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

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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.

Keywords: *S. cerevisiae*, *S. eubayanus*, *S. uvarum*, hybridisation, nitrogen requirement, temperature

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 H₂S 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

68 in nucleotide sequences, they barely show any prezygotic barriers, which enables
69 them to mate and form viable diploids (Morales and Dujon, 2012). Natural hybrids
70 have been isolated from different fermentation processes. The most well-known lager
71 beer yeast *S. pastorianus* is a natural hybrid between *S. cerevisiae* and *S. eubayanus*
72 (Gibson and Liti, 2015). Besides, *S. cerevisiae* x *S. uvarum*, *S. cerevisiae* x *S.*
73 *kudriavzevii*, and even triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. uvarum*, have
74 been isolated from wine, cider and beer (Antunovics et al., 2005; González et al.,
75 2006; Krogerus et al., 2018; Le Jeune et al., 2007; Lopandic et al., 2007; Masneuf et
76 al., 1998). Interspecific hybridisation could be an evolution strategy for better
77 adaptation to the environment or could also be a result of domestication. Apart from
78 natural hybrids, artificial hybridisation can be used as a powerful tool for developing
79 yeast strains with desirable merits for industrial uses. Many hybrid strains have been
80 constructed in laboratories to combine beneficial attributes, such as stress tolerance,
81 aroma production, fermentation rate, sugar utilisation, etc (reviewed by Bisson, 2017;
82 Lopandic, 2018; Sipiczki, 2018; Sipiczki, 2019; Steensels et al., 2014). These
83 artificial hybrids, together with natural interspecific hybrids, not only possess the
84 characters of both parents, but also demonstrate superior performance in many cases
85 (García Ríos et al., 2019; Krogerus et al., 2017; Pérez Través et al., 2015; Sipiczki,
86 2008). Some of them have been successfully used in the fermentation-based food and
87 beverage industry. One example is a hybrid developed in our laboratory between *S.*
88 *cerevisiae* and commercial strain *S. uvarum* Velluto BMV 58 (Lallemand Inc.) to
89 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 Rodriguez 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., 2017). Physiologically, *S. uvarum* produces less acetic acids and ethanol, but more glycerol than *S. cerevisiae* (Castelleri 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 (Gayeveskiy 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; Zamboneli 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., 2006; 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 minutes. 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 Δho spore clone selection. The haploid cells with nourseothricin or hygromycin resistance were again verified by diagnostic PCR, as mentioned above.

The Δho haploid strains from both parental with opposite mating types were mixed on the same spot on complete media (YPD) and grown for 24 hours. 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 *MspI* restriction pattern of the *KEL2* gene (Pérez 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 H₂SO₄ 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 minutes, 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, *ScxSe* and *ScxSu*. 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 more than 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 Figure 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 more than 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 Figure 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.

3.2 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 (Figure 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 (more than 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.

3.3 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 Figure 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 (Figure 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 (Figure 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: *ScxSe* produced a higher level of ethyl esters than *ScxSu*, 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 Figure 1).

3.4 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 Figures 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 (Figure 5). The nitrogen uptake rate of the parental strains was clearly influenced by fermentation temperature. *Sc* showed the quickest nitrogen 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 (Figure 6). Three major groups (A-C) were obtained that, as with aroma production (PCA; Figure 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 (Figure 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 Figure 7. As reported for other *S. cerevisiae* strains, Lys was practically the first amino acid to be consumed by all the strains 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 Figure 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 (Figure 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., 2017; 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

464 analysed (Supplementary Table 6). Specifically, nitrogen availability strongly
465 influenced succinic acid production. A more than 2-fold larger amount of succinic
466 acid was produced when fermentations were carried out at a lower nitrogen
467 concentration. Rollero et al. (2015) analysed the combined effects of nitrogen, lipids
468 and temperature on fermentation metabolites production. Correspondingly, they also
469 pointed out that succinic acid production was negatively regulated by the initial
470 nitrogen concentration. The possible involved mechanism was explained by Camarasa
471 et al. (2003): when nitrogen is limited in media, less glutamate is produced from
472 α -ketoglutarate. Hence succinic acid synthesis acts as a safety valve (Rollero et al.,
473 2015) that directs the accumulated α -ketoglutarate pool to the TCA cycle. Thus
474 nitrogen-limited fermentations could potentially contribute to biological acidification,
475 a very desirable process, if we take into account the current drop in titratable acidity,
476 or the increase in pH, in grape musts and wines as a consequence of climate change
477 (Jones et al., 2005). Acetic acid yield is a substantial wine quality factor because it
478 confers off-flavour. Our results demonstrated that the hybrid strains produced
479 significantly less acetic acid than *Sc*, especially when nitrogen was limited in the SM.
480 This feature could be inherited from cryotolerant parental strains as *S. uvarum* and *S.*
481 *eubayanus* produced lower acetic acid levels. In general, acetic acid production was
482 quite high in our study, which could be due to the absence of lipids in our SM. As
483 previously described (Beltran et al., 2008; Rollero et al., 2015), acetic acid production
484 is largely influenced by lipid concentration, and more acetic acid is released by yeast
485 in the SM than in natural grape must due to the difference in lipid concentration

486 (Beltran et al. 2008).

487 Volatile compounds production was also strongly impacted by the fermentation
488 conditions. The largest amount of volatile compounds was generally produced when
489 fermentations were carried out at 28°C with 60 mg/L YAN. Previously, several
490 authors (Beltran et al., 2005; Carrau et al., 2008; Rollero et al., 2015) have
491 demonstrated the negative relation between nitrogen concentration and higher
492 alcohols production. The larger amount of higher alcohols synthesis under
493 nitrogen-limited conditions could be due mainly to the *de novo* synthesis of
494 branched-chain amino acids (BCAAs) through the anabolic pathway (reviewed by
495 Stewart, 2017). In our study, higher production took place only for higher alcohols,
496 but also for ethyl esters and acetate esters at low nitrogen concentrations. Interestingly,
497 ethyl acetate and diethyl succinate were two compounds with the biggest contribution
498 for the concentration of acetate esters and ethyl esters, respectively. The production of
499 these two compounds was higher when nitrogen was limited and matched the very
500 large succinic and acetic acids productions. Without considering ethyl acetate and
501 diethyl succinate, a positive correlation between acetate esters and ethyl esters
502 production and nitrogen concentration was observed (Supplementary Figure 7), which
503 agrees with the conclusion drawn by several previous studies (Mouret et al., 2014;
504 Rollero et al., 2015). Low-temperature fermentation has normally been applied to
505 better conserve aromas (Beltran et al., 2002; Gamero et al., 2010; Molina et al., 2007;
506 Torija et al., 2003). However, Mouret et al. (2014) mentioned the possible
507 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 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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Competing Interests

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

6. References

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

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

582 Antunovics, Z., Nguyen, H.V., Gaillardin, C., Sipiczki, M., 2005. Gradual genome
 583 stabilisation by progressive reduction of the *Saccharomyces uvarum* genome in an
 584 interspecific hybrid with *Saccharomyces cerevisiae*. *FEMS Yeast Res.* 5, 1141–
 585 1150. <https://doi.org/10.1016/j.femsyr.2005.04.008>

