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Interspecific hybridisation among diverse *Saccharomyces* species: A combined biotechnological solution for low-temperature and nitrogen-limited wine fermentations

Ying Su[a], Amparo Gamero[a,b], María Eugenia Rodríguez[c,d], Christian Ariel Lopes[c,d], Amparo Querol[a], José Manuel Guillamón[a,*]

[a] Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Carrer del Catedràtic Agustín Escardino Benlloch, 7, 46980 Paterna, Valencia, Spain
[b] Área de Tecnología de Alimentos, Facultad de Farmacia, Universitat de València, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain
[c] Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina – Universidad Nacional del Comahue), Buenos Aires 1400, 8300 Neuquén, Argentina
[d] Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, 8303 Cinco Saltos, Río Negro, Argentina

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ABSTRACT

Lack of the prezygotic barrier in the *Saccharomyces* genus facilitates the construction of artificial interspecific hybrids among different *Saccharomyces* species. Hybrids that maintain the interesting features of parental strains have been applied in industry for many beneficial purposes. Two of the most important problems faced by wine makers is nitrogen deficiency in grape must and low-temperature fermentation. In our study, hybrids were constructed by using selected low nitrogen-demanding cryotolerant *S. eubayanus*, *S. uvarum* strains and *S. cerevisiae*. The fermentation capacity of the hybrid strains was tested under four conditions by combining two temperatures, 12 °C and 28 °C, and two nitrogen concentrations, 60 mg/L and 300 mg/L. The hybrid strains obtained combined characters of both parental strains and conferred better fermentation rates under low-temperature or low-nitrogen conditions. The hybrid strains also produced larger amounts of acetate esters and higher alcohols, which increase aroma intensity and complexity in wine. Nitrogen sources were more rapidly consumed by the hybrid strains, which allows greater competition ability under nitrogen-deficiency conditions. Therefore, the interspecific hybridisation between low nitrogen-demanding cryotolerant strains and *S. cerevisiae* is a potential solution for low-temperature or low-nitrogen fermentations.

1. Introduction

Nitrogen is one of the substantial nutrients for yeasts that regulates biomass formation and fermentation activity, and provides important precursors for wine aroma-related compounds, such as higher alcohols, esters and volatile fatty acids (Bely et al., 1990; Bisson, 1991; Swiegers et al., 2005). Lack of nitrogen is one of the main causes of sluggish or stuck wine fermentation and H_2S production (Bell and Henschke, 2005; Giudici and Kunkee, 1994). While nitrogen plays a significant role in fermentation processes and sensory wine characters, yeast assimilable nitrogen (YAN) deficiency in grape must has been well-identified in many wine regions in Europe and elsewhere in the world (Butzke, 1998; Hagen et al., 2008; Henschke and Jiranek, 1993; Nicolini et al., 2004). Many studies have revealed that at least 140 mg/L YAN is needed to achieve complete wine dryness (Bely et al., 1990, Mendes-Ferreira et al., 2004). Technically speaking, a higher YAN concentration is needed to match a higher sugar concentration in grape must. Nitrogen addition to grape must can be one solution, but it is often difficult to handle. Excessive nitrogen additions may lead to the presence of non-assimilated residual nitrogen at the end of fermentation, which leads to microbial instability and ethyl carbamate accumulation in wine (Ough and Amerine, 1988). Therefore, it is of much interest to develop yeast strains with lower nitrogen requirements to complete fermentations.

The *Saccharomyces* genus has been used as an ideal paradigm for hybridisations. In spite of the fact that eight identified species (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, *S. uvarum*, *S. eubayanus* and *S. jurei*) are highly divergent in nucleotide sequences, they barely show any prezygotic barriers, which enables them to mate and form viable diploids (Morales and Dujon, 2012). Natural hybrids have been isolated from different fermentation processes. The most

* Corresponding author.
E-mail address: guillamon@iata.csic.es (J.M. Guillamón).

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well-known lager beer yeast *S. pastorianus* is a natural hybrid between *S. cerevisiae* and *S. eubayanus* (Gibson and Liti, 2015). Besides, *S. cerevisiae* x *S. uvarum*, *S. cerevisiae* x *S. kudriavzevii,* and even triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. uvarum,* have been isolated from wine, cider and beer (Antunovics et al., 2005; González et al., 2006; Krogerus et al., 2018; Le Jeune et al., 2007; Lopandic et al., 2007; Masneuf et al., 1998). Interspecific hybridisation could be an evolution strategy for better adaptation to the environment or could also be a result of domestication. Apart from natural hybrids, artificial hybridisation can be used as a powerful tool for developing yeast strains with desirable merits for industrial uses. Many hybrid strains have been constructed in laboratories to combine beneficial attributes, such as stress tolerance, aroma production, fermentation rate, sugar utilisation, etc. (reviewed by Bisson, 2017; Lopandic, 2018; Sipiczki, 2018; Sipiczki, 2019; Steensels et al., 2014). These artificial hybrids, together with natural interspecific hybrids, not only possess the characters of both parents, but also demonstrate superior performance in many cases (García-Ríos et al., 2019; Krogerus et al., 2017; Pérez-Través et al., 2015; Sipiczki, 2008). Some of them have been successfully used in the fermentation-based food and beverage industry. One example is a hybrid developed in our laboratory between *S. cerevisiae* and commercial strain *S. uvarum* Velluto BMV 58 (Lallemand Inc.) to improve the ethanol tolerance of *S. uvarum,* which has been commercialised as Velluto Evolution (Lallemand Inc.).

Fermentation at lower temperature is becoming increasingly popular because volatile compounds can be better preserved and it, therefore, provides wine with fruitier and fresher notes (Molina et al., 2007). However, cold temperature is one of the major stresses for *S. cerevisiae*, which largely influences the fermentation rate. *S. eubayanus* and *S. uvarum* are two of the most applied species for artificial hybridisation. Their well-known cryophilic nature favours the requirement of low-temperature fermentation. *S. uvarum* strains have been isolated as the dominant strain from different fermentation environments, including wine, cider and apple chicha (reviewed by Rodríguez et al., 2016). This species, together with *S. cerevisiae*, is able to tolerate different stress conditions and allows wine fermentations to finish (Alonso-del-Real et al., 2017b). Physiologically, *S. uvarum* produces less acetic acids and ethanol, but more glycerol than *S. cerevisiae* (Castellari et al., 1994; Masneuf-Pomarède et al., 2010). In particular, this species produces more 2-phenylethanol and 2-phenylethyl acetate, which confer wine a pleasant rose-like odour (González Flores et al., 2017; Masneuf-Pomarède et al., 2010; Stribny et al., 2015). *S. eubayanus* was first isolated and identified by Libkind et al. (2011) from Patagonia. After its identification, it has been isolated in various locations in North America (Peris et al., 2014), China (Bing et al., 2014) and New Zealand (Gayevskiy and Goddard, 2016), and also from other substrates and locations in Patagonia (Rodríguez et al., 2014). However, no isolate has yet been obtained from Europe. Keen research interest has been shown in *S. eubayanus* after its identification. Nowadays, the beer fermented only by *S. eubayanus* can be found on the market.

Several studies have previously constructed hybrids between *S. cerevisiae* and *S. eubayanus*, *S. uvarum* aiming to improve the cryophilic character of *S. cerevisiae* (Diderich et al., 2018; García-Ríos et al., 2019; Hebly et al., 2015; Kishimoto, 1994; Magalhães et al., 2017; Origone et al., 2018; Rainieri et al., 1998; Zambonelli et al., 1997). However, so far, hybridisation has not been used to improve the nitrogen requirements of a wine yeast. In our previous study (Su et al., 2019), we compared the nitrogen requirements of the *S. eubayanus* and *S. uvarum* Patagonian strains to *S. cerevisiae* by carrying out both fermentation and growth experiments with different nitrogen concentrations and several temperatures. Our results revealed that both species, especially the *S. eubayanus* strains, have relatively lower nitrogen requirements than the *S. cerevisiae* control strain. Therefore, the combination of two interesting oenological features, cold tolerance and low-nitrogen requirements, in *S. eubayanus* and *S. uvarum* makes these two species ideal candidates for hybridising with *S. cerevisiae*. Thus, the objective of our study was to improve yeast fermentation activity at a low temperature and a limited nitrogen concentration by constructing hybrids between *S. cerevisiae* and the low nitrogen-demanding cryotolerant strains of *S. eubayanus* and *S. uvarum*. In fact, a low-temperature and nitrogen-limited fermentation is a very challenging situation for wine yeasts. Both conditions produce similar metabolic and transcriptional effects, decreasing biomass yield and fermentation rate during wine fermentation (Beltran et al., 2007; Pizarro et al., 2008).

Different strategies to construct artificial hybrids, including protoplast fusion, rare mating, spore to spore mating, mass mating, etc., have been successful applied (reviewed by Morales and Dujon, 2012; Sipiczki, 2008; Steensels et al., 2014). In this study, we applied the direct cell to cell mating method which is simply mixing cell cultures of two selected stable haploid parents. This approach is not used regularly to develop novel yeast hybrids due to the homothallic nature of most industrial yeast strains, making them unsuited for this approach. However, homothallic strains would be amenable to this approach after genetically disrupting the *HO* endonuclease gene, a gene responsible for mating-type switching (Katz Ezov et al., 2010), making these strains fit for cell-to-cell mating experiments (Steensels et al., 2014). Despite this requires a genetic transformation, which implies that the resulting hybrid is classified as a GMO, we used this direct and quick hybridisation method. We aimed at this stage to get a proof of concept that hybridisation of *S. cerevisiae* with *S. eubayanus* and *S. uvarum* was a feasible method to get strains with good fermentation performance at low temperature and nitrogen-limited conditions. The constructed hybrids were evaluated by conducting fermentations under four different conditions by combining two temperatures and two nitrogen concentrations. Fermentation metabolite and volatile compounds production by different yeast strains was determined. Nitrogen assimilation during fermentation was monitored throughout the fermentation process.

2. Materials and methods

2.1. Strains and media

S. eubayanus (*Se*) strain NPCC1285 was isolated from *A. araucana* seeds and *S. uvarum* (*Su*) strain NPCC1317 was obtained from fermented apple chichi, both from the Patagonia region in Argentina. These strains were identified as low nitrogen-demanding strains in our previous work (Su et al., 2019) and were used as cryotolerant parental strains. Lalvin T73 (Lallemand Inc., Montreal, Canada) was used as the *S. cerevisiae* (*Sc*) parental strain.

