
337 concentration of 140 mg/L. In this assay, since the influence of the temperature has been
338 reduced, a strong effect of nitrogen concentration on fermentation capacity, and hence, in
339 the competition capacity of the yeast species involved in the mixed cultures was observed
340 (Figure 6). The implantation percentages of *S. cerevisiae* at 300 mg/L YAN were around

60-90%, whereas the same at 60 mg/L YAN was only 40-50%, independently from the analysed mixed culture (*S. cerevisiae* + *S. uvarum* or *S. cerevisiae* + *S. eubayanus*). Moreover, at this high nitrogen concentration, the imposition of *S. cerevisiae* is stronger competing with *S. eubayanus* than with *S. uvarum*. Interestingly, with 140 mg/L YAN, the implantation of *S. cerevisiae* kept around 50-60% throughout the fermentation (Figure 6A). This is of great importance, since under this condition, most of the fermentations were able to finish (Figure 6B), and the implantation of the cryotolerant strains remain considerable. Furthermore, the monoculture of *S. eubayanus* also showed the fastest fermentation activity. As at 12ºC, none of the strains finished the nitrogen-limited fermentations (60 mg/L YAN) and the cryotolerant strains exhibited higher CFU counts than *S. cerevisiae* monocultures, regardless of the nitrogen concentration (Figure 6B-C).

Based on the percentage of *S. cerevisiae* at the end of mixed culture fermentation, a multi-factorial ANOVA was performed (Table 1). In the multi-factorial ANOVA, the effects of three parameters have been compared: temperature (12°C, 20°C and 28°C), nitrogen (60mg/L and 300mg/L) and the cryotolerant strain used in the mixed culture (*S.cerevisiae*+*S. eubayanus* and *S. cerevisiae*+*S. uvarum*). According to the results, temperature (T), nitrogen concentration (N) and the combination of T and N had significant and strong influences on the competitiveness of *S. cerevisiae* in the mixed culture; regardless of the cryotolerant species used in combination with *S. cerevisiae*. When combining all the three parameters together, the impact is also significant with P value lower than 0.05.

The analysis of main chemical parameters at the end of fermentations has been carried out for fermentations at 20°C. As mentioned above, no culture was able to complete the

364 fermentation at low nitrogen concentration (60 mg/L), with the presence of high values of
365 residual sugars in the final wines (Supplementary figure 1A). Since not all the
366 fermentations were completed, the production of ethanol and glycerol is shown as yield
367 produced per gram of sugar (glucose + fructose) consumed (Supplementary figure 1B-C).
368 No significant differences were observed in ethanol yield among different nitrogen
369 concentrations and different strains. On the contrary, *S. eubayanus* and *S. uvarum* strains
370 have much higher glycerol yield than *S. cerevisiae*, especially with low nitrogen
371 concentration 60 mg/L YAN. Nitrogen concentration does not have big influence on
372 glycerol yield by *S. cerevisiae*.

373 **4. Discussion**

374 **4.1. Growth and fermentative performance are variable according to species and life**
375 **history of yeasts in monocultures**

376 It has been described that the nitrogen requirement during wine fermentation is a strain-
377 specific feature (Gutiérrez et al., 2012). We demonstrated, for the first time, the existence
378 of a differential behaviour in nitrogen requirement among strains of the cryotolerant species
379 *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*. The effect of different nitrogen concentrations
380 was evidenced in both yeast growth and fermentation rate of the strains. However, a good
381 growth performance was not necessarily associated with a good fermentation performance
382 of a particular yeast, i.e. *S. eubayanus* or *S. uvarum* strains, with the highest and the lowest
383 μ_{max} growth values, showed no differences in their maximum fermentation rate evaluated
384 by CO_2 release. Gutiérrez et al. (2012) and Kemsawasd et al. (2015) have also reported this
385 lack of correlation between growth and fermentation behaviour. In the study of Gutiérrez et
386 al. (2012), the nitrogen requirements of four commercial *S. cerevisiae* wine strains were

387 analysed under growth and fermentation conditions. One of the strains, which showed the
388 poorest growth capacity, had the best fermentation performance. Kemsawasd et al. (2015)
389 investigated the influence of different nitrogen sources on growth and fermentation activity
390 of *S. cerevisiae* and four wine-related non-*Saccharomyces* yeast species (*Lachancea*
391 *thermotolerans*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *Torulaspora*
392 *delbrueckii*), and they have also concluded that a good growth capacity does not always
393 result in a good fermentation behaviour. Therefore, the two parameters, growth and
394 fermentation performance, must be evaluated in order to understand the yeast performance
395 during alcoholic fermentation.
396 Interestingly, most of the strains identified as *S. eubayanus* showed higher μ_{max} values in
397 microplate assays than those belonging to *S. uvarum* species, for all different nitrogen
398 concentrations tested. Even *S. uvarum* strains isolated from the same natural habitats as *S.*
399 *eubayanus* (*A. araucana* trees) showed lower μ_{max} values, similar to those observed for the
400 *S. uvarum* strains isolated from fermentative environments. This observation evidences a
401 clear species-specific behaviour, potentially associated with a particular ecological strategy.
402 The species-specific effect of nitrogen on both growth rate and fermentation performance
403 was also reported by Kemsawasd et al. (2015) for a set of non-*Saccharomyces* species
404 including *Lachancea thermotolerans* and *Metschnikowia pulcherrima*. The authors
405 suggested that some of the differences between the responses of non-*Saccharomyces*
406 species and *S. cerevisiae* could be related to genetic differences shaped by human activity
407 (domestication) of *S. cerevisiae*. Spor et al. (2009) also observed that populations
408 harboured different strategies depending on their ecological niches. These authors found
409 that forest and laboratory strains reach a large carrying capacity (population size) and a

410 small cell size in fermentation, but they have a low reproduction rate in respiration and
411 produce lower quantities of ethanol, suggesting that they store cell resources rather than
412 secreting secondary products. Contrarily, the industrial strains of this species reproduce
413 slowly, reach a small carrying capacity but have a big cell size in fermentation and a high
414 reproduction rate in respiration with higher glucose consumption rates. These two
415 contrasted behaviours were metaphorically defined by the authors (Spor et al., 2009) as
416 ‘‘ant’’ and ‘‘grasshopper’’ strategies of resource utilisation. Similar results were obtained
417 in this work when compared with different strains of *Saccharomyces* non-*cerevisiae* species
418 isolated from diverse habitats. We observed that independently from the nitrogen
419 concentration, both the highest μ_{max} in microplates assays and the highest CFU numbers in
420 competition experiments were observed for *S. eubayanus* strains isolated from natural
421 habitats. It is interesting to note that for the specific case of *S. uvarum* species, including
422 strains from natural habitats and fermentative environments, previous phylogenetic
423 analyses have evidenced the existence of at least two different populations with low genetic
424 flux among the strains of this species, isolated from natural habitats in Patagonia and
425 named Southamerica-A and Southamerica-B/Holartic (Almeida et al., 2014). On the other
426 hand, all strains isolated from fermented beverages in the same region belonged to the
427 Southamerica-A/Holartic subpopulation (Almeida et al., 2014; Rodríguez et al., 2017). This
428 data also supports the fact that the origin, more than the phylogenetics relationships, are
429 involved in the physiological response of the yeast to the analysed culture conditions. In
430 previous work carried out in our laboratory with a set of *S. uvarum* and *S. eubayanus* strains
431 including those used in this work, we also evidenced clear physiological differences

between strains from natural and fermentative habitats (González Flores et al., 2017; Origone et al., 2017).

The effect of nitrogen content on both the growth and fermentative capacity of the evaluated yeasts is a complex issue. Although several works have evaluated the chemical composition of grape must in different winemaking areas, the lack of information about the same in natural habitats makes the evaluation of this phenomenon difficult. Strikingly, the strongest effect of increasing nitrogen concentrations on the maximum fermentative rate was observed for strains associated with industrial fermentations (*S. cerevisiae* T73, *S. uvarum* BMV58 and CECT 12600) as well as for strains strictly associated with natural habitats (S. *kudriavzevii* CR85 and CR90). This was observed for pure culture fermentations with 60, 140 and 300 mg/L YAN. However, the same assay carried out with *S. eubayanus*, the other species associated only with natural habitats, demonstrated only a slight difference in this parameter at increasing nitrogen concentrations. These results suggest that both the yeast species and their ecological life-history are involved in the response strategy of yeasts to this environmental factor. This phenomenon became relevant for the development of mixed cultures for winemaking as it has been demonstrated to enhance the organoleptic complexity of wines. This fact, added to the tendency to produce wines at low temperature as well as to produce more aromatic wines, makes the study of *Saccharomyces* non-*cerevisiae* cryotolerant yeast strains an interesting topic for research, especially in mixed cultures with *S. cerevisiae*. The competition ability of several *S. cerevisiae* strains under different nitrogen concentration has been evaluated (García-Ríos et al., 2014; Lemos Junior et al., 2017; Vendramini et al., 2017); however, little is known

about the competition ability between *S. cerevisiae* versus other species from the *Saccharomyces* genus.

4.2. Low temperature and low nitrogen concentration favour *Saccharomyces* non-*cerevisiae* implantation in mixed cultures

The implantation capacity of two *Saccharomyces* non-*cerevisiae* strains (*S. eubayanus* NPCC 1285 from natural habitats and *S. uvarum* NPCC 1317 from apple chicha), which were selected according to their performance in fermentations with low nitrogen concentrations, was evaluated in mixed cultures with the wine commercial *S. cerevisiae* strain T73 at different temperatures.

Our results indicate that, as expected, increased temperature improves the advantage of *S. cerevisiae*, whereas, conversely, lowered temperature reduces its competitiveness, favouring cryotolerant strains, independently from the nitrogen concentration. This phenomenon was first reported by Arroyo-López et al. (2011) and Salvadó et al. (2011) for competition experiments between different yeast strains, and recently evidenced by Alonso del Real et al. (2017) for competitions between one strain of *S. eubayanus*, *S. uvarum* or *S. kudriavzevii* and *S. cerevisiae*. In our work, we also evidenced that temperature is the most important parameter governing the implantation of both *S. eubayanus* and *S. uvarum* in mixed cultures with *S. cerevisiae*. However, we observed a better competition capacity of our strain *S. eubayanus* NPCC 1285 against *S. cerevisiae* T73 at 20ºC. This fact was particularly observed with an intermediate nitrogen concentration (<140 mg/L). This could be due to the fact that the strains used in competition fermentation were selected because of their low nitrogen requirement, according to the assays previously discussed.