586 Bach, B., Colas, S., Massini, L., Barnavon, L., Vuchot, P., 2011. Effect of nitrogen
 587 addition during alcoholic fermentation on the final content of biogenic amines in
 588 wine. *Ann. Microbiol.* 61, 185–190. <https://doi.org/10.1007/s13213-010-0119-z>

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

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

596 Beltran, G., Rozés, N., Mas, A., Guillamón, J.M., 2007. Effect of low-temperature
 597 fermentation on yeast nitrogen metabolism 809–815.
 598 <https://doi.org/10.1007/s11274-006-9302-6>

599 Beltran, G., Torija, M.J., Novo, M., Ferrer, N., Poblet, M., Guillamón, J.M., Rozès, N.,
 600 Mas, A., 2002. Analysis of yeast populations during alcoholic fermentation: a six
 601 year follow-up study. *Syst Appl Microbiol* 25, 287–93.
 602 <https://doi.org/10.1078/0723-2020-00097>

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

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

609 Bisson, L.F., 2017. Yeast Hybrids in Winemaking. *Catal.* 1, 27–34.
 610 <https://doi.org/10.5344/catalyst.2016.16001>

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

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

617 Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P., Zambonelli, C.,
 618 1994. Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant
 619 *Saccharomyces* strains. *Vitis* 33, 49–52.

620 Christine, L.J., Marc, L., Catherine, D., Claude, E., Jean-Luc, L., Michel, A., Isabelle,
 621 M.P., 2007. Characterization of natural hybrids of *Saccharomyces cerevisiae* and
 622 *Saccharomyces bayanus* var. *uvarum*. *FEMS Yeast Res.* 7, 540–549.
 623 <https://doi.org/10.1111/j.1567-1364.2007.00207.x>

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

628 Gamero, A., Tronchoni, J., Querol, A., Belloch, C., 2013. Production of aroma
 629 compounds by cryotolerant *Saccharomyces* species and hybrids at low and

630 moderate fermentation temperatures. J. Appl. Microbiol. 114, 1405–1414.
 631 <https://doi.org/10.1111/jam.12126>

632 García-Ríos, E., Guillén, A., De la Cerda, R., Pérez-través, L., Querol, A., Guillamón,
 633 J.M., 2019. Improving the cryotolerance of wine yeast by interspecific
 634 hybridization in the genus *Saccharomyces* 9, 1–12.
 635 <https://doi.org/10.3389/fmicb.2018.03232>

636 Gayevskiy, V., Goddard, M.R., 2016. *Saccharomyces eubayanus* and *Saccharomyces*
 637 *arboricola* reside in North Island native New Zealand forests. Environ. Microbiol.
 638 18, 1137–1147. <https://doi.org/10.1111/1462-2920.13107>

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

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

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

647 González Flores, M., Rodríguez, M.E., Oteiza, J.M., Barbagelata, R.J., Lopes, C.A.,
 648 2017. Physiological characterization of *Saccharomyces uvarum* and

649 *Saccharomyces eubayanus* from Patagonia and their potential for cidermaking.
 650 Int. J. Food Microbiol. 249, 9–17.
 651 <https://doi.org/10.1016/j.ijfoodmicro.2017.02.018>

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

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

660 Hagen, K.M., Keller, M., Edwards, C.G., 2008. Survey of biotin, pantothenic acid, and
 661 assimilable nitrogen in winegrapes from the Pacific Northwest. Am. J. Enol. Vitic.
 662 59, 432–436.

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

667 Henschke, P.A., Jiranek, V., 1993. Yeast: Metabolism of nitrogen compounds. In:
 668 Wine Microbiology and Biotechnology. 77–164.
 669 <https://doi.org/10.1089/end.2014.0018>

670 Jolly, N.P., Varela, C., Pretorius, I.S., 2014. Not your ordinary yeast:
 671 Non-Saccharomyces yeasts in wine production uncovered. FEMS Yeast Res. 14,
 672 215–237. <https://doi.org/10.1111/1567-1364.12111>

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

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

679 Krogerus, K., Magalhães, F., Vidgren, V., Gibson, B., 2017. Novel brewing yeast
 680 hybrids: creation and application. Appl. Microbiol. Biotechnol. 101, 65–78.
 681 <https://doi.org/10.1007/s00253-016-8007-5>

682 Krogerus, K., Preiss, R., Gibson, B., 2018. A unique *Saccharomyces cerevisiae* ×
 683 *Saccharomyces uvarum* hybrid isolated from Norwegian farmhouse beer:
 684 characterization and reconstruction. Front. Microbiol. 9, 1–15.
 685 <https://doi.org/10.3389/fmicb.2018.02253>

686 Libkind, D., Hittinger, C.T., Valério, E., Gonçalves, C., Dover, J., Johnston, M.,
 687 Gonçalves, P., Sampaio, J.P., 2011. Microbe domestication and the identification
 688 of the wild genetic stock of lager-brewing yeast. *Proc. Natl. Acad. Sci. U. S. A.*
 689 108, 14539–44. <https://doi.org/10.1073/pnas.1105430108>

690 Lippman, Z.B., Zamir, D., 2007. Heterosis: revisiting the magic. *Trends Genet.* 23, 60–
 691 66. <https://doi.org/10.1016/j.tig.2006.12.006>

692 Lopandic, K., 2018. *Saccharomyces* interspecies hybrids as model organisms for
 693 studying yeast adaptation to stressful environments. *Yeast* 35, 21–38.
 694 <https://doi.org/10.1002/yea.3294>

695 Lopandic, K., Gangl, H., Wallner, E., Tscheik, G., Leitner, G., Querol, A., Borth, N.,
 696 Breitenbach, M., Prillinger, H., Tiefenbrunner, W., 2007. Genetically different
 697 wine yeasts isolated from Austrian vine-growing regions influence wine aroma
 698 differently and contain putative hybrids between *Saccharomyces cerevisiae* and
 699 *Saccharomyces kudriavzevii*. *FEMS Yeast Res.* 7, 953–965.
 700 <https://doi.org/10.1111/j.1567-1364.2007.00240.x>

701 Magalhães, F., Krogerus, K., Vidgren, V., Sandell, M., Gibson, B., 2017. Improved
 702 cider fermentation performance and quality with newly generated *Saccharomyces*
 703 *cerevisiae* × *Saccharomyces eubayanus* hybrids. *J. Ind. Microbiol. Biotechnol.*
 704 <https://doi.org/10.1007/s10295-017-1947-7>

705 Masneuf-Pomarède, I., Bely, M., Marullo, P., Lonvaud-Funel, A., Dubourdieu, D.,
 706 2010. Reassessment of phenotypic traits for *Saccharomyces bayanus* var. *uvarum*
 707 wine yeast strains. *Int. J. Food Microbiol.* 139, 79–86.
 708 <https://doi.org/10.1016/j.ijfoodmicro.2010.01.038>

709 Masneuf, I., Hansen, J., Groth, C., Piskur, J., 1998. New hybrids between
 710 *Saccharomyces* sensu stricto yeast species found among wine and cider
 711 production strains. *Appl. Environ. Microbiol.* 64, 3887–3892.