Yeast extract peptone dextrose (YPD) medium, which contains 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract, was used for yeast propagation.

Fermentations were carried out with synthetic grape must (SM) which was prepared as described by Riou et al. (1997) with some modifications. This medium contains 200 g/L of sugar as a 50% glucose and 50% fructose mix. The concentrations of organic acids were the following: 5 g/L malic acid, 0.5 g/L citric acid and 3 g/L tartaric acid. Minerals and vitamins were supplied at the same concentration as those described by Su et al. (2019). The composition of nitrogen sources in the SM was 40% of ammonium chloride and 60% of amino acids. The composition of amino acids in the 1L stock was 1.5 g L-tyrosine, 13.4 g L-tryptophan, 2.5 g L-isoleucine, 3.4 g aspartic acid, 9.2 g glutamic acid, 28.3 g L-arginine, 3.7 g L-leucine, 5.8 g L-threonine, 1.4 g glycine, 38.4 g L-glutamine, 11.2 g L-alanine, 3.4 g L-valine, 2.4 g L-methionine, 2.9 g L-phenylalanine, 6 g L-serine, 2.6 g L-histidine, 1.3 g L-lysine, 1.5 g L-cysteine and 46.1 g L-proline, which corresponded to 13.75 g/L of Yeast Assimilable Nitrogen (YAN is the sum of the amino acid and ammonium concentrations, except proline). The final pH of the SM was adjusted to 3.3 with sodium hydroxide. Synthetic musts were sterilised by filtration through 0.22 µm pore-sized membrane filters (Thermo scientific, MA, USA).

2.2. Hybrids construction

Firstly, we generated stable haploid versions of the diploid parental strains by deleting one copy of the *HO* gene using the dominant drug resistance markers nourseothricin and hygromycin (Goldstein and McCusker 1999). The *HO* deletion was performed by PCR-mediated gene replacement (short flanking homology method). The deletion cassette was obtained by PCR using the pAG25 plasmid that contains nourseothricin resistance and the pAG32 plasmid that contains hygromycin resistance. Namely, one copy of the *HO* gene of T73 was replaced with *NatMX6* and the *HO* genes of *S. eubayanus* and *S. uvarum* were replaced with *HphMX6*. The primers used for gene deletion are listed in Supplementary Table 1. Transformation was performed by the lithium acetate method (Gietz and Schiestl, 2008). Transformants were selected by resistance to nourseothricin or hygromycin, and correct deletion cassette integration was confirmed by diagnostic PCR using the primers upstream and downstream of the deleted region (Supplementary Table 1).

The successfully transformed diploid parental strains (heterozygous for *HO*) were sporulated on acetate medium (1% potassium acetate, 2% agar) for 5–7 days. Asci were digested with 0.5 mg/mL zymolyase at 37 °C for 30 min. Tetrads were dissected using a micromanipulator (MSM 400; Singer Instruments, Watchet, UK) on YPD agar plates (2% Bacto peptone, 1% yeast extract, 2% glucose, and 2% agar). Viable spore clones were then streaked onto YPD with nourseothricin or hygromycin agar plates for Δ*ho* spore clone selection. The haploid cells with nourseothricin or hygromycin resistance were again verified by diagnostic PCR, as mentioned above.

The Δ*ho* haploid strains from both parental with opposite mating types were mixed on the same spot on complete media (YPD) and grown for 24 h. Cells were then harvested, diluted and plated on YPD plates containing both hygromycin and nourseothricin to select diploid hybrids. These hybrids were then confirmed by the *Msp*I restriction pattern of the *KEL2* gene (Pérez-Través et al., 2015). Eleven hybrids of each combination (*ScxSu* and *ScxSe*) were tested for growth capacity at 60, 140 and 300 mg/L YAN, and no significant differences among the different hybrids were observed (data not shown).

2.3. Fermentation activity analysis

Fermentations were carried out in 100-mL bottles with 80 mL of the SM at two nitrogen concentrations (60 and 300 mg/L YAN) at 12 °C or 28 °C. The SM was inoculated with both pure and mix cultures to a population size of 2×10^6 cells/mL. The mix-culture fermentations were inoculated with 50% of each of the two tested strains. Fermentation kinetics was followed by measuring the density reduction of the SM. Fermentation was considered finished when density went below 998 g/L (Gutiérrez et al., 2012).

Unlike *S. cerevisiae*, *S. uvarum* and *S. eubayanus* strains are less heat-tolerant and are unable to form colonies on YPD plates at 37 °C. Therefore, in the mix-culture fermentations, the percentage of *S. cerevisiae* was determined simply by plating the sample on two YPD plates and incubating at 30 °C and 37 °C. Colony-forming units (CFU) were counted after 2 days of incubation. The CFU number on the 37 °C plates represents the *S. cerevisiae* population and the CFU difference between the two plates represents the population of the non-*cerevisiae* strains in the mixed culture (Su et al., 2019).

2.4. HPLC analysis for residual sugars and fermentation metabolites

At the end of the fermentations, samples were taken and analysed for the main wine chemical parameters, including glucose, fructose, ethanol, glycerol, succinic acid and acetic acid. Samples were first centrifuged to remove yeast cells and the supernatants were diluted 3 times with deionised water to then be filtered through 0.22 µm pore-sized nylon filters (Phenomenex, CA, USA). HPLC, equipped with a refraction index detector and a UV–Visible detector, was used for the analysis. The employed mobile phase was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min. Metabolites were separated by a HyperREZ XP Carbohydrate H+ 8 mm column with a column temperature of 45 °C (Thermo Scientific, MA, USA).

2.5. HPLC analysis for residual amino acids

The analysis of the residual amino acids was carried out in a ultimate 3000® UPLC (Thermo Scientific, MA, USA) equipped with a UV–visible detector (Thermo Scientific, MA, USA). The HPLC analysis method is based on Gómez-Alonso et al. (2007), but with some modifications. The 400 µL samples were derivatised with 12 µL diethylethoxymethylenemalonate (DEEMM), together with 300 µL of methanol. The reactions were performed in screw-cap test tubes in an ultrasonic bath for 30 min, followed by heating at 80 °C for 2 h to degrade any excess DEEMM. After derivatisation, samples were filtrated through 0.22 µm nylon syringe filters (Phenomenex, CA, USA). Chromatographic separation was performed with an Accucore® C18 LC column (Thermo Scientific, MA, USA). The binary gradient was applied (phase A: 25 mM acetate buffer, pH 6.0, and phase B: acetonitrile) at a flow rate of 1.2 mL/min and a column temperature of 30 °C. The gradient is shown in Supplementary Table 2.

2.6. GC analysis for volatile compounds

Higher alcohols and esters were analysed by the headspace solid-phase microextraction (HS-SPME) technique using a 100 µm poly-dimethylsiloxane (PDMS) fibre (Supelco, Sigma-Aldrich, Madrid, Spain). The extraction method was the same as that described by Stribny et al. (2016). 2-heptanone (0.005%) was added as an internal standard. A TRACE GC Ultra® gas chromatograph (Thermo Scientific, MA, USA) with a flame ionization detector (FID) was used, equipped with an HP-INNOWax 30 m × 0.25 mm capillary column coated with a 0.25 µm layer of cross-linked polyethylene glycol (Agilent Technologies, CA, USA). The oven temperature programme was: 5 min at 35 °C, 2 °C/min to 150 °C, 20 °C/min to 250 °C and 2 min at 250 °C. The detector temperature remained constant at 300 °C. Chromatographs were analysed by the Chrom Quest programme. Volatile compounds were identified by the retention time for the reference compounds. Quantification of volatile compounds was determined using the calibration graphs of the corresponding standard volatile compounds.

2.7. Statistical analysis

All the fermentations were carried out in triplicate. Fermentation kinetics was calculated by fitting the density data to the 4-parameter logistic model. The time needed to consume all the sugars (T100) was extracted from the smoothed data. One-way analysis of variance (ANOVA) was conducted with version 7.0 of the Statistica software package. The statistical level of significance was set at $p \leq 0.05$ with a Tukey HSD test. For the nitrogen uptake order analysis, the area under the curve (AUC) value was calculated by using the R statistical software v. 3.0 with the "growth curver" package (Sprouffske, 2018). A heatmap was plotted by Mev MultiExperiment Viewer, and hierarchical clustering was based on Euclidean distance metrics. A principal component analysis (PCA) was performed using the software package LatentiX 2.12 (http://www.latentix.com). Data were autoscale-normalised.

3. Results

3.1. Fermentation kinetics and cell composition in the mix-culture fermentation

Fermentations were carried out at four different conditions with a combination of two temperatures, 12 °C and 28 °C, and two nitrogen

concentrations, 60 mg/L and 300 mg/L YAN. The strains used in these fermentations were parental strains *S. cerevisiae* (*Sc*), *S. eubayanus* (*Se*) and *S. uvarum* (*Su*) and two hybrid strains, *Sc*x*Se* and *Sc*x*Su*. For comparison purposes, the mix-culture fermentations between *S. cerevisiae* and the cryotolerant parental strains (*Sc* + *Se* and *Sc* + *Su*) were also carried out. The results indicated that 12 °C and 60 mg/L YAN formed an extremely harsh condition and none of the tested strains or combinations of strains were able to complete fermentations within 500 h. However, the lowest residual sugar concentration was detected in the *Su* fermentation, which was > 3-fold lower than the *Sc* fermentation (21 vs. 75 g/L; Supplementary Table 3). For the other fermentation conditions, the time needed by the different strains to end fermentations (T100) are shown in Fig. 1 (A–C). As expected, *Sc* displayed such better performance and spent less time to finish fermentations than *Se* and *Su*, mainly under the optimum condition for *S. cerevisiae* (28 °C and 300 mg/L YAN). The cryotolerant strains carried out the fermentations more slowly. They had difficulties to deplete all the sugars at 28 °C and 60 mg/L YAN and > 10 g/L fructose remained in the must (Supplementary Table 3). On the contrary, the hybrid strains displayed outstanding fermentation performance, and even exceeded their *Sc* parental strain by completing fermentations in shortest time under all the conditions. The fermentation time of the mix-culture fermentations was somewhere between that of the *Sc* and cryotolerant strains. In order to determine the competitiveness of each strain in the mix-culture fermentations, the percentage of each inoculated strain was also monitored (Supplementary Fig. 1). Our results showed that temperature strongly influenced the imposition of *Sc* on *Se* and *Su*. *Sc* was less competitive than the cryotolerant strains at 12 °C. Regardless of nitrogen content, at the end of these low temperature fermentations, the percentage of *Sc* represented only 20–30% of the total population. In contrast at 28 °C, the percentage of *Sc* was around 60%–80%. A high nitrogen concentration also favoured the implementation of *Sc*, but the influence was not as determinant as temperature.