476 Several other factors can be related to the competitive capacity of a yeast strain including
477 competition for space and for other specific nutrients, oxygen affinity, production of
478 antagonist compounds as killer toxins or other diffusible molecules, among others (Fleet,
479 2003). In the same way, Pérez-Torrado et al. (2017) demonstrated that both cell-to-cell
480 contact and differential sulphite production and resistance are important factors that
481 determine the dominance of one *S. cerevisiae* strain over another. Additionally, Cheraiti et
482 al. (2005) observed that acetaldehyde production and redox interactions are involved in
483 competitions between *S. cerevisiae* and *S. uvarum*.
484 A particular strategy that seems to adopt *S. eubayanus*, and, to a lesser extent, *S. uvarum*, is
485 a significantly higher CFU number than in *S. cerevisiae* at lower temperatures. This
486 phenomenon was observed in most of the analysed conditions in monocultures (both
487 microplates and competition assays). These differences were more evident at lower
488 nitrogen concentrations, i.e. *S. cerevisiae* evidenced an increase in its population size
489 directly proportional to the nitrogen content in the medium while this response was not in
490 the same magnitude for *S. eubayanus*. Natural environments are usually nutrient poor; in
491 this context, yeast strains able to grow in the presence of low nitrogen concentrations are
492 selected, and this could be the situation for *S. eubayanus*. This species seems not to be able
493 to respond efficiently to increasing nitrogen concentrations. Contrarily, it has been argued
494 that *S. cerevisiae* does not show adaptations to any particular habitat, but rather an ability to
495 survive in a wide range of conditions (Goddard and Greig, 2015) which is consistent with
496 its life history of nomadic generalist that inhabits diverse niches (Jouhten et al., 2016; Liti
497 et al., 2009). Interestingly, it has also been demonstrated that one genomic region that
498 shows strong differentiation between the oak and wine European populations of *S.*

499 *cerevisiae* correspond to a gene coding for a transporter for oligopeptides that can act as
500 nitrogen sources in wines (Marsit et al., 2015). Additionally, Treu et al. (2014) evidenced
501 that gene sequences involved in nitrogen metabolism are more variable among *S. cerevisiae*
502 strains from vineyard than in industrial strains, suggesting a different aptitude in nitrogen
503 uptake and management. Altogether, these data indicate that nitrogen metabolism is
504 strongly dependent on the origin of the yeast strains in both *S. cerevisiae* and
505 *Saccharomyces* non-*cerevisiae* yeasts. The exploitation of these differential metabolic
506 behaviours could be of interest for the development of mixed cultures for rational
507 winemaking.

508 **5. Conclusions**

509 The results obtained in this work evidenced for the first time that both nitrogen content in
510 must and fermentation temperature are important factors involved in the competition of *S.*
511 *eubayanus* and *S. uvarum* in mixed culture fermentations with *S. cerevisiae*. Moreover, *S.*
512 *eubayanus* in mono and mixed culture with *S. cerevisiae* showed the best fermentation
513 performance in synthetic must, at low nitrogen concentration and low fermentation
514 temperature. The employment of this kind of mixed cultures can become a strategy for
515 winemakers to overcome the problem of nitrogen-limited musts.

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COMPETING INTERESTS

The author declares that there are no competing interests regarding the publication of this paper.

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671

673 **FIGURE CAPTIONS**

674 **Figure 1:** Heatmap representation of μ_{max} values of the analysed strains at increasing
675 nitrogen concentrations. Each line corresponds to a strain (indicated at the right of the
676 Figure) and each column to a particular nitrogen concentration (indicated at the top of the
677 Figure). The colour key bar at the top indicates μ_{max} value relative to the average for each
678 particular nitrogen concentration: values higher than the average are in red and values
679 lower than the average are in green. Hierarchical clustering is showed on the left. Colour
680 dots on the right of the Figure indicate both species identity and origin of the strains. The
681 statistical significance is shown in supplementary material table 1.
682
683 **Figure 2:** (A) Maximum fermentation velocity and (B) lag stage of strains from several
684 origins at fermentation performed at different YAN concentrations (■ 60mg/L, ■ 140 mg/L
685 and ■ 300 mg/L). The statistical significance is shown in supplementary material table 3.
686
687 **Figure 3:** Fermentation kinetics at 28°C 60 mg/L YAN (A), 28°C 300 mg/L YAN (B),
688 12°C 60 mg/L YAN (C) and 12°C 300 mg/L YAN (D)
689 — *S. cerevisiae*, — *S. eubayanus*, — *S. uvarum*, ▪▪▪ *S. eubayanus* + *S. cerevisiae*, — — *S.*
690 *uvarum* + *S. cerevisiae.*
691
692 **Figure 4:** Maximum population during stationary phase of fermentation at 28°C (A) and
693 12°C (B). Light green (28°C) and light yellow (12°C) bars represent 60mg/L YAN. Dark
694 green (28°C) and dark yellow (12°C) bars with diagonal lines represent 300mg/L YAN.
695 One-way ANOVA was performed within each nitrogen concentration. Letters on top of the
696 bars indicate significant difference ($p<0.05$).
697
698 **Figure 5:** *S. cerevisiae* growth evolution in mixed culture fermentations at 28°C 60 mg/L
699 YAN (A), 28°C 300 mg/L YAN (B), 12°C 60 mg/L YAN (C) and 12°C 300 mg/L YAN
700 (D). Bars in light colour represent *S. eubayanus* + *S. cerevisiae* and bars in dark colour
701 represent *S. uvarum* + *S. cerevisiae*. Bars with diagonal lines show the value of 300 mg/L
702 YAN. Bars without patterns show the value of 60 mg/L YAN.

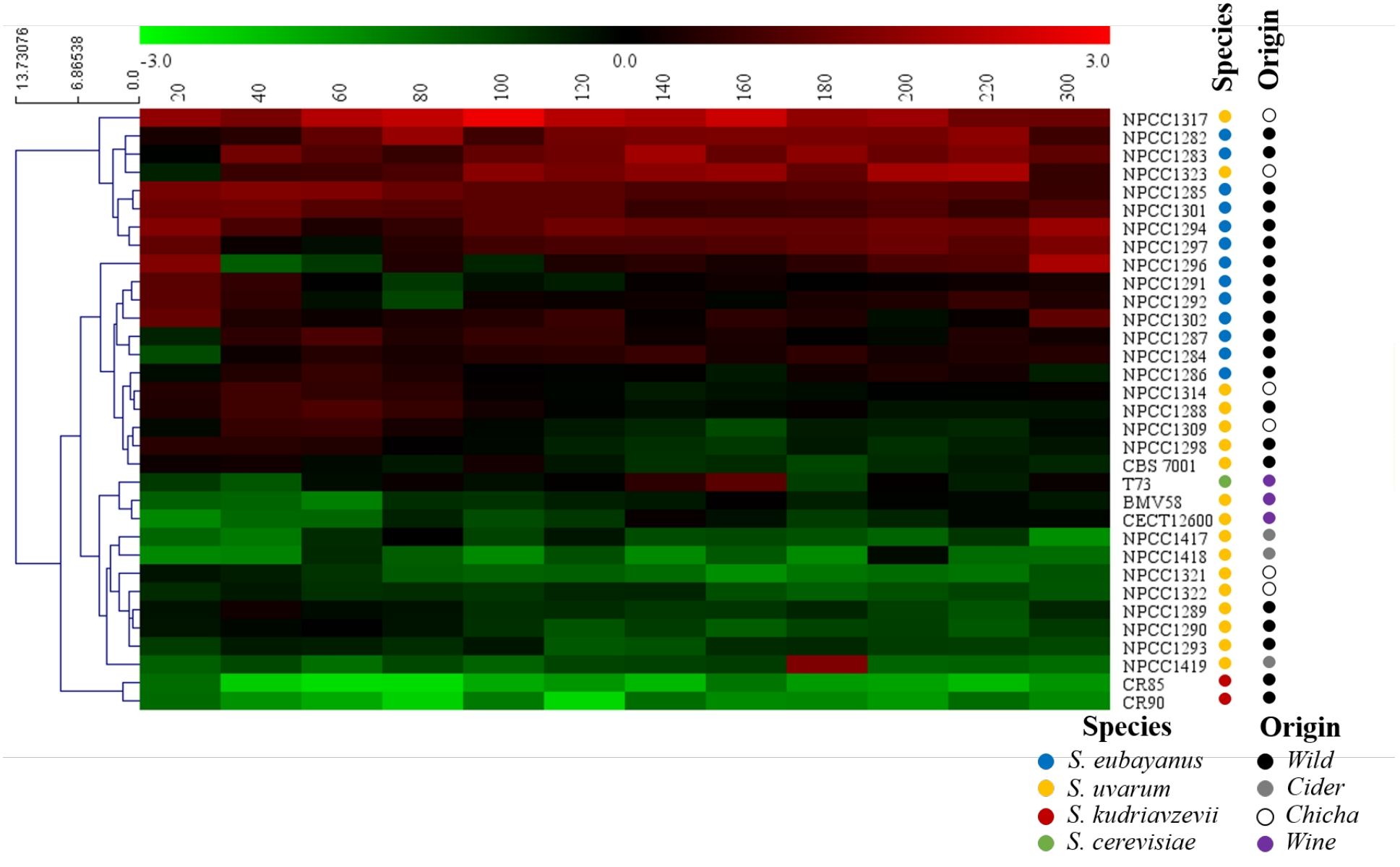
Figure 6: (A) *S. cerevisiae* growth evolution in mixed culture fermentations. Light purple bars represent *S. eubayanus* + *S. cerevisiae* and dark purple bars represent *S. uvarum* + *S. cerevisiae*. 60, 140 and 300 mg/L of YAN are represented by bars without patterns, bars with horizontal lines, and bars with diagonal lines, respectively. (B) Fermentation kinetics at 20°C: — *S. cerevisiae,* — *S. eubayanus*, — *S. uvarum,* ··· *S. eubayanus* + *S. cerevisiae*, — — *S. uvarum* + *S. cerevisiae.* (C) Maximum population during stationary phase of fermentation. Pink bars without pattern represent 60mg/L YAN. Red bars with horizontal lines represent 140mg/L YAN and red bars with diagonal lines represent 300mg/L YAN. One-way ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference ($p<0.05$).

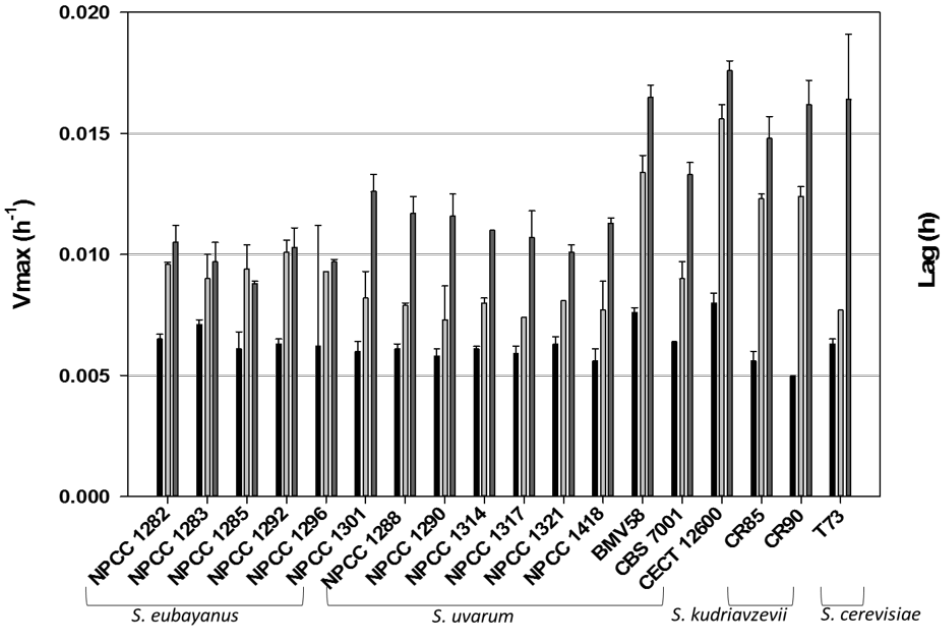
Supplementary figure 1: Chemical characterisation of products obtained after fermentation at 20 °C of synthetic musts containing different nitrogen concentrations (60, 140 and 300 mg/L). ■ 60mg/L, ■ 140 mg/L and ■ 300 mg/L (A) Residual sugar glucose + fructose. (B) Ethanol yield (C) Glycerol yield. One-way ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference ($p<0.05$).