712 Molina, A.M., Swiegers, J.H., Varela, C., Pretorius, I.S., Agosin, E., 2007. Influence of
 713 wine fermentation temperature on the synthesis of yeast-derived volatile aroma
 714 compounds. *Appl. Microbiol. Biotechnol.* 77, 675–687.
 715 <https://doi.org/10.1007/s00253-007-1194-3>

716 Morales, L., Dujon, B., 2012. Evolutionary role of interspecies hybridization and
 717 genetic exchanges in yeasts. *Microbiol. Mol. Biol. Rev.* 76, 721–739.
 718 <https://doi.org/10.1128/MMBR.00022-12>

719 Mouret, J.R., Perez, M., Angenieux, M., Nicolle, P., Farines, V., Sablayrolles, J.M.,
 720 2014. Online-Based kinetic analysis of higher alcohol and ester synthesis during
 721 winemaking fermentations. *Food Bioprocess Technol.* 7, 1235–1245.
 722 <https://doi.org/10.1007/s11947-013-1089-5>

723 Mouret, J.R., Camarasa, C., Angenieux, M., Aguera, E., Perez, M., Farines, V.,
 724 Sablayrolles, J.M., 2014. Kinetic analysis and gas-liquid balances of the

725 production of fermentative aromas during winemaking fermentations: Effect of
 726 assimilable nitrogen and temperature. Food Res. Int. 62, 1–10.
 727 <https://doi.org/10.1016/j.foodres.2014.02.044>

728 Nicolini, G., Larcher, R., Versini, G., 2004. Status of yeast assimilable nitrogen in
 729 Italian grape musts and effects of variety, ripening and vintage. Vitis - J.
 730 Grapevine Res. 43, 89–96.

731 Origone, A.C., Rodríguez, M.E., Oteiza, J.M., Querol, A., Lopes, C.A., 2018.
 732 *Saccharomyces cerevisiae* × *Saccharomyces uvarum* hybrids generated under
 733 different conditions share similar winemaking features. Yeast 35, 157–171.
 734 <https://doi.org/10.1002/yea.3295>

735 Ough, C.S., Amerine, M.A., 1988. Methods for analysis of musts and wines, 2nd
 736 Edition. New York: Wiley-Interscience. pp 172–195.

737 Padilla, B., Zulian, L., Ferreres, À., Pastor, R., Esteve-Zarzoso, B., Beltran, G., Mas,
 738 A., 2017. Sequential inoculation of native non-*Saccharomyces* and
 739 *Saccharomyces cerevisiae* strains for wine making. Front. Microbiol. 8, 1–12.
 740 <https://doi.org/10.3389/fmicb.2017.01293>

741 Pérez-Través, L., Lopes, C.A., Barrio, E., Querol, A., 2012. Evaluation of different
 742 genetic procedures for the generation of artificial hybrids in *Saccharomyces* genus
 743 for winemaking. Int. J. Food Microbiol. 156, 102–111.
 744 <https://doi.org/10.1016/j.ijfoodmicro.2012.03.008>

745 Pérez-Través, L., Lopes, C.A., González, R., Barrio, E., Querol, A., 2015.
 746 Physiological and genomic characterisation of *Saccharomyces cerevisiae* hybrids
 747 with improved fermentation performance and mannoprotein release capacity. Int.
 748 J. Food Microbiol. 205, 30–40. <https://doi.org/10.1016/j.ijfoodmicro.2015.04.004>

749 Peris, D., Sylvester, K., Libkind, D., Gonçalves, P., Sampaio, J.P., Alexander, W.G.,
 750 Hittinger, C.T., 2014. Population structure and reticulate evolution of
 751 *Saccharomyces eubayanus* and its lager-brewing hybrids. Mol. Ecol. 23, 2031–
 752 2045. <https://doi.org/10.1111/mec.12702>

753 Rainieri, S., Zambonelli, C., Giudici, R., Castellari, L., 1998. Characterisation of
 754 thermotolerant *Saccharomyces cerevisiae* hybrids. Biotechnol. Lett. 20, 543–547.
 755 <https://doi.org/10.1023/A:1005389309527>

756 Riou, C., Nicaud, J.M., Barre, P., Gaillardin, C., 1997. Stationary-phase gene
 757 expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast 13, 903–
 758 915.
 759 [https://doi.org/10.1002/\(SICI\)1097-0061\(199708\)13:10<903::AID-YEA145>3.0](https://doi.org/10.1002/(SICI)1097-0061(199708)13:10<903::AID-YEA145>3.0.CO;2-1)
 760 [.CO;2-1](https://doi.org/10.1002/(SICI)1097-0061(199708)13:10<903::AID-YEA145>3.0.CO;2-1)

761 Rodríguez, M.E., Pérez-Través, L., Sangorrín, M.P., Barrio, E., Lopes, C.A., 2014.
 762 *Saccharomyces eubayanus* and *Saccharomyces uvarum* associated with the
 763 fermentation of *Araucaria araucana* seeds in Patagonia. FEMS Yeast Res. 14,
 764 948–965. <https://doi.org/10.1111/1567-1364.12183>

765 Rollero, S., Bloem, A., Camarasa, C., Sanchez, I., Ortiz-Julien, A., Sablayrolles, J.M.,
 766 Dequin, S., Mouret, J.R., 2015. Combined effects of nutrients and temperature on
 767 the production of fermentative aromas by *Saccharomyces cerevisiae* during wine
 768 fermentation. Appl. Microbiol. Biotechnol. 99, 2291–2304.
 769 <https://doi.org/10.1007/s00253-014-6210-9>

770 Sipiczki, M., 2008. Interspecies hybridization and recombination in *Saccharomyces*
 771 wine yeasts. FEMS Yeast Res. 8, 996–1007.
 772 <https://doi.org/10.1111/j.1567-1364.2008.00369.x>

773 Sipiczki, M., 2018. Interspecies hybridisation and genome chimerisation in
 774 *Saccharomyces*: Combining of gene pools of species and its biotechnological
 775 perspectives. Front. Microbiol. 9, 1–20.
 776 <https://doi.org/10.3389/fmicb.2018.03071>

777 Sipiczki, M., 2019. Yeast two- and three-species hybrids and high-sugar fermentation.
 778 Microb. Biotechnol. <https://doi.org/10.1111/1751-7915.13390>

779 Steensels, J., Snoek, T., Meersman, E., Nicolino, M.P., Voordeckers, K., Verstrepen,
 780 K.J., 2014. Improving industrial yeast strains: Exploiting natural and artificial
 781 diversity. FEMS Microbiol. Rev. 38, 947–995.
 782 <https://doi.org/10.1111/1574-6976.12073>

783 Stewart, G., 2017. The Production of secondary metabolites with flavour potential
 784 during brewing and distilling wort fermentations. *Fermentation* 3, 63.
 785 <https://doi.org/10.3390/fermentation3040063>

786 Stribny, J., Gamero, A., Pérez-Torrado, R., Querol, A., 2015. *Saccharomyces*
 787 *kudriavzevii* and *Saccharomyces uvarum* differ from *Saccharomyces cerevisiae*
 788 during the production of aroma-active higher alcohols and acetate esters using
 789 their amino acidic precursors. *Int. J. Food Microbiol.* 205, 41–46.
 790 <https://doi.org/10.1016/j.ijfoodmicro.2015.04.003>