1.1 Metabolites production by different fermentations

At the end of the fermentations, major metabolites were analysed by HPLC (Supplementary Table 3). The yields of ethanol, glycerol, and organic acids by sugar consumption were calculated. No significant differences were observed for the ethanol yields in most of the fermentation conditions (Fig. 2, Supplementary Table 4). However, significant differences were observed for the glycerol yields for the various tested conditions. Generally speaking, *Sc* was a low glycerol producer, whereas the cryotolerant strains gave the highest glycerol yields for all the conditions. Interestingly, under the low-nitrogen condition at 28 °C, the hybrid strains achieved significantly higher glycerol production than their *Sc* parental strain (> 2 g/L higher), but no significant differences were found in the fermentations done with 300 mg/L YAN. Acetic acid synthesis also strongly depended on yeast strains. The hybrid strains produced significantly smaller amounts of acetic acid than *Sc*, especially when nitrogen was limited in the SM. *Su* was the lowest acetic acid producer under all the conditions. Another metabolite whose concentration seemed to be nitrogen-dependent was succinic acid. A negative correlation was found between nitrogen concentration and succinic acid production, with higher yields for all the strains when nitrogen was limited.

3.2. Quantification of volatile compounds

Volatile compounds production was considerably influenced by fermentation temperature, nitrogen concentration and yeast strains (Supplementary Table 5). The concentrations of eleven volatile compounds were determined, and they were classified as ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate, diethyl succinate), acetate esters (ethyl acetate, isoamyl acetate, 2-phenylethyl acetate), and higher alcohols (isobutanol, isoamyl alcohol, benzyl alcohol, 2-phenylethanol). The unfinished fermentations at 12 °C and 60 mg/L of YAN were not included in the comparisons. Supplementary Fig. 2 shows the distribution of these three groups of aroma compounds from all the fermentations under the same condition. Regardless of the inoculated strain, the fermentations carried out with limited nitrogen demonstrated slightly higher acetate esters and ethyl esters production, but these differences were not statistically significant. Higher alcohols production was strongly influenced by fermentation conditions. A much larger amount of higher alcohols was produced when fermentations were carried out with 60 mg/L YAN, with double the concentration of that in the fermentations run with 300 mg/L YAN. A PCA was carried out with the aroma data of all the fermentations (Fig. 3). The first two principal components accounted for 65% of total variance. The topography distribution of the PCA showed that the environmental factors (nitrogen and temperature) more strongly impacted aroma production than the inoculated strain because all the strains were grouped according to fermentation conditions. The only exceptions were strains *Se* and *Su* at 28 °C/300 mg/L YAN, which grouped with the samples at low temperature, which could be interpreted as a consequence of their cryotolerant feature. The fermentations performed with the higher YAN concentration grouped mainly for the higher production of some ethyl esters, isoamyl acetate, and benzyl alcohol, while the fermentations performed with the lower YAN concentration stood out for higher alcohols production.

Se produced the largest amount of acetate esters (mostly ethyl acetate) at 28 °C and *Su* exhibited the same behaviour at 12 °C (Fig. 4; Supplementary Table 5). Conversely, *Sc* and hybrids yielded larger amounts of ethyl esters, mainly diethyl succinate, than the cryotolerant parental strains at low temperature or under low-nitrogen conditions. Significant differences in ester production were observed by the two hybrids: *Sc*x*Se* produced a higher level of ethyl esters than *Sc*x*Su*, regardless of the fermentation conditions. Regarding the higher alcohols, *Sc* and the hybrid strains were the best producers at 28 °C. At 12 °C, hybrids also maintained good higher alcohols production, whereas the synthesis of *Sc* significantly lowered. The production of 2-phenylethyl acetate and 2-phenylethanol was higher for both the cryotolerant and hybrid strains, and occurrence was more obvious for the fermentations conducted at low temperature or at a limited YAN concentration (Supplementary Table 5). The mix-culture fermentations generally gave intermediate production values between the pure culture of *Sc* and the pure cultures of the cryotolerant strains, but behaviour was more irregular because it depended mostly on the degree of imposition of each inoculated strain (Supplementary Fig. 1).

3.3. Nitrogen source uptake order by hybrid strains under different conditions

Nitrogen source preference and nitrogen source assimilation order shed light on the nitrogen metabolism regulation by certain yeast species. In order to explore nitrogen uptake order by hybrids and their parental strains, the residual nitrogen in the must was analysed by HPLC during fermentation. The percentage of nitrogen consumption was plotted against time as the nitrogen consumption curve. The area under the curve (AUC) provided comprehensive information about the time to start consumption, the consumption rate and the maximum consumption percentage. A high AUC value indicates a more rapid complete consumption of certain nitrogen sources. Conversely, a low AUC value denotes slow or incomplete consumption (individual consumption curves are found in Supplementary Figs. 3–6). Although the fermentations run at 12 °C with 60 mg/L YAN did not finish, the nitrogen consumption under this condition was complete. Therefore, this condition was included for the analysis. For an overview of nitrogen consumption efficiency, the AUC value of the total YAN consumption was calculated for the different strains and conditions (Fig. 5). The nitrogen uptake rate of the parental strains was clearly influenced by fermentation temperature. *Sc* showed the quickest nitrogen

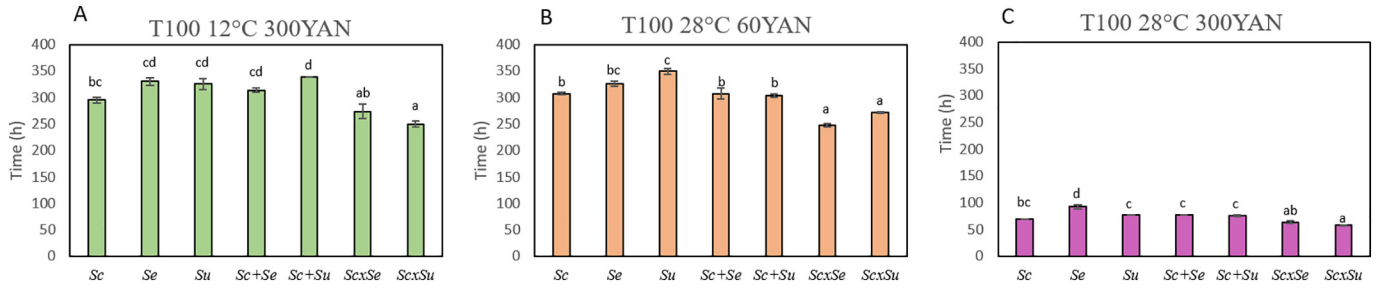

Fig. 1. A-C. Time (hours) required to consume the 100% (T100) of the sugar content in the synthetic must with 12 °C 300 mg/L YAN, 28 °C 60 mg/L YAN, and 28 °C 300 mg/L YAN. The letters at the top of bars indicate the significant difference groups (HSD Tukey test $p \leq 0.05$).

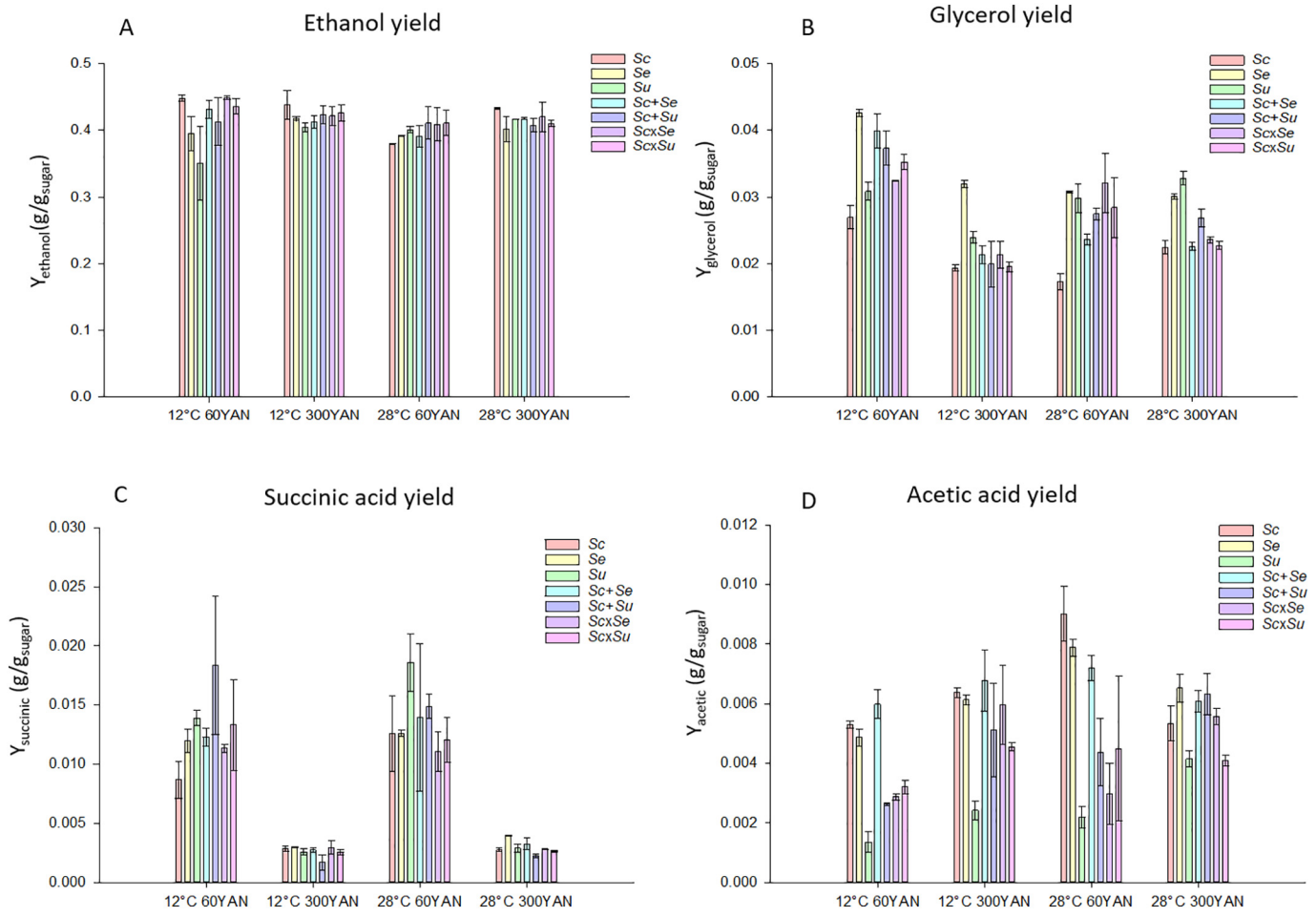

Fig. 2. Yield of major metabolites ethanol, glycerol, succinic acid and acetic acid, at the end of fermentations. 60YAN and 300YAN indicate that the assimilable nitrogen concentration was 60 mg/L and 300 mg/L respectively. Different strains are represented by different coloured bars.