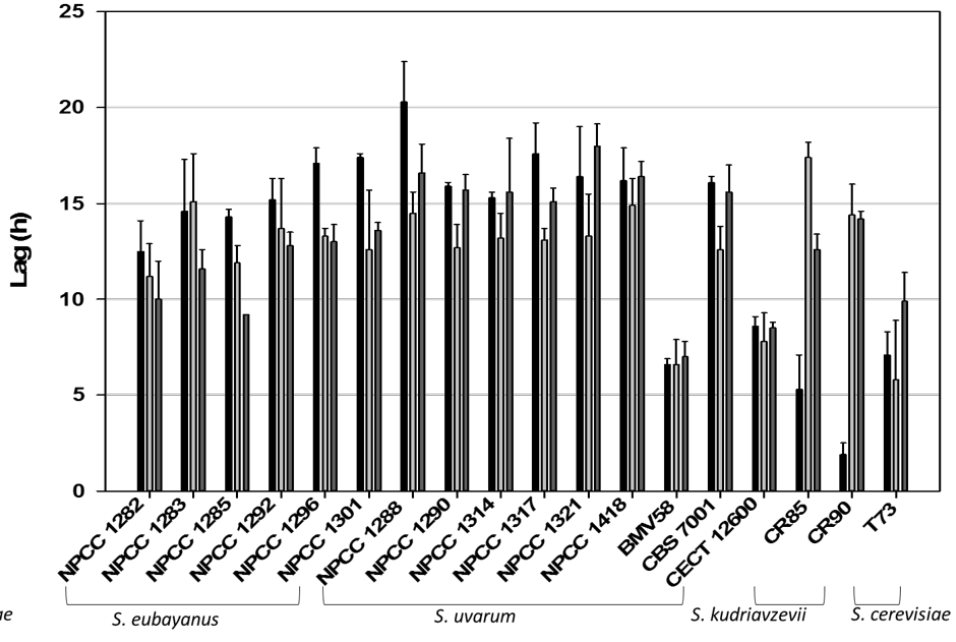
Table 1: F-values and P-values for percentage of *S. cerevisiae* at the end of mix-culture fermentation obtained by a multi-factorial ANOVA

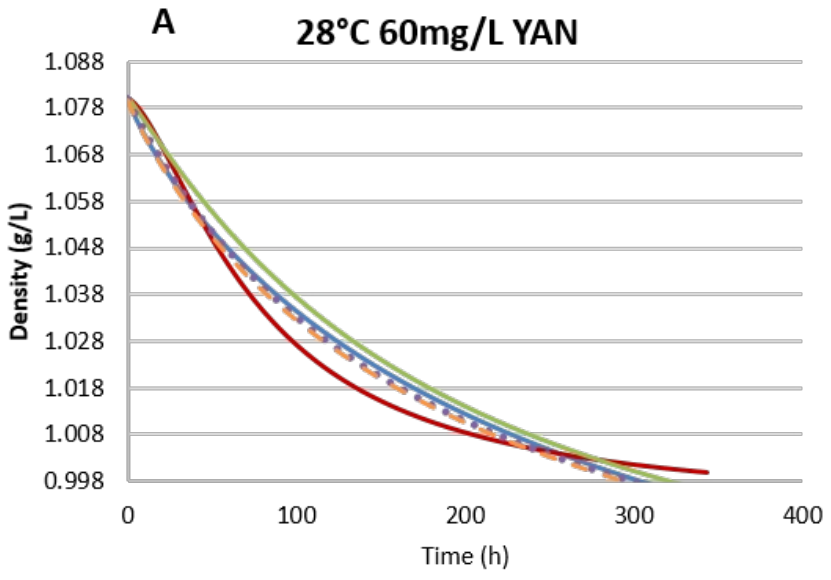
	Sources of variation						
	temp (T)	**N concentration (N)**	**Mix-culture[a] (M)**	**T*N**	**T*M**	**N*M**	**T*N*M**
F value	386.80	9.97	0.10	31.76	3.26	0.18	6.02
P value[b]	$p<0.001$	$p<0.01$	0.76	$p<0.001$	0.076	0.68	$p<0.05$
DF	2	1	1	2	2	1	2

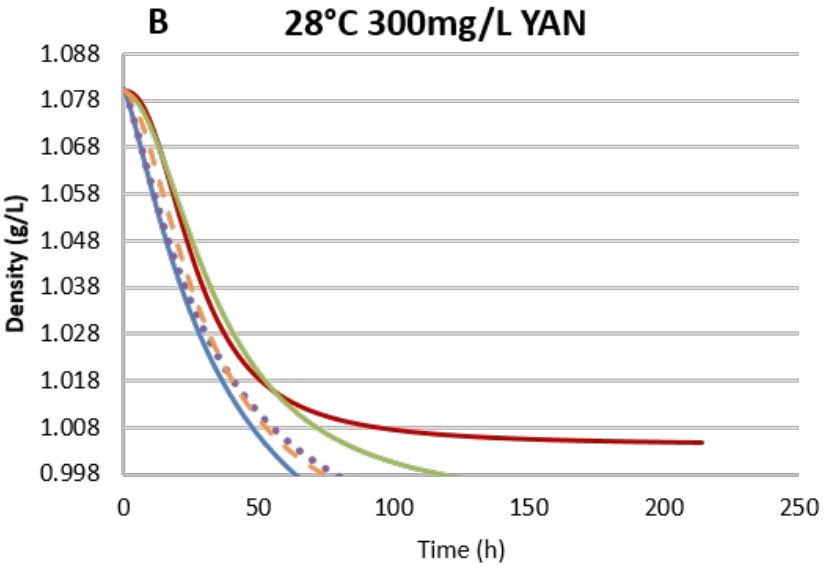
a. There are two mix-culture type which are *S. eubayanus* + *S. cerevisiae* and *S. uvarum* + *S. cereivisiae*
b. The data is considered as significant when p value is less than 0.05.

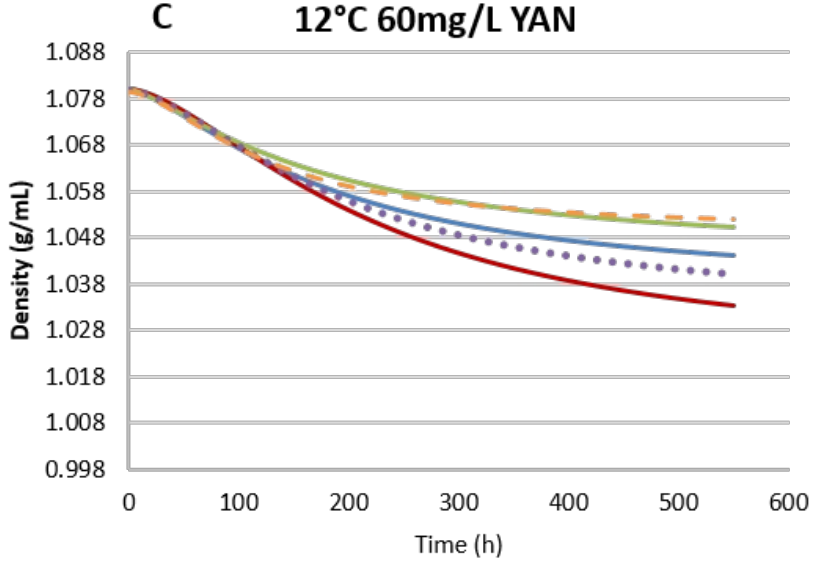

13.73076
6.86538
0.0
-3.0
0.0
3.0
20
40
60
80
100
120
140
160
180
200
220
300
Species
Origin
NPCC1317
NPCC1282
NPCC1283
NPCC1323
NPCC1285
NPCC1301
NPCC1294
NPCC1297
NPCC1296
NPCC1291
NPCC1292
NPCC1302
NPCC1287
NPCC1284
NPCC1286
NPCC1314
NPCC1288
NPCC1309
NPCC1298
CBS 7001
T73
BMV58
CECT12600
NPCC1417
NPCC1418
NPCC1321
NPCC1322
NPCC1289
NPCC1290
NPCC1293
NPCC1419
CR85
CR90
Species
S. eubayanus
S. uvarum
S. kudriavzevii
S. cerevisiae
Origin
Wild
Cider
Chicha
Wine

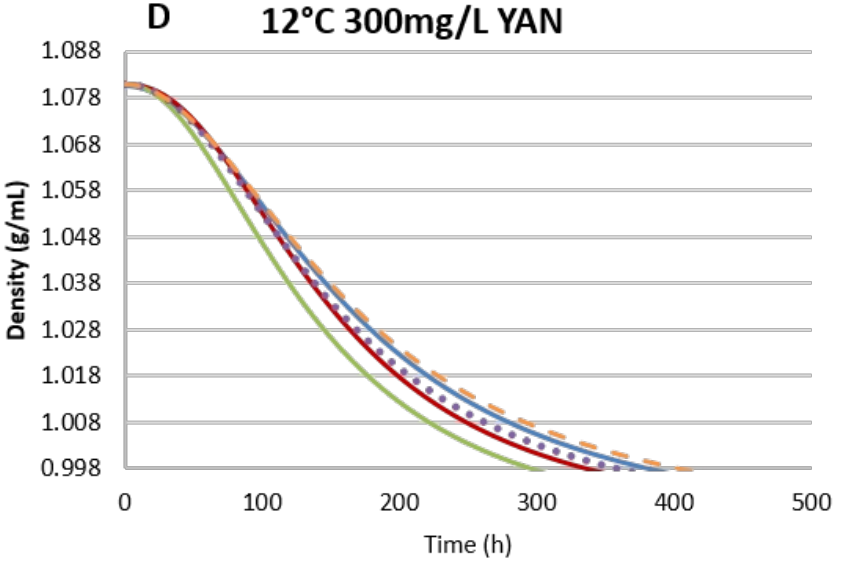

Vmax (h-1)
0.020
0.015
0.010
0.005
0.000
NPCC 1282
NPCC 1283
NPCC 1285
NPCC 1292
NPCC 1296
NPCC 1301
NPCC 1288
NPCC 1290
NPCC 1314
NPCC 1317
NPCC 1321
NPCC 1418
BMV58
CBS 7001
CECT 12600
CR85
CR90
T73
S. eubayanus
S. uvarum
S. kudriavzevii
S. cerevisiae