791 Stribny, J., Querol, A., Pérez-Torrado, R., 2016. Differences in enzymatic properties of
 792 the *Saccharomyces kudriavzevii* and *Saccharomyces uvarum* alcohol
 793 acetyltransferases and their impact on aroma-active compounds production. *Front.*
 794 *Microbiol.* 7, 1–13. <https://doi.org/10.3389/fmicb.2016.00897>

795 Su, Y., Cecilia, A., Eugenia, M., Querol, A., Manuel, J., Ariel, C., 2019. Fermentative
 796 behaviour and competition capacity of cryotolerant *Saccharomyces* species in
 797 different nitrogen conditions. *Int. J. Food Microbiol.* 291, 111–120.
 798 <https://doi.org/10.1016/j.ijfoodmicro.2018.11.020>

799 Swiegers, J.H., Bartowsky, E.J., Henschke, P.A., Pretorius, I.S., 2005. Yeast and
 800 bacterial modulation of wine aroma and flavour. *Aust. J. Grape Wine Res.* 11,
 801 139–173. <https://doi.org/10.1111/j.1755-0238.2005.tb00285.x>

802 Torija, M.J., Rozès, N., Poblet, M., Guillamón, J.M., Mas, A., 2003. Effects of
 803 fermentation temperature on the strain population of *Saccharomyces cerevisiae*.
 804 Int. J. Food Microbiol. 80, 47–53.
 805 [https://doi.org/10.1016/S0168-1605\(02\)00144-7](https://doi.org/10.1016/S0168-1605(02)00144-7)

806 Winge, Ø., Roberts, C., 1949. A gene for diploidization in yeasts. C. R. Trav. Lab.
 807 Carlsberg, Ser. Physiol. 24, 341-346

808 Zhang, B.Q., Luan, Y., Duan, C.Q., Yan, G.L., 2018. Use of *Torulaspora delbrueckii*
 809 Co-fermentation with two *Saccharomyces cerevisiae* Strains with different
 810 aromatic characteristic to improve the diversity of red wine aroma profile. Front.
 811 Microbiol. 9. <https://doi.org/10.3389/fmicb.2018.00606>

812 Zambonelli, C., Passarelli, P., Rainieri, S., Bertolini, L., Giudici, P., Castellari, L.,
 813 1997. Technological properties and temperature response of interspecific
 814 *Saccharomyces* hybrids. J. Sci. Food Agric. 74, 7–12.
 815 [https://doi.org/10.1002/\(SICI\)1097-0010\(199705\)74:1<7::AID-JSFA753>3.0.CO](https://doi.org/10.1002/(SICI)1097-0010(199705)74:1<7::AID-JSFA753>3.0.CO)
 816 ;2-X

Figure captions

Figure 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 $p \leq 0.05$).

Figure 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.

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

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

Figure 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.

Figure 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.

Figure 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.

Supplementary Figure 1. Percentage of *S. cerevisiae* in the mix culture at the end of fermentations. Blue bars represent the mix culture of *S. cerevisiae* and *S. eubayanus*; green bars represent the mix culture of *S. cerevisiae* and *S. uvarum*. 60YAN and 300YAN indicate that the assimilable nitrogen concentration was 60 mg/L and 300 mg/L, respectively.

Supplementary Figure 2. Box plots represent the production distribution of the acetate esters, ethyl esters and higher alcohols of three fermentation conditions.

Supplementary Figure 3. The nitrogen consumption curves of the fermentations carried out at 28°C and 60 mg/L YAN.

Supplementary Figure 4. The nitrogen consumption curves of the fermentations carried out at 28°C and 300 mg/L YAN.

Supplementary Figure 5. The nitrogen consumption curves of the fermentations carried out at 12°C and 60 mg/L YAN.

Supplementary Figure 6. The nitrogen consumption curves of fermentations carried out at 12°C and 300 mg/L YAN.

Supplementary Figure 7. The production distribution of acetate esters without ethyl acetate, and ethyl esters without diethyl succinate by different strains under three fermentation conditions.

PCA Scores and Loadings

Acetate esters (mg/L)

Lutyl esters (mg/L)

Higher alcohols (mg/L)

12°C 60YAN

12°C 300YAN

28°C 60YAN

28°C 300YAN

1	1	1	1	1
2	2	2	2	2
3	3	3	3	3
4	7	6	5	7
5	5	5	4	5
6	14	10	7	11
7	9	12	12	13
8	4	4	11	4
9	8	9	9	10
10	10	7	8	9
11	12	8	14	12
12	13	13	13	14
13	19	15	10	8
14	15	16	18	16
15	16	20	15	18
16	17	17	17	17
17	18	19	19	19
18	20	18	6	6
19	11	14	16	15
20	6	11	20	20

1	2	1	1	1
14	6	2	12	8
17	8	10	15	9
5	9	3	4	5
2	14	13	3	6
8	7	5	13	3
9	4	9	5	7
19	19	19	20	20
6	11	7	2	2
4	12	11	14	12
15	18	14	10	14
10	10	12	7	10
20	17	20	19	18
11	16	15	11	13
16	15	16	16	16
7	1	4	6	4
12	5	8	9	11
3	13	17	17	17
13	3	6	8	15
18	20	18	18	19

1	1	1	1	1
2	3	4	8	6
7	13	7	11	16
3	5	6	5	3
3	5	6	5	3
8	6	2	7	8
5	2	3	3	4
20	20	18	20	20
6	8	8	6	5
19	18	16	18	18
14	15	15	14	10
11	9	11	10	9
17	19	17	19	19
12	16	19	15	11
9	7	9	4	7
16	10	10	13	14
13	11	12	12	13
10	14	13	2	17
18	12	14	16	15
15	17	20	17	12

1	1	1	1	1
4	4	6	5	9
2	10	2	3	4
8	5	9	7	6
11	13	7	12	8
6	6	3	8	10
5	3	5	4	2
20	19	19	20	19
7	11	10	9	11
18	15	14	14	15
14	14	15	16	17
9	7	12	6	7
19	20	20	19	20
15	16	17	17	16
12	12	13	13	13
16	9	8	11	5
10	8	11	10	12
13	17	16	15	14
3	2	4	2	3
17	18	18	18	18

acetate esters

ethyl esters

higher alcohols

- 12°C 300YAN
- 28°C 60YAN
- 28°C 300YAN

Sc 28°C 60YAN *Se* 28°C 60YAN *Su* 28°C 60YAN *Sc+Se* 28°C 60YAN *Sc+Su* 28°C 60YAN *ScxSe* 28°C 60YAN *ScxSu* 28°C 60YAN

Sc 28°C 300YAN *Su* 28°C 300YAN *Se* 28°C 300YAN *Sc+Se* 28°C 300YAN *Sc+Su* 28°C 300YAN *ScxSe* 28°C 300YAN *ScxSu* 28°C 300YAN

Sc 12°C 60YAN *Se* 12°C 60YAN *Su* 12°C 60YAN *Sc+Se* 12°C 60YAN *Sc+Su* 12°C 60YAN *ScxSe* 12°C 60YAN *ScxSu* 12°C 60YAN

Sc 12°C 300YAN *Se* 12°C 300YAN *Su* 12°C 300YAN *Sc+Se* 12°C 300YAN *Sc+Su* 12°C 300YAN *ScxSe* 12°C 300YAN *ScxSu* 12°C 300YAN

acetate esters (w/o ethyl acetate)

ethyl esters (w/o diethyl succinate)