consumption efficiency at a high temperature, while *Se* and *Su* more rapidly consumed total nitrogen at 12 °C. These differences were more pronounced with 300 mg/L YAN because all the strains quickly exhausted total nitrogen under the 60 mg/L YAN conditions. It is noteworthy that the hybrid strains were more similar to the fittest parent at each temperature.

The AUC values of 20 individual nitrogen sources for each fermentation condition were also calculated and ranked from high to low to indicate the uptake order. Ranking orders were represented on a heatmap with hierarchical clustering (Fig. 6). Three major groups (A-C) were obtained that, as with aroma production (PCA; Fig. 3), were determined mainly by the fermentation conditions. Once again, the exception came from the parental *Se* and *Su* at 28 °C and 300 mg/L YAN, which were grouped in cluster C (12 °C and 300 mg/L of YAN). Another interesting exception was the parental *Sc* and the two hybrids at 28 °C and 300 mg/L of YAN, which were not included in any of these major groups and represented the three conditions with a shorter fermentation time.

Two main groups were obtained when this hierarchical clustering was applied to the individual 19 amino acids and ammonium chloride (Fig. 6). Regardless of the fermentation conditions, the green cluster gathered the most rapidly consumed amino acids (the greenest squares) and the yellow cluster grouped all the amino acids of a delayed uptake (the yellowest squares). Black squares represent the amino acids of intermediate consumption. A more detailed consumption order for each individual nitrogen source for the different strains and fermentation conditions is found in Fig. 7. As reported for other *S. cerevisiae* strains, Lys was practically the first amino acid to be consumed by all the strains

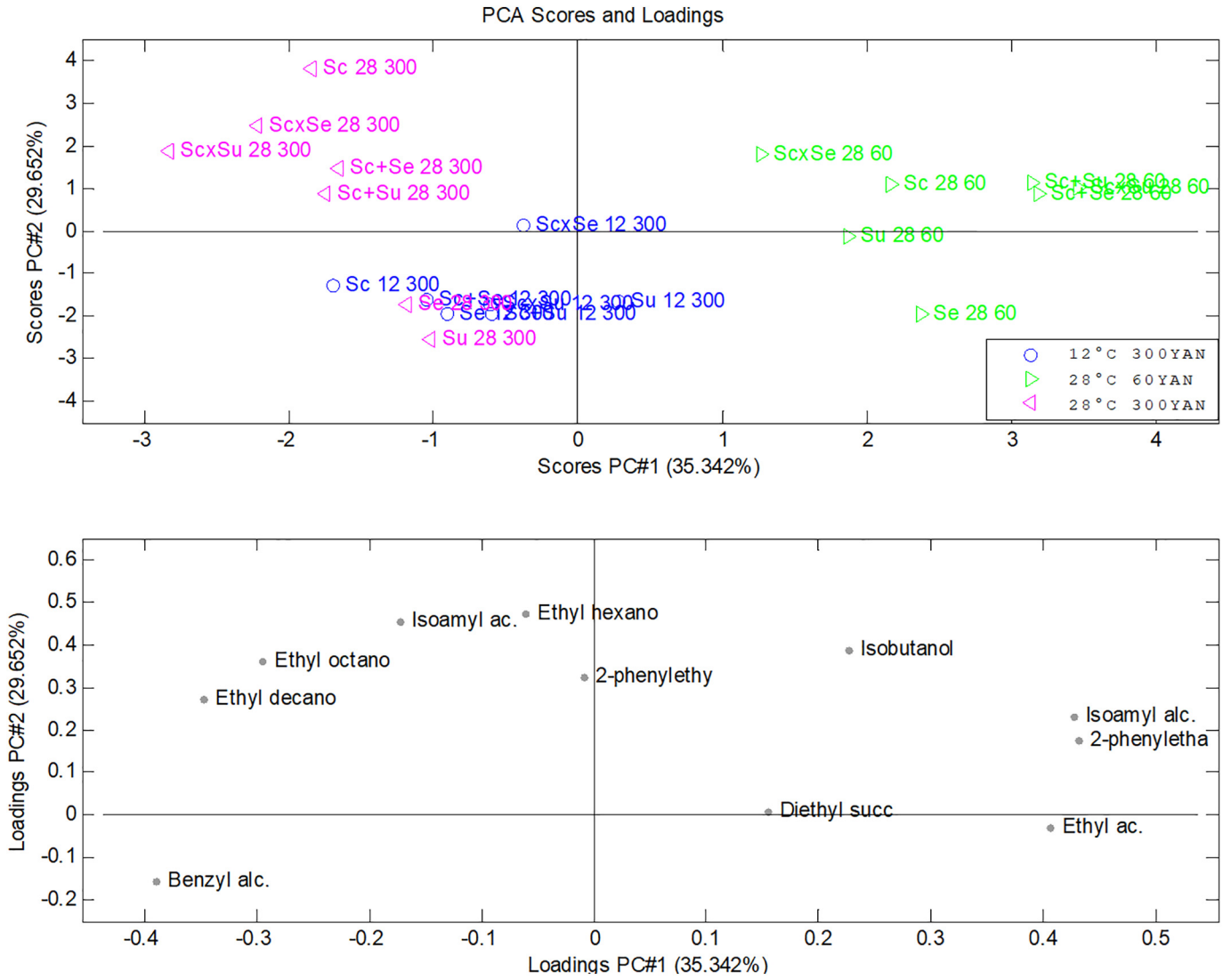

Fig. 3. The PCA plot of the volatile compounds produced by different strains under three fermentation conditions. Scores represent fermentations. Loadings denote different volatile compounds.

and fermentation conditions, followed by other amino acids that were early consumed, e.g. Ile, His, Asp, Leu, Arg, Ser and Thr. Lastly, Pro, Cys, Glu, Ala and Gly were among the last consumed amino acids. From this individual nitrogen source consumption overview, some strain-specificities are worth mentioning. The biggest differences among strains were detected at 28 °C and 300 mg/L YAN as total nitrogen was quickly exhausted in the nitrogen-limiting fermentations. *Sc* was the fastest nitrogen-consuming strain that depleted all the nitrogen sources within 30 h, except for proline and cysteine (Supplementary Fig. 4). The hybrid strains showed similar consumption profiles to *Sc*, and almost all the sources were depleted. Conversely, *Se* and *Su* consumed nitrogen more slowly than *Sc*, with many residual nitrogen sources after 30 h of fermentation. As expected, both nitrogen concentration and fermentation temperature clearly conditioned the uptake profile of the amino acids in the different strains. The cryotolerant strains consumed Arg more quickly in the nitrogen-limiting fermentations than in 300 mg/L of YAN. The Trp consumption performed by all the strains at 12 °C was much slower than that at 28 °C. *Su* consumed Asp more quickly than the other strains at 12 °C. One remarkable finding was the preference Phe consumption by the cryotolerant and hybrid strains. In all the strains, Tyr was very rapidly consumed at 12 °C with 300 mg/L YAN (Fig. 7).

4. Discussion

4.1. Advantage of interspecific hybridisation for yeast strain adaptation to different fermentation environments

Interspecific hybridisation in the *Saccharomyces* genus was successfully applied for yeast strain improvement. Strains were crossed to obtain robust hybrids that carried different favourable traits of parental strains. The hybrid strains between *S. cerevisiae*, and the selected low nitrogen-demanding cryotolerant strains of *S. eubayanus* and *S. uvarum* (Su et al., 2019), were constructed herein. These hybrids showed good adaptation to different fermentation conditions and outcompeted their parental strains in fermentation rate terms. The phenomenon in which hybrids possessed phenotypic superiority over parental strains has been previously reported and is known as heterosis or hybrid vigour (Lippman and Zamir, 2007; reviewed by Steensels et al., 2014). The hybrid process provides a heterozygous advantage to buffer against deleterious recessive alleles and provides genetic plasticity to adapt to variable environmental conditions. Apart from hybridisation, the co- or sequential inoculation of non-*Saccharomyces* or *Saccharomyces* non-*cerevisiae* strains has also been suggested as an oenological practice with multiple purposes, such as increasing aroma complexity, lowering ethanol content and increasing glycerol in wine (Alonso-del-Real et al.,