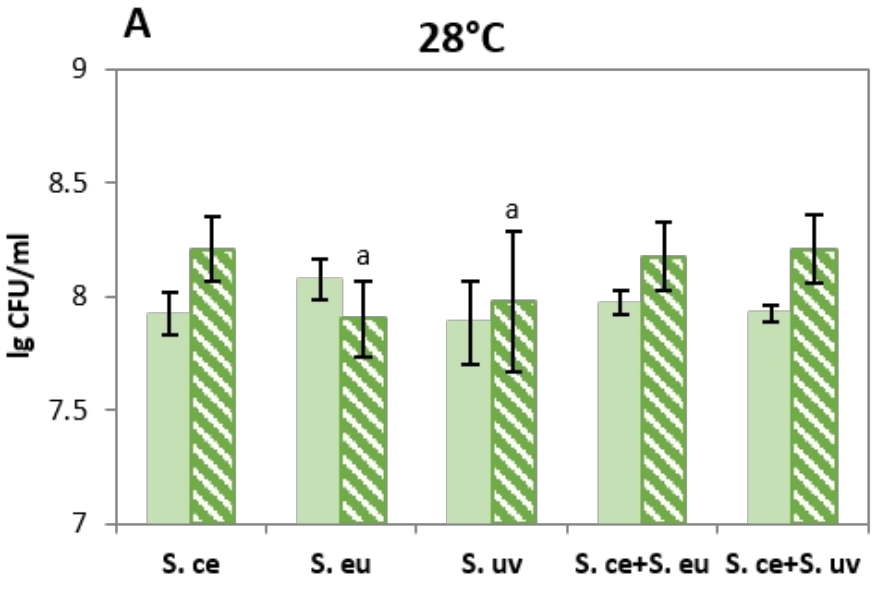

Lag (h)
25
20
15
10
5
0
NPCC 1282
NPCC 1283
NPCC 1285
NPCC 1292
NPCC 1296
NPCC 1301
NPCC 1288
NPCC 1290
NPCC 1314
NPCC 1317
NPCC 1321
NPCC 1418
BMV58
CBS 7001
CECT 12600
CR85
CR90
T73
S. eubayanus
S. uvarum
S. kudriavzevii
S. cerevisiae

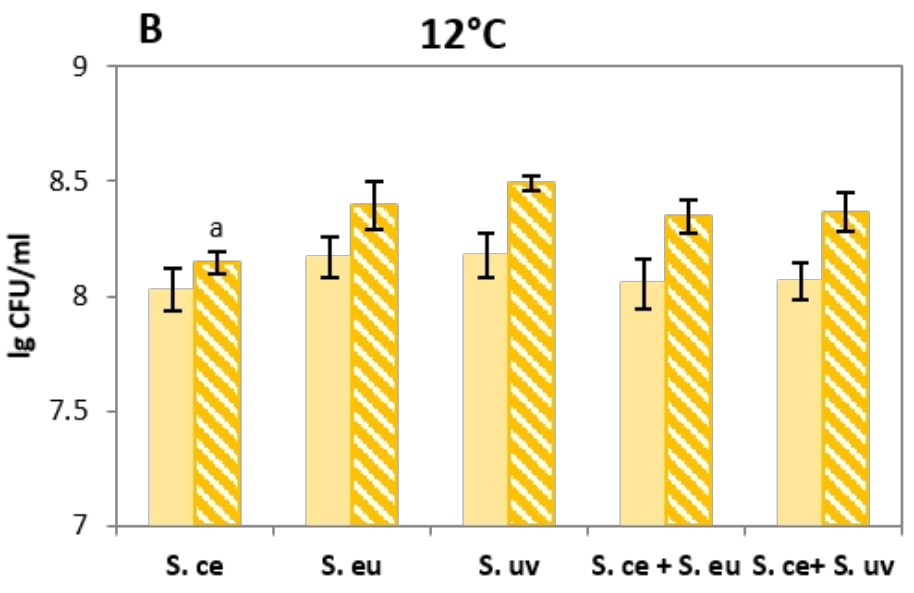

A
28°C 60mg/L YAN
Density (g/L)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0
100
200
300
400
Time (h)

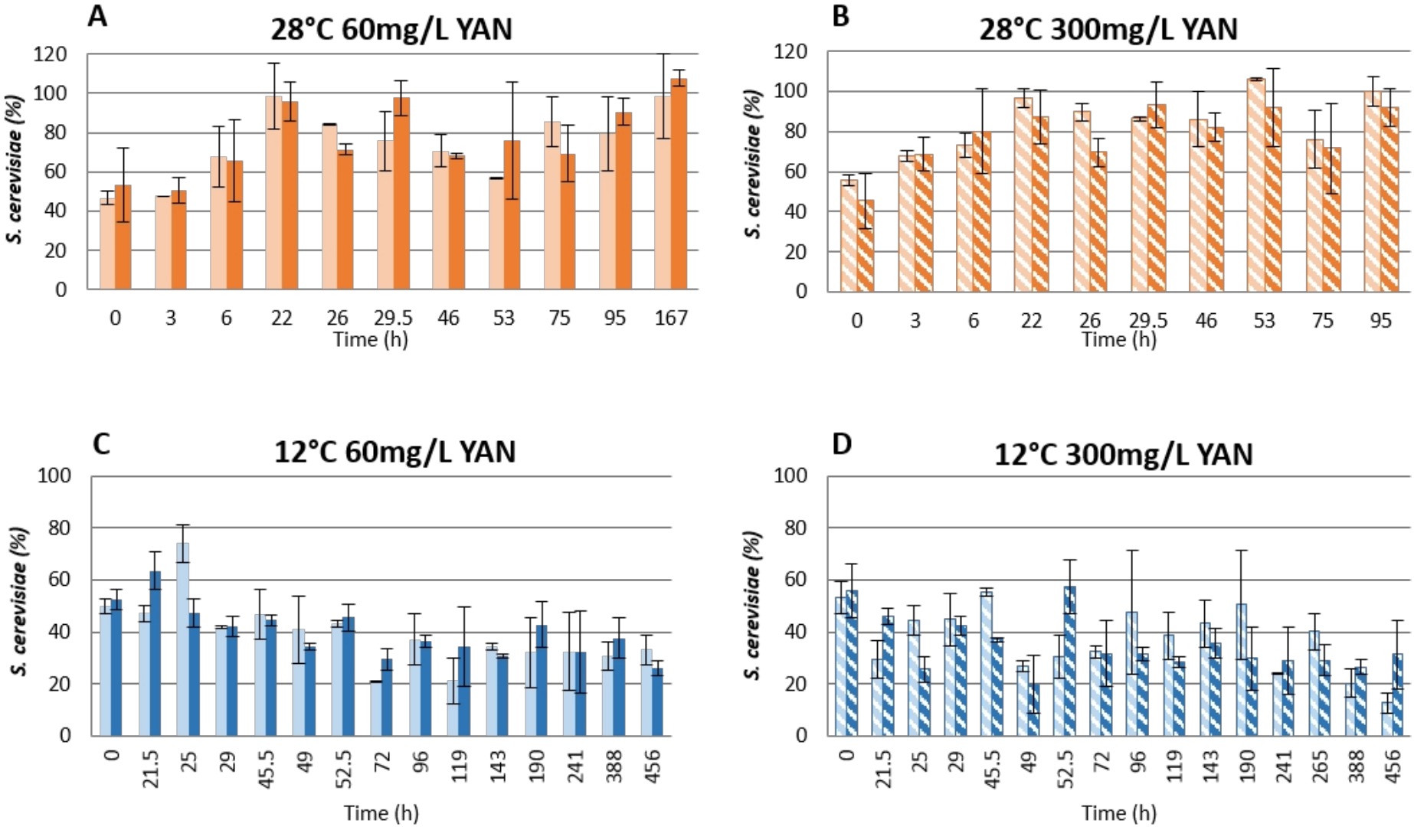

B
28°C 300mg/L YAN
Density (g/L)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0
50
100
150
200
250
Time (h)

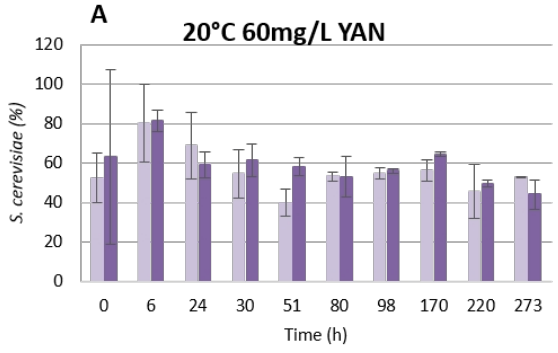

C
12°C 60mg/L YAN
Density (g/mL)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0
100
200
300
400
500
600
Time (h)

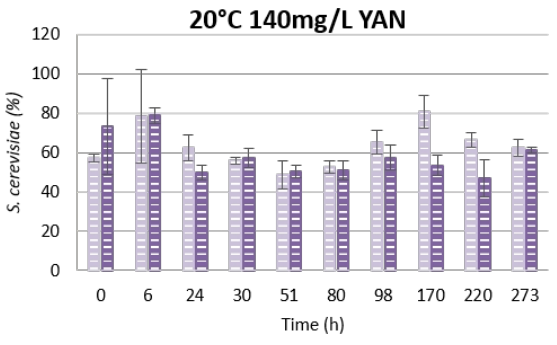

D
12°C 300mg/L YAN
Density (g/mL)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0
100
200
300
400
500
Time (h)