Supplementary Table 1. The primers used for HO gene deletion

Primer	Sequence 5'-3'	Purpose
HO-F	AGACATCGCAAACGTCACGGCTAACTCTTACGTTAT GTGCGCAGATGGCTCGTACGCTGCAGGTCGACG	To amplify the antibiotic resistance cassette to delete <i>HO</i> in pair with the reverse primer
HO-R hyg	ACTCTTATGAGGCCCGCGGACAGCATGAAACTGTA AGATTCCGCCACATTATATCAGATCCACTAGTGGC	To amplify the <i>hphMX4</i> cassette to delete <i>HO</i>
HO-R nat	ACTCTTATGAGGCCCGCGGACAGCATGAAACTGTA AGATTCCGCCACATTACTAGTGGATCTGATATC	To amplify the <i>natMX4</i> cassette to delete <i>HO</i>
Se-HOcp-F	TTGCTTCAGTCGAAGTTTCG	To check the disruption of the <i>HO</i> gene of <i>S. eubayanus</i>
Se-HOcp-R	GTACCAATACGGTCACTCCA	To check the disruption of the <i>HO</i> gene of <i>S. eubayanus</i>
HOcp-F	GAGGTTTGCAGAAGCTTGTGTA	To check the disruption of the <i>HO</i> gene of <i>S. cerevisiae</i> and <i>S. uvarum</i>
HOcp-R	TTGGCGTATTTCTACTCCAGCAT	To check the disruption of the <i>HO</i> gene of <i>S. cerevisiae</i> and <i>S. uvarum</i>

Supplementary Table 2. Eluent gradient for the HPLC determination of amino acids.

Time (min)	0.0	3.0	5.0	11.0	12.5	14.0	18.0	21.0	23.0	25.0	26.0
Phase A(%)	95.0	94.0	92.0	90.0	88.0	82.0	80.0	70.0	60.0	25.0	20.0
Phase B(%)	5.0	6.0	8.0	10.0	12.0	18.0	20.0	30.0	40.0	75.0	80.0

Supplementary Table 3. Chemical composition of synthetic must at the end of fermentation in g/L (different letters indicate significant differences, Tukey HSD test $p \leq 0.05$)

Temp (°C)	YAN (mg/L)	Strain	glucose		fructose		ethanol		glycerol		succinic acid		acetic acid	
12	60	<i>Sc</i>	20.17	± 1.68 ^d	54.95	± 1.53 ^e	56.02	± 1.73 ^a	3.36	± 0.02 ^b	1.09	± 0.13 ^a	0.66	± 0.02 ^b
12	60	<i>Se</i>	9.37	± 0.52 ^b	39.89	± 0.44 ^c	59.67	± 2.29 ^a	6.43	± 0.01 ^d	1.80	± 0.07 ^{ab}	0.74	± 0.03 ^{bc}
12	60	<i>Su</i>	1.11	± 0.012 ^a	20.63	± 0.60 ^a	62.41	± 4.71 ^a	5.50	± 0.10 ^a	2.48	± 0.07 ^{bc}	0.24	± 0.03 ^d
12	60	<i>Sc+Se</i>	15.35	± 0.33 ^c	49.98	± 0.66 ^d	58.17	± 0.49 ^a	5.37	± 0.14 ^a	1.65	± 0.04 ^{ab}	0.81	± 0.04 ^c
12	60	<i>Sc+Su</i>	7.36	± 0.23 ^b	39.39	± 1.68 ^c	63.26	± 1.95 ^a	5.71	± 0.13 ^a	2.80	± 0.41 ^c	0.41	± 0.01 ^a
12	60	<i>ScxSe</i>	3.65	± 0.35 ^a	30.39	± 1.19 ^b	74.58	± 0.44 ^b	5.39	± 0.05 ^a	1.89	± 0.04 ^{ab}	0.48	± 0.01 ^a
12	60	<i>ScxSu</i>	18.95	± 0.53 ^d	53.92	± 0.68 ^{de}	55.45	± 0.22 ^a	4.49	± 0.11 ^c	1.69	± 0.23 ^{ab}	0.41	± 0.01 ^a
12	300	<i>Sc</i>	0.09	± 0.02 ^{ab}	4.64	± 0.58	85.63	± 2.38	3.78	± 0.06 ^a	0.56	± 0.02 ^{ab}	1.25	± 0.02 ^{ab}
12	300	<i>Se</i>	0.11	± 0.06 ^{ab}	5.59	± 2.32	81.21	± 1.29	6.22	± 0.12 ^c	0.57	± 0.00 ^a	1.19	± 0.03 ^{ab}
12	300	<i>Su</i>	0.02	± 0.01 ^{ab}	0.00	± 0.00	80.95	± 0.62	4.79	± 0.09 ^b	0.52	± 0.03 ^a	0.49	± 0.03 ^c
12	300	<i>Sc+Se</i>	0.14	± 0.04 ^b	4.44	± 2.18	80.64	± 0.05	4.17	± 0.08 ^{ab}	0.54	± 0.02 ^{ab}	1.33	± 0.12 ^b
12	300	<i>Sc+Su</i>	0.00	± 0.00 ^a	0.36	± 0.21	84.50	± 1.44	3.98	± 0.35 ^a	0.34	± 0.06 ^b	1.02	± 0.16 ^{ab}
12	300	<i>ScxSe</i>	0.08	± 0.00 ^{ab}	4.74	± 0.29	82.29	± 1.48	4.17	± 0.19 ^{ab}	0.58	± 0.06 ^a	1.16	± 0.13 ^{ab}
12	300	<i>ScxSu</i>	0.02	± 0.01 ^{ab}	1.85	± 0.27	84.48	± 1.09	3.87	± 0.07 ^a	0.51	± 0.02 ^{ab}	0.90	± 0.01 ^a
28	60	<i>Sc</i>	0.00	± 0.00 ^a	0.44	± 0.26 ^a	75.86	± 0.10 ^{abc}	3.45	± 0.13 ^c	2.51	± 0.32	1.80	± 0.09 ^b
28	60	<i>Se</i>	0.24	± 0.06 ^a	11.47	± 1.53 ^b	73.77	± 0.71 ^{ab}	5.80	± 0.06 ^{ab}	2.37	± 0.05	1.49	± 0.01 ^b
28	60	<i>Su</i>	1.23	± 0.51 ^b	22.25	± 3.64 ^c	70.79	± 1.24 ^c	5.25	± 0.07 ^{ab}	3.27	± 0.14	0.39	± 0.04 ^a
28	60	<i>Sc+Se</i>	0.00	± 0.00 ^a	0.53	± 0.30 ^a	78.01	± 1.49 ^{abc}	4.72	± 0.09 ^a	2.78	± 0.62	1.44	± 0.04 ^b
28	60	<i>Sc+Su</i>	0.06	± 0.00 ^a	3.99	± 0.09 ^a	80.52	± 2.35 ^{ab}	5.39	± 0.09 ^{ab}	2.92	± 0.10	0.86	± 0.11 ^a
28	60	<i>ScxSe</i>	0.02	± 0.01 ^a	1.56	± 0.37 ^a	81.15	± 2.30 ^{ab}	6.37	± 0.43 ^b	2.20	± 0.17	0.59	± 0.10 ^a
28	60	<i>ScxSu</i>	0.00	± 0.00 ^a	0.00	± 0.00 ^a	82.19	± 1.88 ^b	5.69	± 0.45 ^{ab}	2.41	± 0.19	0.90	± 0.24 ^a
28	300	<i>Sc</i>	0.00	± 0.00 ^a	0.69	± 0.40 ^a	86.34	± 0.26 ^a	4.47	± 0.09 ^a	0.55	± 0.02 ^{abc}	1.07	± 0.06 ^b
28	300	<i>Se</i>	0.31	± 0.12 ^b	11.52	± 2.53 ^c	75.67	± 2.82 ^b	5.67	± 0.04 ^b	0.75	± 0.01 ^d	1.23	± 0.06 ^b
28	300	<i>Su</i>	0.02	± 0.01 ^a	2.53	± 0.35 ^{ab}	82.39	± 0.15 ^a	6.48	± 0.11 ^c	0.58	± 0.03 ^{ab}	0.82	± 0.03 ^a
28	300	<i>Sc+Se</i>	0.16	± 0.03 ^{ab}	5.53	± 0.74 ^b	81.13	± 0.19 ^{ab}	4.39	± 0.04 ^a	0.64	± 0.04 ^b	1.18	± 0.03 ^b
28	300	<i>Sc+Su</i>	0.00	± 0.00 ^a	1.02	± 0.02 ^{ab}	81.19	± 0.98 ^{ab}	5.35	± 0.13 ^b	0.46	± 0.01 ^c	1.26	± 0.07 ^b
28	300	<i>ScxSe</i>	0.04	± 0.01 ^a	2.36	± 0.16 ^{ab}	83.06	± 2.24 ^a	4.67	± 0.05 ^a	0.56	± 0.01 ^{ab}	1.10	± 0.03 ^b
28	300	<i>ScxSu</i>	0.00	± 0.00 ^a	0.00	± 0.00 ^a	82.06	± 0.46 ^{ab}	4.55	± 0.06 ^a	0.53	± 0.01 ^{ac}	0.82	± 0.02 ^a