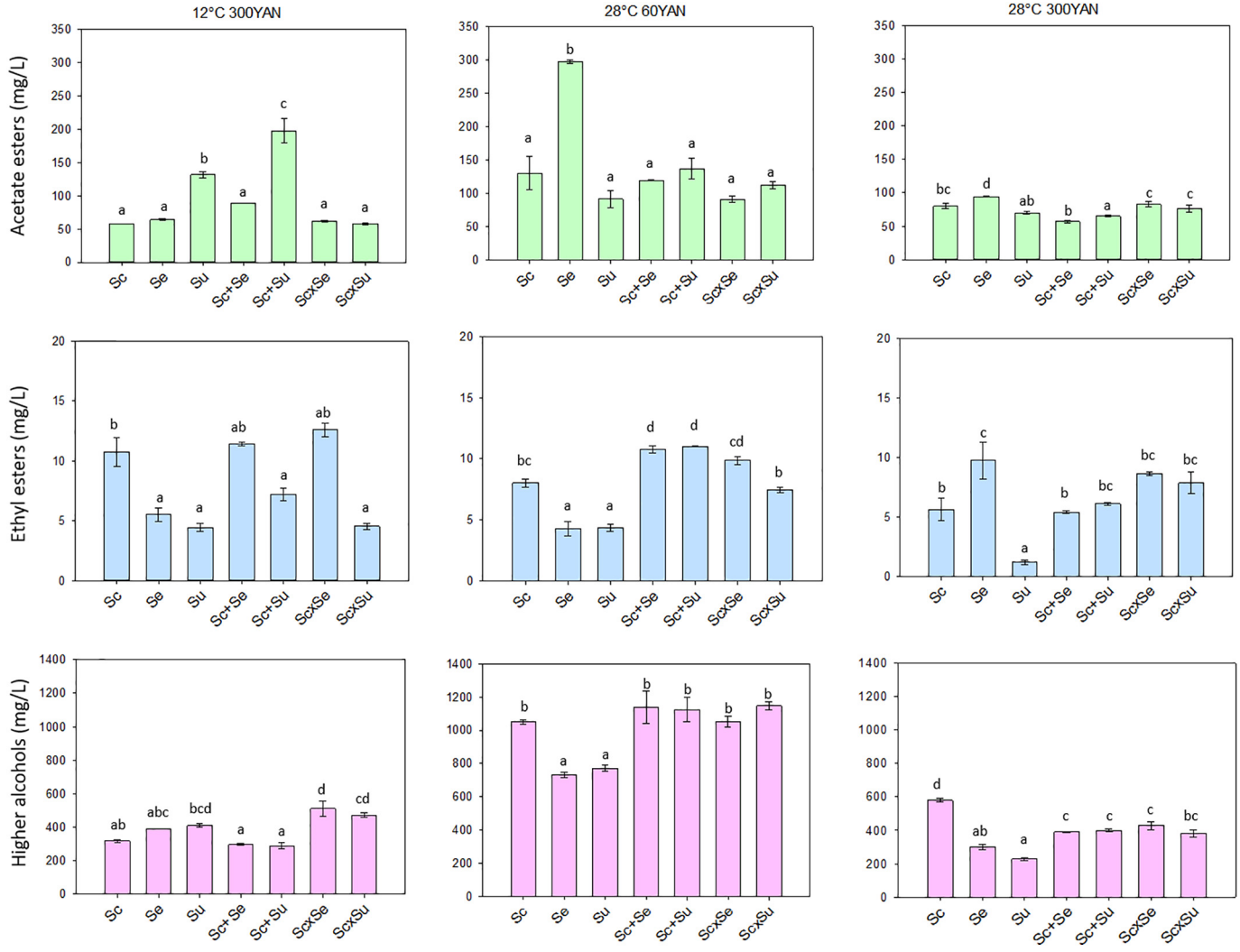

Fig. 4. Production (mg/L) of acetate esters, ethyl esters and higher alcohols by different strains under three fermentation conditions.

2017a; Jolly et al., 2014; Padilla et al., 2017; Zhang et al., 2018). For comparison purposes, mix-culture fermentations were also included herein. As in our previous work (Su et al., 2019), we observed that the population of different strains was strongly influenced by both fermentation temperature and nitrogen concentration. Low temperature favoured the growth of *S. eubayanus* and *S. uvarum*, while higher temperature gave rise to *S. cerevisiae* dominating in fermentation, especially at a high nitrogen concentration. Generally speaking, the mix-culture fermentation behaviour was similar to that of the strains with a higher carrying capacity. The intermediate metabolites production level between both strains in the mix-culture fermentation was also observed. Although the mix-culture fermentation improved the fermentation rate under suboptimum conditions, they were not as efficient as the hybrid strains. For applications in industry, the instability of the co- or sequential inoculation could be more difficult to manage, and wine quality could be hard to predict. Therefore, interspecific hybridisation could be a better alternative to improve yeast fermentation capacity.

4.2. Influence of fermentation conditions on yeast metabolism

Fermentation conditions have greatly impact yeast metabolism. The combination of two temperatures and two YAN concentrations was applied in the present study to explore the behaviour of the hybrid strains. The factorial ANOVA illustrated that the fermentation temperature, the initial nitrogen concentration, strains and the combination of different factors significantly impacted most of the metabolites herein analysed (Supplementary Table 6). Specifically, nitrogen availability strongly influenced succinic acid production. A > 2-fold larger amount of succinic acid was produced when fermentations were carried out at a lower nitrogen concentration. Rollero et al. (2015) analysed the combined effects of nitrogen, lipids and temperature on fermentation metabolites production. Correspondingly, they also pointed out that succinic acid production was negatively regulated by the initial nitrogen concentration. The possible involved mechanism was explained by Camarasa et al. (2003): when nitrogen is limited in media, less glutamate is produced from α-ketoglutarate. Hence succinic acid synthesis acts as a safety valve (Rollero et al., 2015) that directs the accumulated α-ketoglutarate pool to the TCA cycle. Thus nitrogen-limited fermentations could potentially contribute to biological acidification, a very desirable process, if we take into account the current drop in titratable acidity, or the increase in pH, in grape musts and wines as a consequence of climate change (Jones et al., 2005). Acetic acid yield is a substantial wine quality factor because it confers off-flavour. Our results demonstrated that the hybrid strains produced significantly less acetic acid than *Sc*, especially when nitrogen was limited in the SM. This feature could be inherited from cryotolerant parental strains as *S. uvarum* and *S. eubayanus* produced lower acetic acid levels. In general, acetic acid production was quite high in our study, which could be due to the absence of lipids in our SM. As previously described (Beltran et al., 2008; Rollero et al., 2015), acetic acid

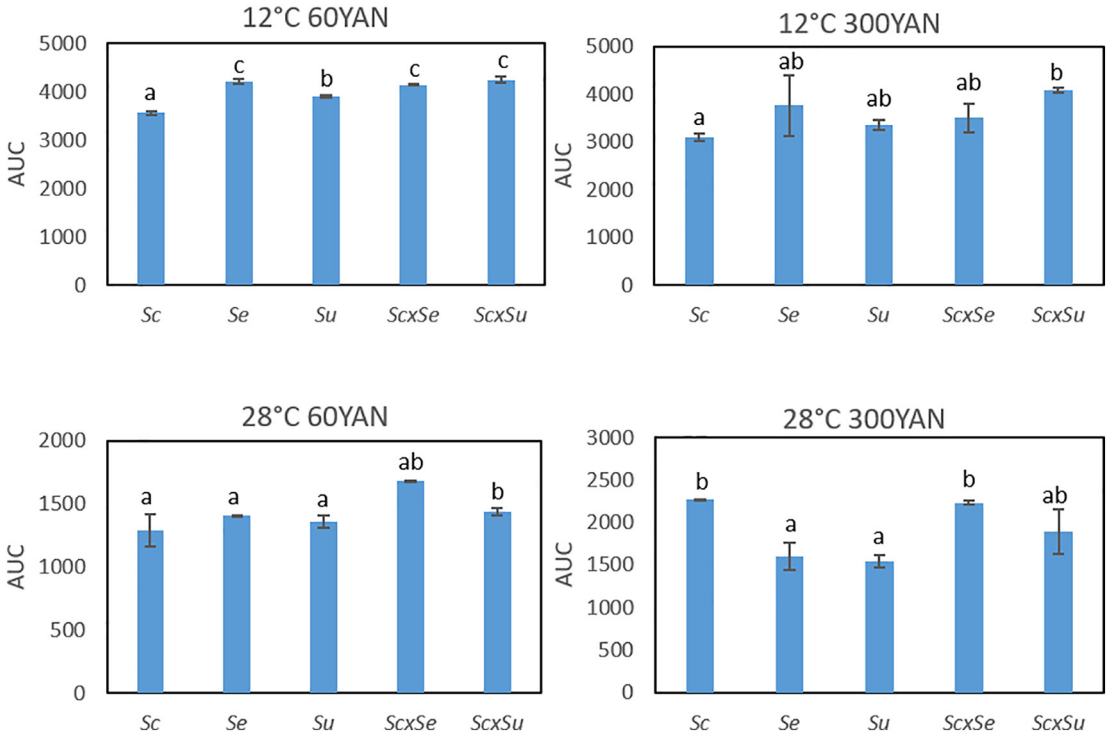

Fig. 5. The AUC value of total nitrogen consumption for the different strains under each fermentation condition. AUC provides the summary information of the maximum consumption percentage of YAN during the time-lapse analysed.

production is largely influenced by lipid concentration, and more acetic acid is released by yeast in the SM than in natural grape must due to the difference in lipid concentration (Beltran et al., 2008).

Volatile compounds production was also strongly impacted by the fermentation conditions. The largest amount of volatile compounds was generally produced when fermentations were carried out at 28 °C with 60 mg/L YAN. Previously, several authors (Beltran et al., 2007; Carrau et al., 2008; Rollero et al., 2015) have demonstrated the negative relation between nitrogen concentration and higher alcohols production. The larger amount of higher alcohols synthesis under nitrogen-limited conditions could be due mainly to the de novo synthesis of branched-chain amino acids (BCAAs) through the anabolic pathway (reviewed by Stewart, 2017). In our study, higher production took place only for higher alcohols, but also for ethyl esters and acetate esters at low nitrogen concentrations. Interestingly, ethyl acetate and diethyl succinate were two compounds with the biggest contribution for the concentration of acetate esters and ethyl esters, respectively. The production of these two compounds was higher when nitrogen was limited and matched the very large succinic and acetic acids productions. Without considering ethyl acetate and diethyl succinate, a positive correlation between acetate esters and ethyl esters production and nitrogen concentration was observed (Supplementary Fig. 7), which agrees with the conclusion drawn by several previous studies (Mouret et al., 2014a; Rollero et al., 2015). Low-temperature fermentation has normally been applied to better conserve aromas (Beltran et al., 2002; Gamero et al., 2013; Molina et al., 2007; Torija et al., 2003). However, Mouret et al. (2014a, 2014b) mentioned the possible overestimated influence of temperature on ester synthesis. Our results illustrate that the initial nitrogen concentration, instead of temperature, seems to more strongly impact volatile compounds formation. Nevertheless, as the fermentations performed at 12 °C and 60 mg/L YAN did not finish, this evidence may not be sufficient to reach this conclusion.