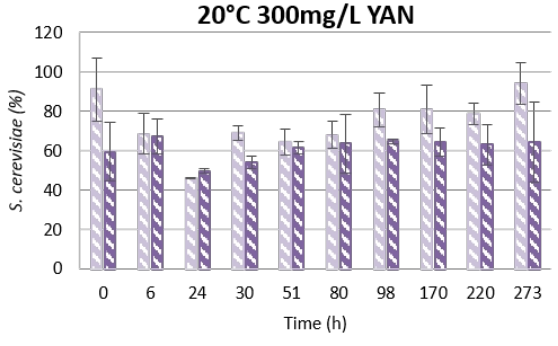

A
28°C
lg CFU/ml
9
8.5
8
7.5
7
a
a
S. ce
S. eu
S. uv
S. ce+S. eu
S. ce+S. uv

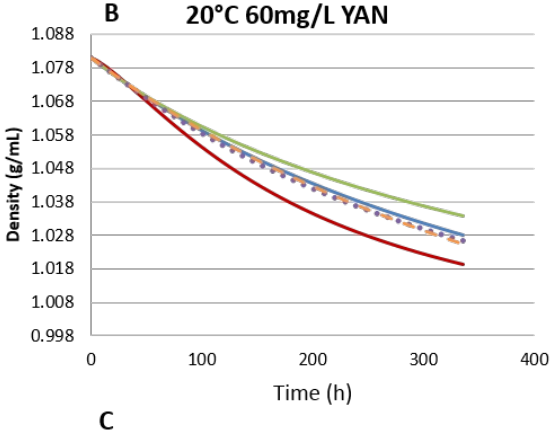

B
12°C
lg CFU/ml
9
8.5
8
7.5
7
a
S. ce
S. eu
S. uv
S. ce + S. eu
S. ce+ S. uv

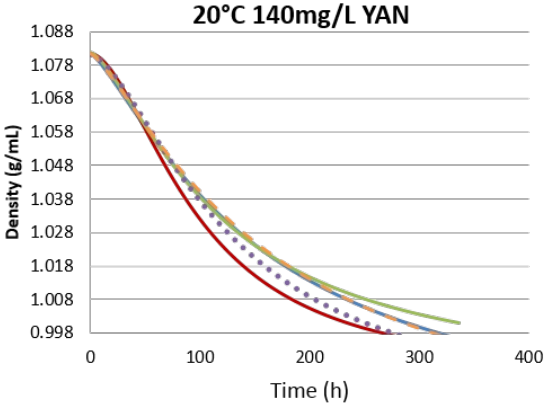

A
28°C 60mg/L YAN
S. cerevisiae (%)
120
100
80
60
40
20
0
0 3 6 22 26 29.5 46 53 75 95 167
Time (h)
B
28°C 300mg/L YAN
S. cerevisiae (%)
120
100
80
60
40
20
0
0 3 6 22 26 29.5 46 53 75 95
Time (h)
C
12°C 60mg/L YAN
S. cerevisiae (%)
100
80
60
40
20
0
0 21.5 25 29 45.5 49 52.5 72 96 119 143 190 241 388 456
Time (h)
D
12°C 300mg/L YAN
S. cerevisiae (%)
100
80
60
40
20
0
0 21.5 25 29 45.5 49 52.5 72 96 119 143 190 241 265 388 456
Time (h)

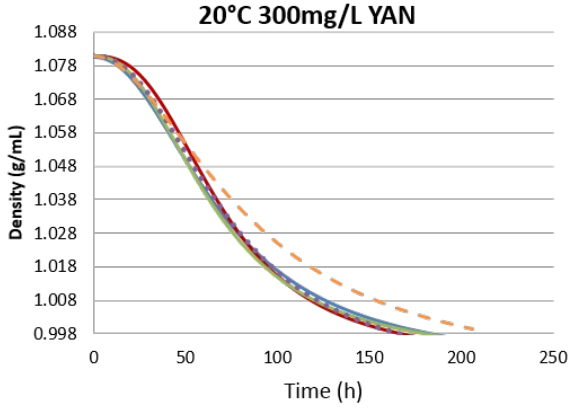

A
20°C 60mg/L YAN
S. cerevisiae (%)
120
100
80
60
40
20
0
0 6 24 30 51 80 98 170 220 273
Time (h)

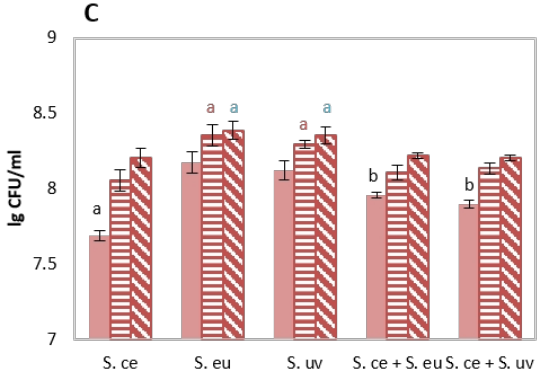

20°C 140mg/L YAN
S. cerevisiae (%)
120
100
80
60
40
20
0
0 6 24 30 51 80 98 170 220 273
Time (h)

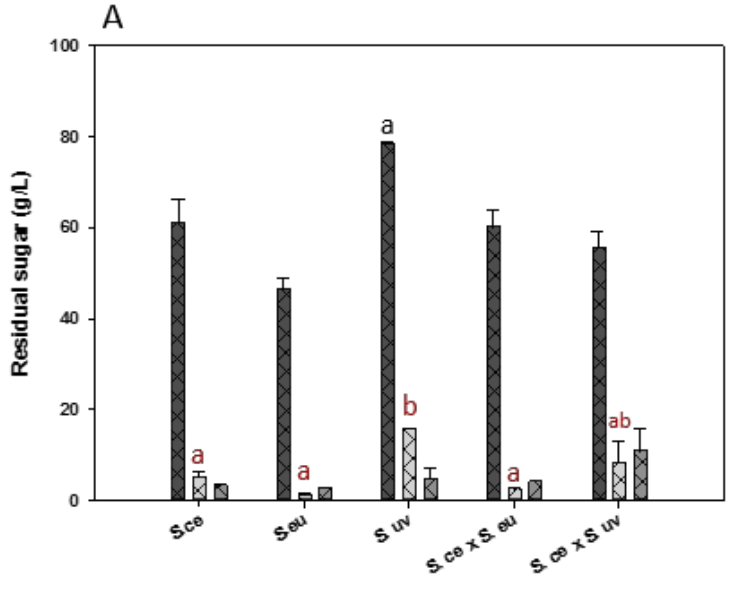

20°C 300mg/L YAN
S. cerevisiae (%)
120
100
80
60
40
20
0
0 6 24 30 51 80 98 170 220 273
Time (h)

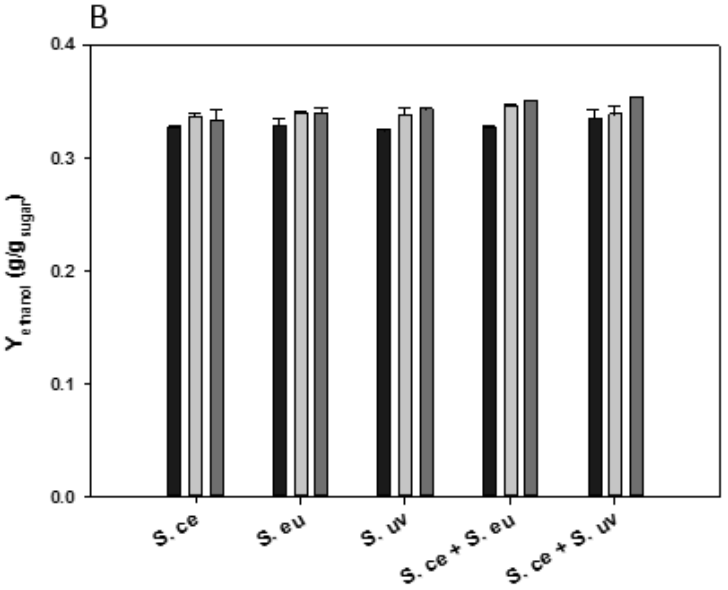

B
20°C 60mg/L YAN
Density (g/mL)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0 100 200 300 400
Time (h)

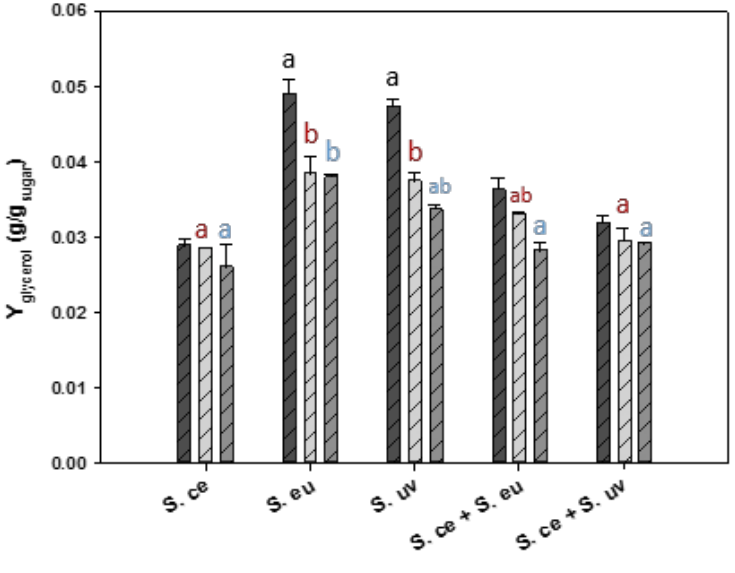

20°C 140mg/L YAN
Density (g/mL)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0 100 200 300 400
Time (h)


20°C 300mg/L YAN
Density (g/mL)
1.088
1.078
1.068
1.058
1.048
1.038
1.028
1.018
1.008
0.998
0 50 100 150 200 250
Time (h)


C
lg CFU/ml
9
8.5
8
7.5
7
a a a a b b
S. ce
S. eu
S. uv
S. ce + S. eu
S. ce + S. uv


A
Residual sugar (g/L)
100
80
60
40
20
0
a
a
a
b
a
ab
Sce
Seu
S uv
S. ce x S. eu
S. ce x S uv


B
$Y_{ethanol}$ (g/g_{sugar})
0.4
0.3
0.2
0.1
0.0
S. ce
S. eu
S. uv
S. ce + S. eu
S. ce + S. uv


C
$Y_{glycerol}$ (g/g_{sugar})
0.06
0.05
0.04
0.03
0.02
0.01
0.00
a
a
a
b
b
a
b
ab
ab
a
a
a
S. ce
S. eu
S. uv
S. ce + S. eu
S. ce + S. uv

Suppl Mat Table 1: Maximum growth rate (μ_{max}) of different *Saccharomyces* strains in synthetic media containing increasing nitrogen concentrations.