Supplementary Table 4. The metabolite production yield at the end of fermentation (g/g_{sugar}) (different letters indicate significant differences, Tukey HSD test p≤0.05).

Temp (°C)	YAN (mg/L)	Strain	ethanol yield		glycerol yield		succinic acid yield		acetic acid yield	
12	60	<i>Sc</i>	0.45	± 0.00 ^a	0.03	± 0.00 ^f	8.69x10 ⁻³	± 0.78x10 ^{-3a}	5.30x10 ⁻³	± 0.06x10 ^{-3b}
12	60	<i>Se</i>	0.40	± 0.01 ^{ab}	0.04	± 0.00 ^e	11.97x10 ⁻³	± 0.51x10 ^{-3a}	4.87x10 ⁻³	± 0.14x10 ^{-3b}
12	60	<i>Su</i>	0.35	± 0.03 ^b	0.03	± 0.00 ^a	13.90x10 ⁻³	± 0.32x10 ^{-3ab}	1.36x10 ⁻³	± 0.18x10 ^{-3c}
12	60	<i>Sc+Se</i>	0.43	± 0.01 ^a	0.04	± 0.00 ^{de}	12.28x10 ⁻³	± 0.38x10 ^{-3ab}	5.99x10 ⁻³	± 0.25x10 ^{-3d}
12	60	<i>Sc+Su</i>	0.41	± 0.02 ^{ab}	0.04	± 0.00 ^{cd}	18.35x10 ⁻³	± 2.92x10 ^{-3b}	2.64x10 ⁻³	± 0.02x10 ^{-3a}
12	60	<i>ScxSe</i>	0.45	± 0.00 ^a	0.03	± 0.00 ^{ab}	11.35x10 ⁻³	± 0.17x10 ^{-3a}	2.88x10 ⁻³	± 0.05x10 ^{-3a}
12	60	<i>ScxSu</i>	0.44	± 0.01 ^a	0.04	± 0.00 ^{bc}	13.33x10 ⁻³	± 1.92x10 ^{-3ab}	3.21x10 ⁻³	± 0.11x10 ^{-3a}
12	300	<i>Sc</i>	0.44	± 0.01 ^b	0.02	± 0.00 ^a	2.85x10 ⁻³	± 0.11x10 ^{-3a}	6.37x10 ⁻³	± 0.08x10 ^{-3ab}
12	300	<i>Se</i>	0.42	± 0.00 ^{ab}	0.03	± 0.00 ^c	2.95x10 ⁻³	± 0.02x10 ^{-3a}	6.13x10 ⁻³	± 0.08x10 ^{-3ab}
12	300	<i>Su</i>	0.40	± 0.00 ^a	0.02	± 0.00 ^b	2.59x10 ⁻³	± 0.14x10 ^{-3a}	2.43x10 ⁻³	± 0.16x10 ^{-3c}
12	300	<i>Sc+Se</i>	0.41	± 0.00 ^{ab}	0.02	± 0.00 ^{ab}	2.75x10 ⁻³	± 0.08x10 ^{-3a}	6.79x10 ⁻³	± 0.51x10 ^{-3b}
12	300	<i>Sc+Su</i>	0.42	± 0.01 ^{ab}	0.02	± 0.00 ^a	1.70x10 ⁻³	± 0.32x10 ^{-3b}	5.11x10 ⁻³	± 0.79x10 ^{-3ab}
12	300	<i>ScxSe</i>	0.42	± 0.01 ^{ab}	0.02	± 0.00 ^{ab}	2.97x10 ⁻³	± 0.29x10 ^{-3a}	5.95x10 ⁻³	± 0.66x10 ^{-3ab}
12	300	<i>ScxSu</i>	0.43	± 0.01 ^{ab}	0.02	± 0.00 ^a	0.25x10 ⁻³	± 0.10x10 ^{-3a}	4.56x10 ⁻³	± 0.07x10 ^{-3a}
28	60	<i>Sc</i>	0.38	± 0.00	0.02	± 0.00 ^c	12.56x10 ⁻³	± 1.60x10 ^{-3ab}	9.02x10 ⁻³	± 0.46x10 ^{-3b}
28	60	<i>Se</i>	0.39	± 0.00	0.03	± 0.00 ^a	12.60x10 ⁻³	± 0.13x10 ^{-3ab}	7.89x10 ⁻³	± 0.14x10 ^{-3b}
28	60	<i>Su</i>	0.40	± 0.00	0.03	± 0.00 ^a	18.61x10 ⁻³	± 1.21x10 ^{-3b}	2.20x10 ⁻³	± 0.18x10 ^{-3a}
28	60	<i>Sc+Se</i>	0.39	± 0.01	0.02	± 0.00 ^b	13.96x10 ⁻³	± 3.12x10 ^{-3ab}	7.21x10 ⁻³	± 0.21x10 ^{-3b}
28	60	<i>Sc+Su</i>	0.41	± 0.01	0.03	± 0.00 ^{ab}	14.88x10 ⁻³	± 0.51x10 ^{-3ab}	4.38x10 ⁻³	± 0.57x10 ^{-3a}
28	60	<i>ScxSe</i>	0.41	± 0.01	0.03	± 0.00 ^a	11.06x10 ⁻³	± 0.82x10 ^{-3a}	2.98x10 ⁻³	± 0.51x10 ^{-3a}
28	60	<i>ScxSu</i>	0.41	± 0.01	0.03	± 0.00 ^{ab}	12.05x10 ⁻³	± 0.93x10 ^{-3ab}	4.50x10 ⁻³	± 1.21x10 ^{-3a}
28	300	<i>Sc</i>	0.43	± 0.00 ^b	0.02	± 0.00 ^a	2.75x10 ⁻³	± 0.07x10 ^{-3abc}	5.34x10 ⁻³	± 0.29x10 ^{-3a}
28	300	<i>Se</i>	0.40	± 0.01 ^a	0.03	± 0.00 ^c	3.97x10 ⁻³	± 0.02x10 ^{-3d}	6.53x10 ⁻³	± 0.23x10 ^{-3b}
28	300	<i>Su</i>	0.42	± 0.00 ^{ab}	0.03	± 0.00 ^d	2.91x10 ⁻³	± 0.17x10 ^{-3ab}	4.15x10 ⁻³	± 0.14x10 ^{-3c}
28	300	<i>Sc+Se</i>	0.42	± 0.00 ^{ab}	0.02	± 0.00 ^a	3.27x10 ⁻³	± 0.24x10 ^{-3b}	6.09x10 ⁻³	± 0.18x10 ^{-3ab}
28	300	<i>Sc+Su</i>	0.41	± 0.00 ^{ab}	0.03	± 0.00 ^b	2.29x10 ⁻³	± 0.07x10 ^{-3c}	6.33x10 ⁻³	± 0.35x10 ^{-3ab}
28	300	<i>ScxSe</i>	0.42	± 0.01 ^{ab}	0.02	± 0.00 ^a	2.83x10 ⁻³	± 0.03x10 ^{-3ab}	5.57x10 ⁻³	± 0.14x10 ^{-3ab}
28	300	<i>ScxSu</i>	0.41	± 0.00 ^{ab}	0.02	± 0.00 ^a	2.64x10 ⁻³	± 0.04x10 ^{-3ac}	4.10x10 ⁻³	± 0.09x10 ^{-3c}