Comparing with *S. cerevisiae*, the cryotolerant parental strains produced larger amounts of acetate esters and smaller quantities of ethyl esters and higher alcohols. Among the acetate esters, a bigger amount of ethyl acetate was detected, which will confer wines a solvent-like off-flavour. For this reason, the *S. eubayanus* and *S. uvarum* strains used in our study may not be perfect strains for pure culture fermentations in sensory character terms. However, the hybrid strains, mainly *ScxSe*, produced bigger amounts of ethyl esters and higher alcohols. These compounds are highly desirable in wine because they are the main donors of fruity and floral aromas. Moreover, the higher production of both 2-phenylethyl acetate and 2-phenylethanol by the hybrid strains provided the wine a pleasant rose-like odour, which enhanced aroma complexity.

4.3. Nitrogen requirement and sequential utilisation by hybrid strains

In our previous work (Su et al., 2019), competition fermentations were carried out between *S. cerevisiae* and the cryotolerant strains at low nitrogen concentrations. The result revealed that the selected cryotolerant strains outcompeted *S. cerevisiae* at low nitrogen concentrations, with fermentation temperature no higher than 20 °C. However, the cryotolerant strains displayed poor fermentation capacity at 28 °C. The hybrid strains demonstrated outstanding fermentation behaviour at 28 °C with only 60 mg/L YAN, while the cryotolerant parental strains did not manage to deplete all the fermentable sugars under the same condition. During fermentations, nitrogen sources were consumed more rapidly by the hybrid strains than by the parental strains, which indicates better ability to compete in the uptake of key nutrients. Under the nitrogen-limiting condition, this strategy could ensure an optimum population size.

For *S. cerevisiae*, the nitrogen source uptake order is regulated mainly by nitrogen catabolite repression (NCR) and the Ssy1p-Ptr3p-Ssy5 (SPS) mechanism. As *S. eubayanus* and *S. uvarum* are phylogenetically closely related to *S. cerevisiae*, an alike regulation system is also expected. Similar nitrogen source uptake order has been observed among *S. cerevisiae*, cryotolerant strains and hybrid strains. In our study, fermentations were conducted under the same condition, and grouped together on the heatmap of nitrogen uptake order. Therefore, instead of yeast strains, the fermentation conditions more substantially influenced the nitrogen source uptake order. Notably, phenylalanine

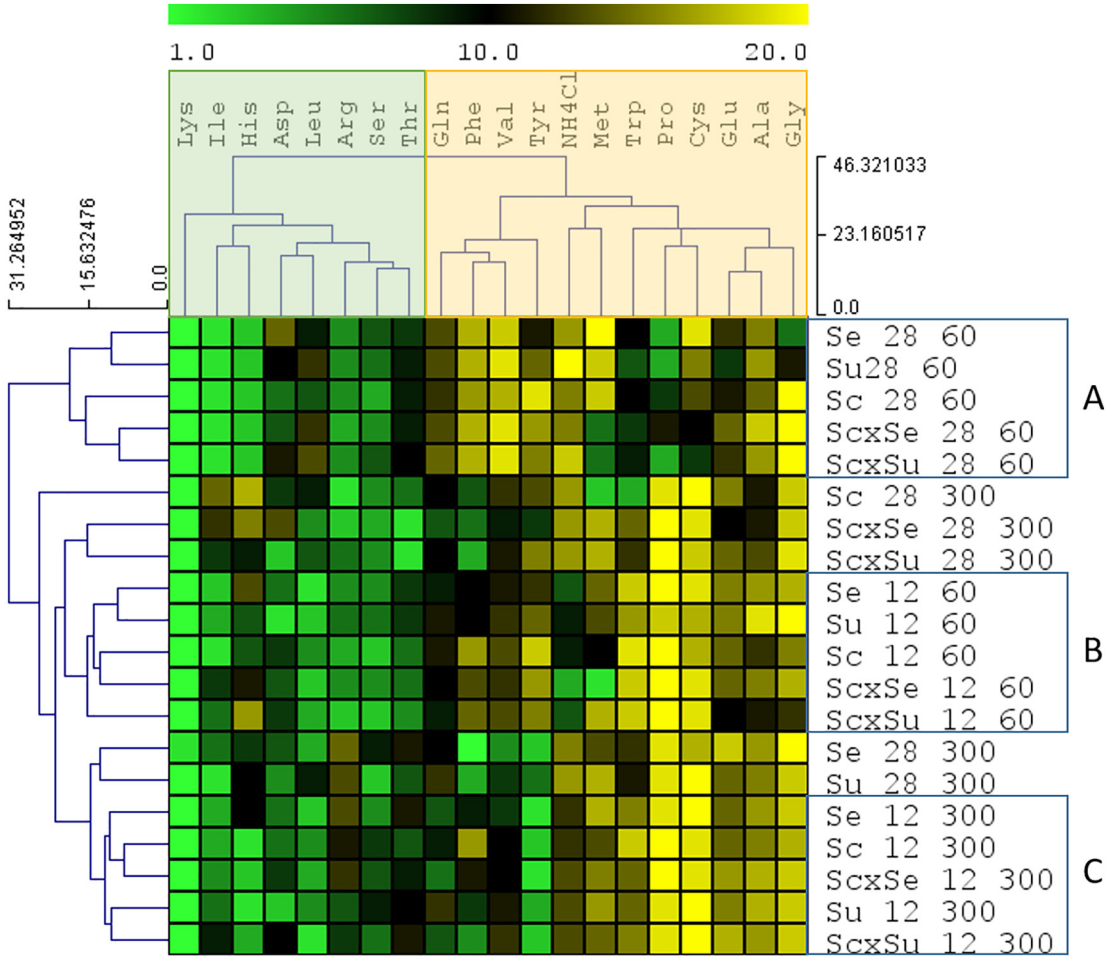

Fig. 6. The heatmap of the consumption order of different nitrogen sources with a hierarchical cluster calculated by Euclidean distance. The colour scheme from green to yellow indicates the consumption order from the 1st to the 20th. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

28°C 60YAN

	Sc	Se	Su	ScxSe	ScxSu
Lys	1	1	1	1	1
Ile	2	2	2	2	2
His	3	3	3	3	3
Ser	4	7	6	5	7
Arg	5	5	5	4	5
Asp	6	14	10	7	11
Leu	7	9	12	12	13
Pro	8	4	4	11	4
Thr	9	8	9	9	10
Trp	10	10	7	8	9
Glu	11	12	8	14	12
Gln	12	13	13	13	14
Cys	13	19	15	10	8
Ala	14	15	16	18	16
NH_4Cl	15	16	20	15	18
Phe	16	17	17	17	17
Val	17	18	19	19	19
Met	18	20	18	6	6
Tyr	19	11	14	16	15
Gly	20	6	11	20	20

28°C 300YAN

	Sc	Se	Su	ScxSe	ScxSu
Lys	1	2	1	1	1
Ile	14	6	2	12	8
His	17	8	10	15	9
Ser	5	9	3	4	5
Arg	2	14	13	3	6
Asp	8	7	5	13	3
Leu	9	4	9	5	7
Pro	19	19	19	20	20
Thr	6	11	7	2	2
Trp	4	12	11	14	12
Glu	15	18	14	10	14
Gln	10	10	12	7	10
Cys	20	17	20	19	18
Ala	11	16	15	11	13
NH_4Cl	16	15	16	16	16
Phe	7	1	4	6	4
Val	12	5	8	9	11
Met	3	13	17	17	17
Tyr	13	3	6	8	15
Gly	18	20	18	18	19

12°C 60YAN

	Sc	Se	Su	ScxSe	ScxSu
Lys	1	1	1	1	1
Ile	2	3	4	8	6
His	7	13	7	11	16
Ser	3	5	6	5	3
Arg	3	5	6	5	3
Asp	8	6	2	7	8
Leu	5	2	3	3	4
Pro	20	20	18	20	20
Thr	6	8	8	6	5
Trp	19	18	16	18	18
Glu	14	15	15	14	10
Gln	11	9	11	10	9
Cys	17	19	17	19	19
Ala	12	16	19	15	11
NH_4Cl	9	7	9	4	7
Phe	16	10	10	13	14
Val	13	11	12	12	13
Met	10	14	13	2	17
Tyr	18	12	14	16	15
Gly	15	17	20	17	12

12°C 300YAN

	Sc	Se	Su	ScxSe	ScxSu
Lys	1	1	1	1	1
Ile	4	4	6	5	9
His	2	10	2	3	4
Ser	8	5	9	7	6
Arg	11	13	7	12	8
Asp	6	6	3	8	10
Leu	5	3	5	4	2
Pro	20	19	19	20	19
Thr	7	11	10	9	11
Trp	18	15	14	14	15
Glu	14	14	15	16	17
Gln	9	7	12	6	7
Cys	19	20	20	19	20
Ala	15	16	17	17	16
NH_4Cl	12	12	13	13	13
Phe	16	9	8	11	5
Val	10	8	11	10	12
Met	13	17	16	15	14
Tyr	3	2	4	2	3
Gly	17	18	18	18	18

Fig. 7. The uptake order of different nitrogen sources under four fermentation conditions. The ranking is based on AUC values. A lower ranking number represents early consumption; a higher ranking number denotes late consumption. The colour scheme from yellow to green represents the ranking from low to high, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was consumed relatively more quickly by the *S. eubayanus*, *S. uvarum* and hybrid strains, which corresponded to higher productions of 2-phenylethanol and 2-phenylethyl acetate by these strains via the Ehrlich pathway.

5. Conclusions

For the first time, this work used selected low nitrogen-demanding cryotolerant strains for hybrid construction with *S. cerevisiae*. The obtained hybrid strains showed good adaptation to low-temperature and low-nitrogen fermentation conditions and better fermentation performance than the parental strains. A high fermentation speed not only saves time and energy for wineries, but also prevents contamination by undesirable microorganisms during fermentation. Hybrid strains are very promising for conducting fermentations under stressful conditions. We are well aware that our constructed strains are genetically modified and unable to be directly used in industry. However, as a result of these preliminary insights, we are now constructing non-GMO strains by applying natural auxotrophy and temperature as selection markers, as previously reported by Magalhães et al. (2017). Due to the inherent instability of interspecific yeast hybrids, the constructed hybrids will be subjected to a genomic stabilisation process after several generations of vegetative propagation. This process of stabilisation can be exploited for obtaining segregants (evolved hybrids) that display a high range of phenotypes (Sipiczki, 2018).