Species	Strains	YAN concentration (mg/L)											
		20	**40**	**60**	**80**	**100**	**120**	**140**	**160**	**180**	**200**	**220**	**300**
S. eubayanus	**NPCC1282**	0.08±0.01^{ab}	0.09±0.00^{ab}	0.11±0.01^{ab}	0.14±0.01^{abcd}	0.12±0.01^{abc}	0.14±0.01^{def}	0.15±0.01^{abcd}	0.15±0.00^{bcd}	0.15±0.01^{bc}	0.15±0.01^{de}	0.16±0.00^{f}	0.13±0.01^{defghi}
	NPCC1283	0.08±0.01^{ab}	0.11±0.01^{b}	0.11±0.0^{ab}	0.12±0.01^{abc}	0.13±0.01^{abc}	0.14±0.01^{def}	0.16±0.02^{de}	0.15±0.01^{bc}	0.16±0.02^{bcd}	0.14±0.00^{bcd}	0.15±0.00^{ef}	0.14±0.01^{efghi}
	NPCC1284	0.06±0.02^{ab}	0.09±0.01^{ab}	0.10±0.01^{a}	0.11±0.00^{ab}	0.12±0.01^{abc}	0.12±0.01^{abcdef}	0.13±0.03^{abcd}	0.13±0.01^{abc}	0.13±0.03^{abc}	0.12±0.02^{abcd}	0.13±0.01^{abcdef}	0.12±0.02^{cdefghi}
	NPCC1285	0.10±0.00^{ab}	0.11±0.00^{b}	0.12±0.00^{a}	0.13±0.01^{abcd}	0.13±0.00^{abc}	0.14±0.01^{cdef}	0.13±0.00^{abcd}	0.14±0.01^{abc}	0.14±0.01^{abc}	0.14±0.00^{abcd}	0.14±0.00^{bcdef}	0.13±0.00^{cdefghi}
	NPCC1286	0.08±0.01^{ab}	0.09±0.01^{ab}	0.10±0.01^{a}	0.11±0.01^{ab}	0.11±0.01^{ab}	0.11±0.02^{abcd}	0.11±0.01^{abc}	0.11±0.02^{abc}	0.12±0.01^{abc}	0.12±0.01^{abcd}	0.13±0.01^{abcdef}	0.10±0.01^{abcde}
	NPCC1287	0.07±0.00^{ab}	0.09±0.01^{ab}	0.11±0.02^{ab}	0.11±0.00^{abc}	0.12±0.02^{abc}	0.12±0.01^{abcdef}	0.12±0.00^{abc}	0.13±0.01^{abc}	0.12±0.00^{abc}	0.11±0.02^{abcd}	0.13±0.01^{abcdef}	0.12±0.01^{bcdefgh}
	NPCC1291	0.09±0.00^{ab}	0.01±0.00^{ab}	0.09±0.02^{a}	0.09±0.01^{ab}	0.10±0.01^{ab}	0.10±0.01^{abcd}	0.12±0.01^{abc}	0.12±0.01^{abc}	0.12±0.00^{abc}	0.12±0.01^{abcd}	0.12±0.01^{abcdef}	0.12±0.00^{cdefgh}
	NPCC1292	0.10±0.02^{ab}	0.09±001^{ab}	0.09±0.01^{a}	0.09±0.01^{ab}	0.11±0.01^{ab}	0.11±0.00^{abcd}	0.12±0.01^{abc}	0.12±0.01^{abc}	0.13±0.01^{abc}	0.12±0.01^{abcd}	0.13±0.01^{bcdef}	0.12±0.01^{cdefgh}
	NPCC1294	0.10±0.01^{ab}	0.10±0.00^{ab}	0.10±0.00^{a}	0.11±0.00^{abc}	0.13±0.00^{abc}	0.14±0.01^{def}	0.14±0.00^{abcd}	0.14±0.00^{bc}	0.14±0.00^{abc}	0.14±0.00^{bcd}	0.15±0.01^{cdef}	0.15±0.01^{ij}
	NPCC1296	0.10±0.02^{ab}	0.06±0.01^{a}	0.08±0.01^{a}	0.11±0.01^{ab}	0.10±0.02^{ab}	0.12±0.00^{abcde}	0.13±0.01^{abc}	0.13±0.01^{abc}	0.13±0.00^{abc}	0.13±0.01^{abcd}	0.14±0.01^{cdef}	0.16±0.01^{i}
	NPCC1297	0.10±0.02^{ab}	0.09±0.00^{ab}	0.09±0.01^{a}	0.11±0.12^{abc}	0.12±0.01^{abc}	0.13±0.00^{cdef}	0.13±0.01^{abcd}	0.14±0.01^{abc}	0.14±0.00^{abc}	0.14±0.00^{bcd}	0.14±0.00^{cdef}	0.15±0.00^{ghi}
	NPCC1301	0.10±0.02^{ab}	0.11±0.02^{b}	0.11±0.02^{ab}	0.12±0.01^{abcd}	0.13±0.01^{abc}	0.13±0.01^{cdef}	0.13±0.01^{abc}	0.13±0.01^{abc}	0.14±0.00^{abc}	0.14±0.01^{abcd}	0.14±0.01^{bcdef}	0.13±0.01^{efghi}
	NPCC1302	0.10±0.01^{ab}	0.09±0.02^{ab}	0.09±0.02^{a}	0.11±0.01^{ab}	0.12±0.02^{abc}	0.13±0.00^{abcdef}	0.12±0.00^{abc}	0.13±0.01^{abc}	0.13±0.01^{abc}	0.11±0.01^{abcd}	0.12±0.01^{abcdef}	0.14±0.00^{efghi}
S. uvarum	**NPCC1288**	0.08±0.00^{ab}	0.10±0.00^{ab}	0.11±0.00^{ab}	0.12±0.00^{abc}	0.11±0.01^{ab}	0.11±0.00^{abcd}	0.11±0.00^{abc}	0.12±0.00^{abc}	0.12±0.01^{abc}	0.11±0.00^{abcd}	0.11±0.00^{abcdef}	0.11±0.01^{abcdef}
	NPCC1289	0.08±0.00^{ab}	0.09±0.01^{ab}	0.09±0.00^{a}	0.10±0.01^{ab}	0.10±0.00^{ab}	0.10±0.00^{abcd}	0.10±0.00^{abc}	0.11±0.01^{abc}	0.11±0.00^{abc}	0.10±0.00^{abc}	0.10±0.01^{abc}	0.10±0.00^{abcdef}
	NPCC1290	0.07±0.01^{ab}	0.08±0.01^{ab}	0.09±0.01^{a}	0.10±0.02^{ab}	0.09±0.01^{ab}	0.09±0.00^{ab}	0.10±0.01^{abc}	0.09±0.01^{ab}	0.10±0.02^{abc}	0.10±0.02^{abc}	0.10±0.01^{ab}	0.10±0.01^{abcd}
	NPCC1293	0.07±0.02^{ab}	0.08±0.01^{ab}	0.08±0.01^{a}	0.09±0.01^{ab}	0.10±0.02^{ab}	0.08±0.00^{a}	0.09±0.01^{ab}	0.11±0.00^{ab}	0.11±0.00^{abc}	0.10±0.01^{abc}	0.10±0.01^{abc}	0.09±0.00^{abcd}
	NPCC1298	0.09±0.01^{ab}	0.09±0.00^{ab}	0.10±0.00^{a}	0.10±0.00^{ab}	0.11±0.00^{ab}	0.10±0.01^{abcd}	0.10±0.01^{abc}	0.10±0.00^{ab}	0.11±0.01^{abc}	0.10±0.00^{abc}	0.11±0.01^{abcdef}	0.11±0.00^{abcde}
	NPCC1309	0.08±0.00^{ab}	0.10±0.01^{ab}	0.10±0.01^{a}	0.11±0.02^{ab}	0.11±0.02^{ab}	0.10±0.00^{abcd}	0.10±0.00^{abc}	0.10±0.01^{ab}	0.11±0.01^{abc}	0.11±0.01^{abcd}	0.11±0.01^{abcd}	0.11±0.01^{abcdefg}
	NPCC1314	0.08±0.01^{ab}	0.10±0.01^{ab}	0.10±0.01^{a}	0.11±0.01^{abc}	0.11±0.01^{ab}	0.11±0.01^{abcd}	0.11±0.01^{abc}	0.11±0.01^{abc}	0.11±0.00^{abc}	0.11±0.00^{abc}	0.12±0.01^{abcdef}	0.11±0.01^{bcdefg}

Suppl Mat Table 1 (*cont.*): Maximum growth rate (μ_{max}) of different *Saccharomyces* strains in synthetic media containing increasing nitrogen concentrations.

	Strain	YAN concentration (mg/L)											
		20	**40**	**60**	**80**	**100**	**120**	**140**	**160**	**180**	**200**	**220**	**300**
	NPCC1317	0.10±0.00^{b}	0.11±0.01^{b}	0.13±0.01^{ab}	0.15±0.00^{bcd}	0.17±0.02^{cd}	0.16±0.02^{ef}	0.16±0.02^{de}	0.17±0.01^{cd}	0.16±0.00^{cd}	0.16±0.00^{d}	0.12±0.00^{def}	0.14±0.00^{fghi}
	NPCC1321	0.08±0.01^{ab}	0.08±0.00^{ab}	0.08±0.01^{a}	0.08±0.00^{a}	0.08±0.01^{a}	0.08±0.00^{a}	0.08±0.00^{a}	0.08±0.01^{a}	0.09±0.00^{a}	0.09±0.01^{a}	0.09±0.01^{a}	0.09±0.01^{abc}
	NPCC1322	0.07±0.01^{ab}	0.08±0.01^{ab}	0.08±0.02^{a}	0.09±0.01^{ab}	0.09±0.00^{ab}	0.10±0.01^{abcd}	0.10±0.01^{abc}	0.10±0.01^{ab}	0.09±0.01^{ab}	0.09±0.01^{ab}	0.10±0.01^{abc}	0.09±0.00^{abc}
	NPCC1323	0.07±0.01^{ab}	0.10±0.01^{ab}	0.11±002^{a}	0.12±0.00^{abc}	0.14±0.00^{bc}	0.14±0.01^{bcde}	0.15±0.00^{abc}	0.16±0.01^{abc}	0.14±0.01^{abc}	0.16±0.01^{abcd}	0.17±0.01c^{def}	0.13±0.01^{abcde}
	NPCC1417	0.06±0.00^{ab}	0.06±0.01^{a}	0.08±0.02^{a}	0.10±0.01^{ab}	0.09±0.00^{ab}	0.10±0.01^{abcd}	0.09±0.01^{abc}	0.10±0.00^{ab}	0.10±0.02^{ab}	0.09±0.01^{abc}	0.11±0.01^{abcde}	0.07±0.01^{a}
	NPCC1418	0.05±0.02^{a}	0.05±0.01^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.09±0.01^{ab}	0.09±0.01^{abcd}	0.07±0.00^{a}	0.10±0.03^{ab}	0.09±0.01^{ab}	0.11±0.04^{abcd}	0.09±0.01^{ab}	0.08±0.00^{abc}
	NPCC1419	0.06±0.00^{ab}	0.07±0.02^{a}	0.07±0.00^{a}	0.09±0.02^{ab}	0.08±0.00^{a}	0.9±0.02^{abc}	0.09±0.01^{ab}	0.10±0.02^{ab}	0.15±0.05^{abc}	0.09±0.00^{a}	0.09±0.00^{ab}	0.08±0.01^{ab}
	CBS 7001	0.08±0.001^{ab}	0.09±0.01^{ab}	0.09±0.03^{a}	0.10±0.02^{ab}	0.11±0.03^{ab}	0.10±0.02^{abcd}	0.10±0.02^{abc}	0.11±0.01^{ab}	0.10±0.01^{abc}	0.10±0.03^{abc}	0.11±0.03^{abcde}	0.10±0.01^{abcde}
	BMV58	0.06±0.01	0.06±0.02^{a}	0.06±0.05^{a}	0.09±0.08^{ab}	0.09±0.02^{ab}	0.10±0.01^{abcd}	0.11±0.02^{abc}	0.12±0.07^{abc}	0.11±0.01^{abc}	0.11±0.01^{abcd}	0.12±0.01^{abcdef}	0.10±0.00^{abcde}
	CECT12600	0.05±0.00^{a}	0.06±0.01^{a}	0.07±0.00^{a}	0.10±0.00^{ab}	0.09±0.00^{ab}	0.09±0.00^{abcd}	0.12±0.00^{abc}	0.11±0.00^{abc}	0.10±0.00^{abc}	0.11±0.01^{abcd}	0.12±0.00^{abcdef}	0.11±0.01^{abcdef}
S. kudriavzevii	**CR85**	0.06±0.00^{ab}	0.04±0.01^{a}	0.04±0.02^{a}	0.05±0.00^{a}	0.06±0.01^{a}	0.07±0.01^{a}	0.06±0.02^{a}	0.09±0.00^{a}	0.08±0.01^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.07±0.00^{a}
	CR90	0.06±0.03^{ab}	0.05±0.01^{a}	0.05±0.02^{a}	0.05±0.01c^{a}	0.08±0.01^{a}	0.05±0.01^{a}	0.08±0.02^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.08±0.01^{a}	0.09±0.01^{a}	0.07±0.01^{a}
S. cerevisiae	**T73**	0.07±0.00^{ab}	0.06±0.00^{a}	0.09±0.01^{a}	0.11±0.00^{abc}	0.10±0.00^{ab}	0.11±0.01^{abcd}	0.13±0.01^{abc}	0.14±0.01^{abc}	0.10±0.01^{abc}	0.12±0.00^{abcd}	0.11±0.00^{abcde}	0.11±0.01^{abcdef}

Different superscript letters in the same column indicate significant differences (ANOVA and Tukey T. n=3).