Supplementary Table 5. Concentration of volatile compounds in mg/L (different letters indicate significant differences, Tukey HSD test $p \leq 0.05$).

Temp (°C)	YAN (mg/L)	strain	ethyl acetate		isoamyl acetate		2-phenylethyl acetate		ethyl hexanoate		ethyl octanoate	
12	60	<i>Sc</i>	29.75	± 6.86 ^a	0.35	± 0.10 ^{ab}	0.00	± 0.00 ^d	0.23	± 0.01 ^b	0.27	± 0.02 ^d
12	60	<i>Se</i>	50.57	± 4.15 ^{ab}	0.63	± 0.03 ^c	1.18	± 0.05 ^c	0.21	± 0.01 ^b	0.06	± 0.01 ^c
12	60	<i>Su</i>	58.42	± 5.09 ^b	0.28	± 0.01 ^a	2.82	± 0.07 ^e	0.33	± 0.00 ^a	0.10	± 0.01 ^c
12	60	<i>Sc+Se</i>	37.23	± 7.22 ^{ab}	0.53	± 0.01 ^{bc}	0.75	± 0.00 ^b	0.32	± 0.04 ^a	0.16	± 0.00 ^{ab}
12	60	<i>Sc+Su</i>	38.58	± 1.94 ^{ab}	0.23	± 0.01 ^a	1.05	± 0.05 ^c	0.48	± 0.00 ^c	0.15	± 0.00 ^a
12	60	<i>ScxSe</i>	41.90	± 4.71 ^{ab}	0.33	± 0.03 ^a	0.36	± 0.02 ^a	0.36	± 0.00 ^a	0.22	± 0.01 ^{bd}
12	60	<i>ScxSu</i>	35.10	± 3.85 ^{ab}	0.26	± 0.00 ^a	0.56	± 0.04 ^{ab}	0.51	± 0.01 ^c	0.18	± 0.01 ^{ab}
12	300	<i>Sc</i>	56.21	± 0.10 ^{ab}	0.83	± 0.00 ^{ab}	0.21	± 0.01 ^a	0.74	± 0.03 ^e	0.90	± 0.05 ^c
12	300	<i>Se</i>	61.80	± 1.62 ^{ab}	1.18	± 0.10 ^b	0.69	± 0.01 ^c	0.32	± 0.01 ^a	0.42	± 0.03 ^a
12	300	<i>Su</i>	129.29	± 4.68 ^c	1.13	± 0.12 ^{ab}	1.11	± 0.05 ^d	0.59	± 0.07 ^{cde}	0.37	± 0.01 ^a
12	300	<i>Sc+Se</i>	92.57	± 4.92 ^b	0.87	± 0.04 ^{ab}	0.30	± 0.00 ^{ab}	0.40	± 0.02 ^{ab}	0.69	± 0.03 ^b
12	300	<i>Sc+Su</i>	195.67	± 18.45 ^d	0.70	± 0.16 ^a	0.36	± 0.05 ^{ab}	0.46	± 0.04 ^{abc}	0.36	± 0.05 ^a
12	300	<i>ScxSe</i>	67.17	± 7.32 ^{ab}	1.05	± 0.10 ^{ab}	0.44	± 0.05 ^b	0.67	± 0.00 ^{de}	0.80	± 0.02 ^{bc}
12	300	<i>ScxSu</i>	55.62	± 1.67 ^a	0.66	± 0.03 ^a	0.37	± 0.00 ^{ab}	0.53	± 0.01 ^{bcd}	0.43	± 0.01 ^a
28	60	<i>Sc</i>	127.72	± 24.53 ^a	2.22	± 0.23 ^b	0.55	± 0.02 ^b	0.81	± 0.04 ^a	0.58	± 0.02 ^a
28	60	<i>Se</i>	294.96	± 2.29 ^b	1.12	± 0.10 ^{cd}	0.96	± 0.03 ^a	0.14	± 0.01 ^d	0.37	± 0.01 ^b
28	60	<i>Su</i>	88.38	± 12.93 ^a	0.83	± 0.07 ^c	2.30	± 0.05 ^c	0.36	± 0.01 ^c	0.42	± 0.03 ^b
28	60	<i>Sc+Se</i>	116.83	± 0.35 ^a	1.84	± 0.19 ^{ab}	0.97	± 0.08 ^a	0.51	± 0.01 ^{bc}	0.52	± 0.05 ^{ab}
28	60	<i>Sc+Su</i>	124.25	± 15.36 ^a	1.48	± 0.17 ^{acd}	1.07	± 0.03 ^a	0.74	± 0.05 ^{ab}	0.65	± 0.03 ^a
28	60	<i>ScxSe</i>	88.69	± 4.22 ^a	1.83	± 0.06 ^{ab}	0.96	± 0.13 ^a	0.88	± 0.01 ^a	1.03	± 0.02 ^c
28	60	<i>ScxSu</i>	119.35	± 4.71 ^a	1.53	± 0.18 ^{abd}	1.06	± 0.01 ^a	0.73	± 0.10 ^{ab}	0.62	± 0.01 ^a
28	300	<i>Sc</i>	71.70	± 3.57 ^a	5.92	± 0.24 ^c	2.01	± 0.00 ^{bc}	1.03	± 0.07 ^b	1.01	± 0.10 ^{ab}
28	300	<i>Se</i>	92.52	± 0.52 ^b	0.63	± 0.02 ^b	0.93	± 0.09 ^a	0.23	± 0.00 ^c	0.71	± 0.04 ^{bc}
28	300	<i>Su</i>	68.02	± 1.44 ^a	0.97	± 0.18 ^b	1.01	± 0.12 ^a	0.22	± 0.02 ^c	0.31	± 0.05 ^d
28	300	<i>Sc+Se</i>	65.50	± 1.86 ^a	2.76	± 0.10 ^a	1.08	± 0.05 ^a	0.81	± 0.04 ^{ab}	0.65	± 0.07 ^{bd}
28	300	<i>Sc+Su</i>	60.68	± 1.19 ^a	2.98	± 0.02 ^a	1.45	± 0.08 ^{ab}	0.68	± 0.03 ^a	0.85	± 0.07 ^{abc}
28	300	<i>ScxSe</i>	76.96	± 3.62 ^{ab}	3.43	± 0.07 ^a	2.09	± 0.18 ^c	0.94	± 0.08 ^{ab}	1.18	± 0.02 ^a
28	300	<i>ScxSu</i>	72.19	± 5.54 ^a	3.16	± 0.01 ^a	1.40	± 0.07 ^a	0.80	± 0.08 ^{ab}	1.18	± 0.03 ^a