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Declaration of competing interest

The authors declare that there are no competing interests regarding the publication of this paper.

References

Alonso-del-Real, J., Contreras-Ruiz, A., Castiglioni, G.L., Barrio, E., Querol, A., 2017a. The use of mixed populations of *Saccharomyces cerevisiae* and *S. kudriavzevii* to reduce ethanol content in wine: Limited Aeration, inoculum proportions, and sequential inoculation. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.02087.

Alonso-del-Real, J., Lairón-Peris, M., Barrio, E., Querol, A., 2017b. Effect of temperature on the prevalence of *Saccharomyces* non *cerevisiae* species against a *S. cerevisiae* wine strain in wine fermentation: competition, physiological fitness, and influence in final wine composition. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.00150.

Antunovics, Z., Nguyen, H.V., Gaillardin, C., Sipiczki, M., 2005. Gradual genome stabilisation by progressive reduction of the *Saccharomyces uvarum* genome in an interspecific hybrid with *Saccharomyces cerevisiae*. FEMS Yeast Res. 5, 1141–1150. https://doi.org/10.1016/j.femsyr.2005.04.008.

Bell, S.J., Henschke, P.A., 2005. Implications of nitrogen nutrition for grapes, fermentation and wine. Aust. J. Grape Wine Res. 11, 242–295. https://doi.org/10.1111/j.1755-0238.2005.tb00028.x.

Beltran, G., Torija, M.J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J.M., Rozès, N., Mas, A., 2002. Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. Syst. Appl. Microbiol. 25, 287–293. https://doi.org/10.1078/0723-2020-00097.

Beltran, G., Rozés, N., Mas, A., Guillamón, J.M., 2007. Effect of Low-temperature Fermentation on Yeast Nitrogen Metabolism. pp. 809–815. https://doi.org/10.1007/s11274-006-9302-6.

Beltran, G., Novo, M., Guillamón, J.M., Mas, A., Rozès, N., 2008. Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. Int. J. Food Microbiol. 121, 169–177. https://doi.org/10.1016/j.ijfoodmicro.2007.11.030.

Bely, M., Sablayrolles, J.M., Barre, P., 1990. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. 70, 246–252. https://doi.org/10.1016/0922-338X(90)90057-4.

Bing, J., Han, P.J., Liu, W.Q., Wang, Q.M., Bai, F.Y., 2014. Evidence for a far east Asian origin of lager beer yeast. Curr. Biol. 24, R380–R381. https://doi.org/10.1016/j.cub.2014.04.031.

Bisson, L.F., 1991. Influence of nitrogen on yeast and fermentation of grapes In: Proceedings of the International Symposium on nitrogen in grapes and wine. American Society for Enology and Viticulture Davis, CA. Seattle, USA, pp. 78–89.

Butzke, C.E., 1998. Survey of yeast assimilable nitrogen status in musts from California, Oregon, and Washington. Am. J. Enol. Vitic. 49, 220–224.

Camarasa, C., Grivet, J.P., Dequin, S., 2003. Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways of succinate formation in Saccharomyces cerevisiae during anaerobic fermentation. Microbiology 149, 2669–2678. https://doi.org/10.1099/mic.0.26007-0.

Carrau, F.M., Medina, K., Farina, L., Boido, E., Henschke, P.A., Dellacassa, E., 2008. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: effects of yeast assimilable nitrogen on two model strains. FEMS Yeast Res. 8, 1196–1207. https://doi.org/10.1111/j.1567-1364.2008.00412.x.

Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P., Zambonelli, C., 1994. Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant *Saccharomyces* strains. Vitis 33, 49–52.

Diderich, J.A., Weening, S.M., Van Den Broek, M., Pronk, J.T., Daran, J.M.G., 2018. Selection of Pof- *saccharomyces eubayanus* variants for the construction of *S. cerevisiae* × *S. eubayanus* hybrids with reduced 4-vinyl guaiacol formation. Front. Microbiol. 9, 1–17. https://doi.org/10.3389/fmicb.2018.01640.

Gamero, A., Tronchoni, J., Querol, A., Belloch, C., 2013. Production of aroma compounds by cryotolerant *Saccharomyces* species and hybrids at low and moderate fermentation temperatures. J. Appl. Microbiol. 114, 1405–1414. https://doi.org/10.1111/jam.12126.

García-Ríos, E., Guillén, A., De la Cerda, R., Pérez-través, L., Querol, A., Guillamón, J.M., 2019. Improving the Cryotolerance of Wine Yeast by Interspecific Hybridization in the Genus *Saccharomyces*. vol. 9. pp. 1–12. https://doi.org/10.3389/fmicb.2018.03232.

Gayevskiy, V., Goddard, M.R., 2016. *Saccharomyces eubayanus* and *Saccharomyces arboricola* reside in North Island native New Zealand forests. Environ. Microbiol. 18, 1137–1147. https://doi.org/10.1111/1462-2920.13107.

Gibson, B., Liti, G., 2015. *Saccharomyces pastorianus*: genomic insights inspiring innovation for industry. Yeast 32, 17–27. https://doi.org/10.1002/yea.3033.

Gietz, R.D., Schiestl, R.H., 2008. High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nat. Protoc. 2, 31–35. https://doi.org/10.1038/nprot.2007.13.

Giudici, P., Kunkee, R.E., 1994. The effect of nitrogen deficiency and sulfur-containing amino acids on the reduction of sulfate to hydrogen sulfide by wine yeasts. Am. J. Enol. Vitic. 45, 107–112.

Goldstein, A.L., McCusker, J.H., 1999. Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 15, 1541–1553. https://doi.org/10.1002/(SICI)1097-0061. (199910)15:14 < 1541::AID-YEA476 > 3.0.CO;2-K.

Gómez-Alonso, S., Hermosín-Gutiérrez, I., García-Romero, E., 2007. Simultaneous HPLC analysis of biogenic amines, amino acids, and ammonium ion as aminoenone derivatives in wine and beer samples. J. Agric. Food Chem. 55, 608–613. https://doi.org/10.1021/jf062820m.

González Flores, M., Rodríguez, M.E., Oteiza, J.M., Barbagelata, R.J., Lopes, C.A., 2017. Physiological characterization of Saccharomyces uvarum and *Saccharomyces eubayanus* from Patagonia and their potential for cidermaking. Int. J. Food Microbiol. 249, 9–17. https://doi.org/10.1016/j.ijfoodmicro.2017.02.018.

González, S.S., Barrio, E., Gafner, J., Querol, A., 2006. Natural hybrids from *Saccharomyces cerevisiae*, *Saccharomyces bayanus* and *Saccharomyces kudriavzevii* in wine fermentations. FEMS Yeast Res. 6, 1221–1234. https://doi.org/10.1111/j.1567-1364.2006.00126.x.

Gutiérrez, A., Chiva, R., Sancho, M., Beltran, G., Arroyo-López, F.N., Guillamón, J.M., 2012. Nitrogen requirements of commercial wine yeast strains during fermentation of a synthetic grape must. Food Microbiol. 31, 25–32. https://doi.org/10.1016/j.fm.2012.02.012.

Hagen, K.M., Keller, M., Edwards, C.G., 2008. Survey of biotin, pantothenic acid, and assimilable nitrogen in winegrapes from the Pacific Northwest. Am. J. Enol. Vitic. 59, 432–436.

Hebly, M., Brickwedde, A., Bolat, I., Driessen, M.R.M., Hulster, E.A.F. De, Broek, M. Van Den, Pronk, J.T., Geertman, J., Daran, J., Daran-lapujade, P., 2015. S. cerevisiae × S. eubayanus interspecific hybrid: the best of both worlds and beyond. FEMS Yeast Res. 15, fov005. doi:https://doi.org/10.1093/femsyr/fov005.

Henschke, P.A., Jiranek, V., 1993. Yeast: metabolism of nitrogen compounds. In: Wine Microbiology and Biotechnology, pp. 77–164. https://doi.org/10.1089/end.2014.0018.

Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast: non-Saccharomyces yeasts in wine production uncovered. FEMS Yeast Res. 14, 215–237. https://doi.org/10.1111/1567-1364.12111.

Jones, G.V., White, M.A., Cooper, O.R., Storchmann, K., 2005. Climate change and global wine quality. Clim. Chang. 73, 319–343. https://doi.org/10.1007/s10584-005-4704-2.

Katz Ezov, T., Chang, S.L., Frenkel, Z., Segrè, A.V., Bahalul, M., Murray, A.W., Leu, J.Y., Korol, A., Kashi, Y., 2010. Heterothallism in Saccharomyces cerevisiae isolates from nature: Effect of HO locus on the mode of reproduction. Mol. Ecol. 19, 121–131. https://doi.org/10.1111/j.1365-294X.2009.04436.x.

Kishimoto, M., 1994. Fermentation characteristics of hybrids between the cryophilic wine yeast *Saccharomyces bayanus* and the mesophilic wine yeast *Saccharomyces cerevisiae*. J. Ferment. Bioeng. 77, 432–435. https://doi.org/10.1016/0922-338X(94)90019-1.