Values expressed in h^{-1}.

Parameters obtained from OD measurements after fitting to modified Gompertz equation.

Suppl Mat Table 2: Lag phase time (λ) of different *Saccharomyces* strains in synthetic media containing increasing nitrogen concentrations.

Species	Strains	YAN concentration (mg/L)											
		20	40	60	80	100	120	140	160	180	200	220	300
S.eubayanus	**NPCC1282**	6.21±0.38^{abcdefg}	7.98±0.79^{abcdef}	9.52±0.48^{ghi}	9.37±0.42^{defghi}	12.10±0.08^{hij}	10.43±1.32^{abcdefg}	12.88±0.39^{bcdefg}	11.67±0.90^{eghijkl}	10.99±0.50^{fghijk}	13.48±2.08^{hik}	10.23±0.44^{cdefgh}	8.00±0.54^{abcdefg}
	NPCC1283	6.87±0.69^{cdefg}	8.91±0.92^{bcdefg}	10.11±0.19^{ghi}	12.11±0.54^{hi}	12.89±1.50^{ij}	12.89±0.61^{cdefgh}	13.95±1.06^{defg}	13.88±0.96^{ijl}	13.25±1.51^{ijk}	11.69±0.58^{fghij}	12.02±0.70^{fgh}	10.19±1.36^{defghi}
	NPCC1284	13.88±1.54^{h}	10.95±0.66^{efgh}	10.84±0.98^{hi}	13.87±0.03^{i}	14.80±0.18^{j}	15.46±0.55^{efgh}	16.49±0.73^{fg}	14.09±0.79^{ijkl}	18.94±1.20^{lm}	13.57±0.54^{hijkl}	14.75±1.50^{gh}	13.27±0.93^{hi}
	NPCC1285	4.41±0.04^{abcd}	4.62±0.03^{abcd}	4.78±0.12^{abcde}	4.90±0.09^{abcd}	5.07±0.06^{abc}	5.29±0.06^{abcde}	5.12±0.07^{abcd}	5.09±0.06^{abcd}	5.37±0.24^{abcd}	5.20±0.13^{abcd}	5.44±0.16^{abcd}	4.45±0.23^{abc}
	NPCC1286	5.28±0.06^{abcde}	5.47±0.33^{abcdf}	7.23±0.15^{bcdefgh}	7.92±0.38^{bcdefgh}	8.70±0.33^{bcdefghi}	8.88±0.02^{abcdefg}	9.15±0.22^{abcdef}	9.33±0.21^{bcdefghj}	8.63±0.19^{bcdefghi}	8.93±0.27^{bcdefghi}	8.86±0.17^{bcdefh}	10.86±1.28^{efghi}
	NPCC1287	5.70±0.16^{abcdef}	5.57±0.14^{abcdef}	6.09±0.66^{abcdefgh}	6.32±0.69^{abcdef}	6.13±0.72^{abcde}	6.19±1.01^{abcde}	6.58±1.03^{abcdef}	7.30±1.07^{abcdefgh}	6.42±0.74^{abcdef}	7.52±0.96^{abcdefgi}	6.91±1.03^{abcdef}	6.42±1.06^{abcdef}
	NPCC1291	9.03±1.68^{fg}	8.09±0.60^{abcdefg}	10.35±0.24^{hi}	11.98±0.46^{hi}	11.93±0.42^{ghij}	12.79±0.44^{bcdefgh}	12.91±0.83^{bcdefg}	11.78±0.54^{eghjkl}	11.40±0.75^{hijk}	12.12±1.03^{ghijk}	10.28±0.60^{cdefgh}	9.49±0.26^{cdefghi}
	NPCC1292	6.74±0.70^{cdefg}	7.42±0.87^{abcdef}	9.35±1.65^{fghi}	10.50±0.94^{fghi}	10.63±0.84^{efghij}	10.35±0.69^{abcdefg}	13.39±0.66^{cdefg}	13.02±0.48^{hijkl}	13.71±0.80^{jkl}	10.99±0.53^{efghij}	10.33±1.00^{cdefgh}	8.30±0.65^{abcdefg}
	NPCC1294	4.87±0.10^{abcd}	5.31±0.08^{abcdf}	6.55±0.25^{abcdefgh}	6.50±0.41^{abcdefg}	7.00±0.47^{abcdefg}	7.24±0.83^{abcdef}	6.54±0.11^{abcdef}	6.69±0.39^{abcdefg}	6.41±0.20^{abcdef}	7.07±0.39^{abcdefg}	6.26±0.34^{abcde}	5.60±0.02^{abcdef}
	NPCC1296	6.71±0.27^{bcdefg}	10.79±0.77^{egh}	10.34±0.86^{hi}	8.93±0.44^{cdefghi}	9.89±0.10^{cdefghij}	9.43±0.12^{abcdefg}	9.42±0.20^{abcdef}	12.22±0.89^{ghijkl}	12.45±1.19^{hijk}	10.06±0.27^{defghij}	11.32±0.98^{efgh}	9.41±0.91^{cdefghi}
	NPCC1297	5.00±0.07^{abcde}	6.20±0.10^{abcdef}	8.22±0.90^{cdefghi}	7.14±0.32^{abcdefg}	7.52±0.29^{abcdefgh}	7.26±0.24^{abcdef}	8.38±1.21^{abcdef}	8.06±0.21^{abcdefghij}	8.45±0.69^{bcdefgh}	8.18±0.66^{abcdefghi}	7.83±0.05^{abcdef}	6.09±0.21^{abcdef}
	NPCC1301	4.92±0.13^{abcde}	4.98±0.20^{abcd}	5.06±0.22^{abcdef}	5.03±0.25^{abcd}	5.05±0.21^{abc}	5.14±0.21^{abcde}	5.30±0.46^{abcd}	5.71±0.61^{abcde}	6.18±1.19^{abcde}	5.96±1.08^{abcde}	5.34±0.51^{abc}	5.21±0.29^{abcde}
	NPCC1302	7.99±0.36^{defg}	9.00±1.35^{cdefg}	9.82±1.04^{ghi}	9.85±0.30^{efghi}	10.06±1.38^{defghij}	8.95±1.11^{abcdefg}	11.76±0.94^{abcdef}	9.85±0.37^{cdefghj}	11.17±1.46^{ghijk}	12.14±0.94^{ghijk}	11.82±1.56^{fgh}	9.48±0.50^{cdefghi}
S.uvarum	**NPCC1288**	3.13±0.25^{ab}	2.98±0.23^{a}	2.98±0.20^{ab}	3.03±0.11^{a}	3.19±0.35^{a}	3.22±0.33^{a}	3.13±0.22^{a}	3.09±0.11^{a}	3.05±0.18^{a}	3.03±0.09^{a}	2.99±0.10^{a}	2.91±0.06^{a}
	NPCC1289	3.07±0.32^{abc}	2.98±0.11^{a}	2.67±0.00^{ab}	2.52±0.13^{a}	2.82±0.41^{a}	2.96±0.30^{abc}	2.56±0.01^{a}	2.87±0.15^{ab}	3.77±0.54^{ab}	3.50±0.02^{abc}	4.18±0.21^{ab}	4.51±0.02^{abcde}
	NPCC1290	3.84±0.3^{abc}	3.73±0.37^{ab}	3.96±0.50^{abc}	3.81±0.56^{ab}	4.26±0.46^{ab}	3.89±0.41^{abc}	4.23±0.30^{ab}	4.40±0.31^{abc}	5.32±0.37^{abcd}	5.19±0.59^{abcd}	5.09±1.00^{abc}	5.50±1.31^{abcdef}
	NPCC1293	5.35±0.18^{abcde}	5.62±0.22^{abcdef}	6.13±0.39^{abcdefgh}	8.41±2.02^{bcdefgh}	9.68±2.33^{cdefghij}	17.84±6.05^{gh}	7.90±1.47^{abcdef}	6.19±0.70^{abcdef}	6.38±0.72^{abcdef}	9.38±1.53^{cdefghij}	7.79±0.62^{abcdef}	10.15±0.87^{cdefghi}
	NPCC1298	3.07±0.25^{a}	2.99±0.24^{a}	3.23±0.24^{ab}	3.22±0.18^{a}	3.98±0.34^{ab}	3.45±0.33^{ab}	6.04±2.63^{abcd}	3.57±0.38^{ab}	3.58±0.17^{a}	3.75±0.37^{ab}	3.58±0.35^{ab}	3.09±0.09^{a}
	NPCC1309	5.12±0.14^{abcde}	5.63±0.69^{abcdef}	5.74±0.56^{abcdefg}	5.77±0.53^{abcde}	5.64±0.20^{abcde}	5.56±0.14^{abcde}	4.67±0.62^{abc}	5.62±0.09^{abcde}	5.41±0.71^{abcd}	5.60±0.35^{abcde}	5.67±0.27^{abcd}	6.24±0.70^{abcdefg}
	NPCC1314	5.88±1.05^{abcdefg}	6.02±1.09^{abcdef}	6.84±1.95^{abcdefgh}	6.82±1.97^{abcdefg}	7.16±2.28^{abcdefgh}	7.23±2.37^{abcdef}	7.50±2.45^{abcdef}	6.81±1.91^{abcdefg}	6.48±1.42^{abcdefg}	6.99±1.86^{abcdefg}	6.21±0.85^{abcde}	6.14±0.93^{abcdef}
	NPCC1317	2.93±0.046^{a}	3.94±1.06^{abc}	2.85±0.02^{a}	2.96±0.09^{a}	3.21±0.04^{a}	3.28±0.05^{a}	3.26±0.06^{a}	3.39±0.04^{ab}	3.21±0.07^{a}	3.24±0.08^{a}	4.42±1.20^{ab}	3.40±0.08^{ab}
	NPCC1321	4.45±0.63^{abcd}	4.49±0.51^{abcd}	4.31±0.40^{abcd}	4.51±0.44^{abc}	4.46±0.47^{ab}	4.83±0.52^{abcd}	4.84±0.52^{abc}	4.82±0.25^{abc}	5.27±0.46^{abcd}	5.54±0.38^{abcd}	6.03±0.36^{abcde}	6.11±0.32^{abcdef}
	NPCC1322	6.74±0.65^{cdefg}	7.41±0.88^{abcdef}	8.75±0.93^{efghi}	8.29±0.57^{bcdefgh}	8.52±0.74^{bcdefghi}	11.33±5.04^{abcdefg}	13.89±5.09^{defg}	10.83±2.78^{defghijk}	10.20±1.79^{efghijk}	9.69±2.02^{defghij}	10.70±1.86^{defgh}	11.95±1.60^{ghi}
	NPCC1323	6.14±0.46^{abcdefg}	7.02±0.42^{abcdef}	8.35±0.81^{defghi}	8.39±0.32^{bcdefgh}	8.18±0.87^{abcdefghi}	9.29±1.23^{abcdefg}	8.10±0.31^{abcdef}	7.67±0.44^{abcdefghj}	9.39±0.22^{cdefghij}	7.31±0.63^{abcdefg}	7.34±0.67^{abcdef}	5.59±0.68^{abcdef}
	NPCC1417	26.86±1.80^{i}	13.30±3.64^{gh}	8.54±1.30^{defghi}	9.30±1.72^{defghi}	14.58±1.30^{j}	8.15±0.65^{abcdef}	13.80±4.33^{defg}	11.57±3.31^{efghijkl}	9.53±1.44^{defghij}	9.17±1.70^{bcdefghi}	8.09±0.28^{abcdef}	8.89±3.30^{bcdefghi}
	NPCC1418	9.47±0.30^{g}	20.06±1.29^{i}	19.41±0.35^{j}	21.26±1.65^{j}	24.21±1.35^{k}	20.93±2.07^{h}	21.78±2.15^{g}	17.25±1.86^{l}	23.37±0.83^{m}	19.26±1.20^{l}	14.68±3.12^{g}	11.09±2.21^{fghi}
	NPCC1419	8.94±0.08^{efg}	10.29±1.22^{defgh}	12.76±2.07^{i}	12.61±2.48^{hi}	11.73±0.80^{fghij}	17.12±0.75^{fgh}	16.44±1.85^{efg}	16.48±0.27^{jl}	14.87±0.55^{kl}	17.75±1.84^{kl}	15.95±0.70^{g}	10.93±0.40^{defghi}
	CBS7001	15.21±1.73^{h}	14.99±0.49^{hi}	10.84±1.95^{hi}	11.72±0.01^{ghi}	14.83±0.69^{j}	15.05±0.54^{defgh}	13.62±0.36^{bcdefg}	14.53±0.27^{ijl}	14.27±0.78^{jkl}	15.25±0.92^{jkl}	15.13±0.39^{g}	15.21±0.01^{i}
	BMV58	4.51±0.01^{abcd}	4.62±0.03^{abcdf}	4.54±0.01^{abcdef}	4.58±0.10^{abcd}	4.57±0.01^{abcd}	4.56±0.06^{abcde}	4.56±0.00^{abcd}	14.55±0.60^{ijl}	4.54±0.00^{abcd}	4.58±0.03^{abcd}	4.68±0.02^{abc}	4.63±0.05^{abcde}
	CECT12600	5.07±0.01^{abcde}	4.84±0.04^{abcd}	4.95±0.10^{abcde}	4.97±0.11^{abcd}	5.10±0.03^{abcd}	5.01±0.05^{abcde}	5.02±0.00^{abcd}	5.09±0.01^{abcd}	5.18±0.04^{abcd}	4.83±0.11^{abcd}	5.05±0.04^{abc}	5.03±0.04^{abcd}
S.kudriavzevii	**CR85**	5.46±0.05^{abcdefg}	5.67±0.09^{abcdef}	5.73±0.16^{abcdefgh}	5.55±0.29^{abcdef}	5.46±0.05^{abcde}	5.56±0.04^{abcde}	5.48±0.04^{abcde}	5.64±0.05^{abcdefg}	5.43±0.06^{abcde}	5.67±0.00^{abcdef}	5.88±0.12^{abcde}	5.58±0.17^{abcdefg}
	CR90	5.38±0.06^{abcdef}	5.47±0.30^{abcdef}	5.78±0.18^{abcdefgh}	5.99±0.16^{abcdefg}	5.71±0.03^{abcdef}	5.79±0.51^{abcdef}	5.73±0.03^{abcdef}	5.69±0.03^{abcdefg}	5.79±0.05^{abcdef}	5.90±0.05^{abcdef}	5.58±0.14^{abcde}	6.28±0.08^{abcdefgh}
S.cerevisiae	**T73**	4.14±0.02^{abcd}	4.12±0.01^{abcd}	4.22±0.03^{abcde}	4.17±0.00^{abcd}	4.37±0.02^{abc}	4.15±0.01^{abcde}	4.19±0.00^{abcd}	4.15±0.00^{abc}	4.19±0.03^{abc}	4.39±0.24^{abcd}	4.84±0.11^{abcd}	4.11±0.02^{abcd}