Krogerus, K., Magalhães, F., Vidgren, V., Gibson, B., 2017. Novel brewing yeast hybrids:

creation and application. Appl. Microbiol. Biotechnol. 101, 65–78. https://doi.org/10.1007/s00253-016-8007-5.
Krogerus, K., Preiss, R., Gibson, B., 2018. A unique *Saccharomyces cerevisiae* × *Saccharomyces uvarum* hybrid isolated from Norwegian farmhouse beer: characterization and reconstruction. Front. Microbiol. 9, 1–15. https://doi.org/10.3389/fmicb.2018.02253.
Le Jeune, C., Lollier, M., Demuyter, C., Erny, C., Legras, J.L., Aigle, M., Masneuf-Pomarède, I., 2007. Characterization of natural hybrids of Saccharomyces cerevisiae and Saccharomyces bayanus var. uvarum. FEMS Yeast Res. 7, 540–549. https://doi.org/10.1111/j.1567-1364.2007.00207.x.
Libkind, D., Hittinger, C.T., Valério, E., Gonçalves, C., Dover, J., Johnston, M., Gonçalves, P., Sampaio, J.P., 2011. Microbe domestication and the identification of the wild genetic stock of lager-brewing yeast. Proc. Natl. Acad. Sci. U. S. A. 108, 14539–14544. https://doi.org/10.1073/pnas.1105430108.
Lippman, Z.B., Zamir, D., 2007. Heterosis: revisiting the magic. Trends Genet. 23, 60–66. https://doi.org/10.1016/j.tig.2006.12.006.
Lopandic, K., 2018. *Saccharomyces* interspecies hybrids as model organisms for studying yeast adaptation to stressful environments. Yeast 35, 21–38. https://doi.org/10.1002/yea.3294.
Lopandic, K., Gangl, H., Wallner, E., Tscheik, G., Leitner, G., Querol, A., Borth, N., Breitenbach, M., Prillinger, H., Tiefenbrunner, W., 2007. Genetically different wine yeasts isolated from Austrian vine-growing regions influence wine aroma differently and contain putative hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii*. FEMS Yeast Res. 7, 953–965. https://doi.org/10.1111/j.1567-1364.2007.00240.x.
Magalhães, F., Krogerus, K., Vidgren, V., Sandell, M., Gibson, B., 2017. Improved cider fermentation performance and quality with newly generated *Saccharomyces cerevisiae* × *Saccharomyces eubayanus* hybrids. J. Ind. Microbiol. Biotechnol. https://doi.org/10.1007/s10295-017-1947-7.
Masneuf, I., Hansen, J., Groth, C., Piskur, J., 1998. New hybrids between *Saccharomyces* sensu stricto yeast species found among wine and cider production strains. Appl. Environ. Microbiol. 64, 3887–3892.
Masneuf-Pomarède, I., Bely, M., Marullo, P., Lonvaud-Funel, A., Dubourdieu, D., 2010. Reassessment of phenotypic traits for *Saccharomyces bayanus var. uvarum* wine yeast strains. Int. J. Food Microbiol. 139, 79–86. https://doi.org/10.1016/j.ijfoodmicro.2010.01.038.
Mendes-Ferreira, A., Mendes-Faia, A., Leão, C., 2004. Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium concentrations and its implications in winemaking industry. J. Appl. Microbiol. 97, 540–545. https://doi.org/10.1111/j.1365-2672.2004.02331.x.
Molina, A.M., Swiegers, J.H., Varela, C., Pretorius, I.S., Agosin, E., 2007. Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. Appl. Microbiol. Biotechnol. 77, 675–687. https://doi.org/10.1007/s00253-007-1194-3.
Morales, L., Dujon, B., 2012. Evolutionary role of interspecies hybridization and genetic exchanges in yeasts. Microbiol. Mol. Biol. Rev. 76, 721–739. https://doi.org/10.1128/MMBR.00022-12.
Mouret, J.R., Camarasa, C., Angenieux, M., Aguera, E., Perez, M., Farines, V., Sablayrolles, J.M., 2014a. Kinetic analysis and gas-liquid balances of the production of fermentative aromas during winemaking fermentations: effect of assimilable nitrogen and temperature. Food Res. Int. 62, 1–10. https://doi.org/10.1016/j.foodres.2014.02.044.
Mouret, J.R., Perez, M., Angenieux, M., Nicolle, P., Farines, V., Sablayrolles, J.M., 2014b. Online-based kinetic analysis of higher alcohol and ester synthesis during winemaking fermentations. Food Bioprocess Technol. 7, 1235–1245. https://doi.org/10.1007/s11947-013-1089-5.
Nicolini, G., Larcher, R., Versini, G., 2004. Status of yeast assimilable nitrogen in Italian grape musts and effects of variety, ripening and vintage. Vitis - J. Grapevine Res. 43, 89–96.
Origone, A.C., Rodríguez, M.E., Oteiza, J.M., Querol, A., Lopes, C.A., 2018. Saccharomyces cerevisiae × Saccharomyces uvarum hybrids generated under different conditions share similar winemaking features. Yeast 35, 157–171. https://doi.org/10.1002/yea.3295.
Ough, C.S., Amerine, M.A., 1988. Methods for Analysis of Musts and Wines, 2nd edition. Wiley-Interscience, New York, pp. 172–195.
Padilla, B., Zulian, L., Ferreres, À., Pastor, R., Esteve-Zarzoso, B., Beltran, G., Mas, A., 2017. Sequential inoculation of native non-*Saccharomyces* and *Saccharomyces cerevisiae* strains for wine making. Front. Microbiol. 8, 1–12. https://doi.org/10.3389/fmicb.2017.01293.
Pérez-Través, L., Lopes, C.A., González, R., Barrio, E., Querol, A., 2015. Physiological and genomic characterisation of *Saccharomyces cerevisiae* hybrids with improved fermentation performance and mannoprotein release capacity. Int. J. Food Microbiol. 205, 30–40. https://doi.org/10.1016/j.ijfoodmicro.2015.04.004.
Peris, D., Sylvester, K., Libkind, D., Gonçalves, P., Sampaio, J.P., Alexander, W.G., Hittinger, C.T., 2014. Population structure and reticulate evolution of *Saccharomyces eubayanus* and its lager-brewing hybrids. Mol. Ecol. 23, 2031–2045. https://doi.org/10.1111/mec.12702.
Pizarro, F.J., Jewett, M.C., Nielsen, J., Agosin, E., 2008. Growth temperature exerts differential physiological and transcriptional responses in laboratory and wine strains of Saccharomyces cerevisiae. Appl. Environ. Microbiol. 74, 6358–6368. https://doi.org/10.1128/AEM.00602-08.
Rainieri, S., Zambonelli, C., Giudici, R., Castellari, L., 1998. Characterisation of thermotolerant *Saccharomyces cerevisiae* hybrids. Biotechnol. Lett. 20, 543–547. https://doi.org/10.1023/A:1005389309527.
Riou, C., Nicaud, J.M., Barre, P., Gaillardin, C., 1997. Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast 13, 903–915. https://doi.org/10.1002/(SICI)1097-0061(199708)13:10<903::AID-YEA145>3.0.CO;2-1.
Rodríguez, M.E., Pérez-Través, L., Sangorrín, M.P., Barrio, E., Lopes, C.A., 2014. *Saccharomyces eubayanus* and *Saccharomyces uvarum* associated with the fermentation of Araucaria araucana seeds in Patagonia. FEMS Yeast Res. 14, 948–965. https://doi.org/10.1111/1567-1364.12183.
Rodríguez, M.E., Origone, A.C., Flores, M.G., Lopes, C.A., 2016. Saccharomyces in Traditional and Industrial Fermentations from Patagonia. In: Olivera, N., Libkind, D., Donati, E. (Eds.), Biology and Biotechnology of Patagonian Microorganisms Springer, Cham. https://doi.org/10.1007/978-3-319-42801-7_15.
Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M., Dequin, S., Mouret, J.R., 2015. Combined effects of nutrients and temperature on the production of fermentative aromas by *Saccharomyces cerevisiae* during wine fermentation. Appl. Microbiol. Biotechnol. 99, 2291–2304. https://doi.org/10.1007/s00253-014-6210-9.
Sipiczki, M., 2008. Interspecies hybridization and recombination in *Saccharomyces* wine yeasts. FEMS Yeast Res. 8, 996–1007. https://doi.org/10.1111/j.1567-1364.2008.00369.x.
Sipiczki, M., 2018. Interspecies hybridisation and genome chimerisation in Saccharomyces: combining of gene pools of species and its biotechnological perspectives. Front. Microbiol. 9, 1–20. https://doi.org/10.3389/fmicb.2018.03071.
Sipiczki, M., 2019. Yeast two- and three-species hybrids and high-sugar fermentation. Microb. Biotechnol. https://doi.org/10.1111/1751-7915.13390.
Sprouffske, K., 2018. growthcurver: Simple Metrics to Summarize Growth Curves. R package version 0.3.0. https://CRAN.R-project.org/package=growthcurver.
Steensels, J., Snoek, T., Meersman, E., Nicolino, M.P., Voordeckers, K., Verstrepen, K.J., 2014. Improving industrial yeast strains: exploiting natural and artificial diversity. FEMS Microbiol. Rev. 38, 947–995. https://doi.org/10.1111/1574-6976.12073.
Stewart, G., 2017. The production of secondary metabolites with flavour potential during brewing and distilling wort fermentations. Fermentation 3, 63. https://doi.org/10.3390/fermentation3040063.
Stribny, J., Gamero, A., Pérez-Torrado, R., Querol, A., 2015. *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* differ from *Saccharomyces cerevisiae* during the production of aroma-active higher alcohols and acetate esters using their amino acidic precursors. Int. J. Food Microbiol. 205, 41–46. https://doi.org/10.1016/j.ijfoodmicro.2015.04.003.
Stribny, J., Querol, A., Pérez-Torrado, R., 2016. Differences in enzymatic properties of the *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* alcohol acetyltransferases and their impact on aroma-active compounds production. Front. Microbiol. 7, 1–13. https://doi.org/10.3389/fmicb.2016.00897.
Su, Y., Cecilia, A., Eugenia, M., Querol, A., Manuel, J., Ariel, C., 2019. Fermentative behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions. Int. J. Food Microbiol. 291, 111–120. https://doi.org/10.1016/j.ijfoodmicro.2018.11.020.
Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine Res. 11, 139–173. https://doi.org/10.1111/j.1755-0238.2005.tb00285.x.
Torija, M.J., Rozès, N., Poblet, M., Guillamón, J.M., Mas, A., 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. Int. J. Food Microbiol. 80, 47–53. https://doi.org/10.1016/S0168-1605(02)00144-7.
Zambonelli, C., Passarelli, P., Rainieri, S., Bertolini, L., Giudici, P., Castellari, L., 1997. Technological properties and temperature response of interspecific *Saccharomyces* hybrids. J. Sci. Food Agric. 74, 7–12. https://doi.org/10.1002/(SICI)1097-0010(199705)74:1<7::AID-JSFA753>3.0.CO;2-X.
Zhang, B.Q., Luan, Y., Duan, C.Q., Yan, G.L., 2018. Use of *Torulaspora delbrueckii* co-fermentation with two *Saccharomyces cerevisiae* strains with different aromatic characteristic to improve the diversity of red wine aroma profile. Front. Microbiol. 9. https://doi.org/10.3389/fmicb.2018.00606.