Different superscript letters in the same column indicate significant differences (ANOVA and Tukey T. n=3).
Values are expressed in hours.
Parameters obtained from OD measurements after calculating by modified Gompertz equation.

Suppl Mat Table 3: Maximum fermentation rate (V_{max}) of different *Saccharomyces* strains evaluated by CO_2 release in synthetic media containing increasing nitrogen concentrations.

Species	Strains	YAN concentration (mg/L)					
		60		140		300	
		V_{max} (h^{-1})	λ_{co2} (h)	V_{max} (h^{-1})	λ_{co2} (h)	V_{max} (h^{-1})	λ_{co2} (h)
S. eubayanus	**NPCC 1282**	0.0065 ± 0.0002^{abcd}	12.5 ± 1.6^{cd}	0.0096 ± 0.00008^{ab}	11.2 ± 1.7^{abcd}	0.0105 ± 0.0007^{abc}	10.0 ± 2.0^{abcd}
	NPCC 1283	0.0071 ± 0.0002^{bcd}	14.6 ± 2.7^{de}	0.009 ± 0.001^{ab}	15.1 ± 2.5^{de}	0.0097 ± 0.0008^{ab}	11.6 ± 1.0^{bcde}
	NPCC 1285	0.0061 ± 0.0007^{abcd}	14.3 ± 0.4^{de}	0.0094 ± 0.001^{ab}	11.9 ± 0.9^{abcde}	0.0088 ± 0.0001^{a}	9.2 ± 0.0^{abc}
	NPCC 1292	0.0063 ± 0.0002^{abc}	15.2 ± 1.1^{de}	0.0101 ± 0.0005^{bc}	13.7 ± 2.6^{de}	0.0103 ± 0.0008^{ab}	12.8 ± 0.7^{cdef}
	NPCC 1296	0.0062 ± 0.005^{abcd}	17.1 ± 0.8^{ef}	0.0093 ± 0.0^{ab}	13.3 ± 0.4^{cde}	0.0097 ± 0.0001^{ab}	13.0 ± 0.9^{cdef}
	NPCC 1301	0.0060 ± 0.0004^{abc}	17.4 ± 0.2^{def}	0.0082 ± 0.0011^{ab}	12.6 ± 3.1^{bcde}	0.0126 ± 0.0007^{bcd}	13.6 ± 0.4^{defg}
S. uvarum	**NPCC 1288**	0.0061 ± 0.0002^{abc}	20.3 ± 2.1^{f}	0.0079 ± 0.0001^{ab}	14.5 ± 1.1^{de}	0.0117 ± 0.0007^{abc}	16.6 ± 1.5^{gh}
	NPCC 1290	0.0058 ± 0.0003^{ab}	15.9 ± 0.2^{def}	0.0073 ± 0.0014^{a}	12.7 ± 1.2^{cde}	0.0116 ± 0.0009^{abc}	15.7 ± 0.8^{fgh}
	NPCC 1314	0.0061 ± 0.0001^{abc}	15.3 ± 0.3^{de}	0.008 ± 0.0002^{ab}	13.2 ± 1.3^{de}	0.011 ± 0.0^{abc}	15.6 ± 2.8^{fgh}
	NPCC 1317	0.0059 ± 0.0003^{ab}	17.6 ± 1.6^{ef}	0.0074 ± 0.0^{a}	13.1 ± 0.6^{de}	0.0107 ± 0.0011^{abc}	15.1 ± 0.7^{fgh}
	NPCC 1321	0.0063 ± 0.0003^{abc}	16.4 ± 2.6^{def}	0.0081 ± 0.0^{ab}	13.3 ± 2.2^{cde}	0.0101 ± 0.0003^{abc}	18.0 ± 1.15^{h}
	NPCC 1418	0.0056 ± 0.0005^{ab}	16.2 ± 1.7^{def}	0.0077 ± 0.0012^{ab}	14.9 ± 1.4^{de}	0.0113 ± 0.0002^{abc}	16.4 ± 0.8^{gh}
	BMV58	0.0076 ± 0.0002^{cd}	6.6 ± 0.3^{eb}	0.0134 ± 0.0007^{de}	6.6 ± 1.3^{ab}	0.0165 ± 0.0005^{fg}	7.0 ± 0.8^{a}
	CBS 7001	0.0064 ± 0.0^{abcd}	16.1 ± 0.3^{def}	0.009 ± 0.0007^{ab}	12.6 ± 1.2^{cde}	0.0133 ± 0.0005^{cde}	15.6 ± 1.4^{fgh}
	CECT 12600	0.008 ± 0.0004^{d}	8.6 ± 0.5^{bc}	0.0156 ± 0.0006^{e}	7.8 ± 1.5^{abc}	0.0176 ± 0.0004^{g}	8.5 ± 0.3^{ab}
S. kudriavzevii	**CR85**	0.0056 ± 0.0004^{ab}	5.3 ± 1.8^{ab}	0.0123 ± 0.0002^{cd}	17.4 ± 0.8^{e}	0.0148 ± 0.0009^{def}	12.6 ± 0.8^{cdef}
	CR90	0.005 ± 0.0^{a}	1.9 ± 0.6^{a}	0.0124 ± 0.0004^{cd}	14.4 ± 1.6^{de}	0.0162 ± 0.001^{efg}	$14.2 \pm 0.4e^{fgh}$
S. cerevisiae	**T73**	0.0063 ± 0.0002^{abcd}	7.1 ± 1.2^{bc}	0.0077 ± 0.0^{ab}	5.8 ± 3.1^{a}	0.0164 ± 0.0027^{efg}	9.9 ± 1.5^{abcd}

Different superscript letters in the same column indicate significant differences (ANOVA and Tukey T. n=3).