Temp	YAN	strain	ethyl decanoate	diethyl succinate	isobutanol	isoamyl alcohol	benzyl alcohol	2-phenylethanol
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Supplementary Table 5. Concentration of volatile compounds in mg/L (continued) (different letters indicate significant differences, Tukey HSD

test $p\leq0.05$).

(°C)	(mg/L)																			
12	60	Sc	0.41	±	0.01 ^c	5.65	±	0.31 ^b	42.69	±	0.30 ^{ab}	205.15	±	17.62 ^a	nd*	78.94	±	4.66 ^a	nd*: not detected	
12	60	Se	0.09	±	0.01 ^b	3.30	±	0.44 ^a	54.92	±	0.06 ^{cd}	420.49	±	25.58 ^b	nd	113.29	±	3.17 ^{ab}		
12	60	Su	0.22	±	0.01 ^a	2.79	±	0.15 ^a	39.08	±	0.21 ^a	188.43	±	5.57 ^a	nd	108.81	±	1.55 ^{ab}		
12	60	Sc+Se	0.14	±	0.01 ^b	2.89	±	0.36 ^a	61.62	±	0.97 ^{de}	336.36	±	7.31 ^b	nd	131.68	±	2.49 ^b		
12	60	Sc+Su	0.27	±	0.03 ^a	2.06	±	0.45 ^a	47.47	±	2.29 ^{abc}	155.63	±	7.01 ^a	nd	73.00	±	1.39 ^{ac}		
12	60	ScxSe	0.41	±	0.00 ^c	6.10	±	0.01 ^b	67.39	±	1.97 ^e	334.89	±	31.75 ^b	nd	133.75	±	21.46 ^b		
12	60	ScxSu	0.24	±	0.02 ^a	2.72	±	0.16 ^a	49.83	±	3.74 ^{bc}	203.79	±	6.95 ^a	nd	63.11	±	2.03 ^c		
12	300	Sc	0.64	±	0.03 ^a	8.45	±	1.12 ^c	58.60	±	0.78 ^a	198.41	±	3.18 ^{bc}	10.11	±	0.25	51.41	±	2.69 ^a
12	300	Se	0.44	±	0.01 ^{bc}	4.16	±	0.50 ^a	60.67	±	1.14 ^a	253.39	±	2.30 ^{ac}	10.98	±	0.59	63.66	±	1.19 ^a
12	300	Su	0.83	±	0.05 ^a	2.88	±	0.22 ^a	39.82	±	1.86 ^b	295.98	±	15.84 ^a	9.74	±	1.36	108.89	±	8.36 ^b
12	300	Sc+Se	0.84	±	0.08 ^a	6.99	±	0.01 ^{bc}	58.33	±	1.80 ^a	205.99	±	7.63 ^{bc}	11.22	±	0.54	41.54	±	3.72 ^a
12	300	Sc+Su	0.41	±	0.02 ^b	4.48	±	0.42 ^{ab}	50.20	±	7.94 ^{ab}	179.55	±	14.48 ^b	11.59	±	0.27	54.29	±	8.79 ^a
12	300	ScxSe	0.82	±	0.03 ^a	6.68	±	0.54 ^{bc}	115.36	±	0.19 ^d	299.97	±	28.53 ^a	10.04	±	0.75	70.67	±	0.88 ^a
12	300	ScxSu	0.62	±	0.00 ^{abc}	3.03	±	0.25 ^a	81.56	±	0.54 ^c	286.25	±	9.41 ^a	7.75	±	3.97	123.65	±	5.08 ^b
28	60	Sc	0.30	±	0.00 ^a	6.29	±	0.11 ^a	154.84	±	2.74 ^a	703.89	±	9.93 ^a	nd	190.94	±	0.44 ^c		
28	60	Se	0.25	±	0.02 ^a	4.80	±	0.57 ^{ac}	45.40	±	1.14 ^e	391.24	±	3.47 ^b	nd	267.23	±	10.04 ^a		
28	60	Su	0.29	±	0.00 ^a	3.26	±	0.16 ^c	81.95	±	1.43 ^b	341.62	±	2.09 ^b	nd	353.34	±	16.52 ^b		
28	60	Sc+Se	0.36	±	0.04 ^a	9.26	±	0.25 ^b	149.94	±	7.45 ^a	687.72	±	69.92 ^a	nd	301.63	±	18.82 ^{ab}		
28	60	Sc+Su	0.34	±	0.00 ^a	9.19	±	0.05 ^b	142.55	±	1.25 ^a	599.33	±	57.28 ^a	nd	297.98	±	14.35 ^{ab}		
28	60	ScxSe	1.13	±	0.12 ^b	6.86	±	0.65 ^{ab}	116.50	±	7.89 ^{cd}	660.82	±	20.77 ^a	nd	277.43	±	3.29 ^a		
28	60	ScxSu	0.30	±	0.09 ^a	5.84	±	0.79 ^a	99.80	±	1.92 ^{bc}	634.59	±	15.85 ^a	nd	243.24	±	7.46 ^{ac}		
28	300	Sc	1.17	±	0.27 ^{ab}	2.45	±	0.50 ^{ab}	123.76	±	4.87 ^d	337.03	±	27.09 ^d	8.70	±	2.19	63.50	±	1.27 ^a
28	300	Se	1.64	±	0.29 ^a	7.18	±	1.23 ^c	53.45	±	1.05 ^{bc}	125.55	±	1.12 ^{bc}	10.42	±	2.26	114.53	±	14.56 ^{ab}
28	300	Su	0.68	±	0.12 ^b	0.00	±	0.00 ^b	44.33	±	2.85 ^b	103.10	±	1.10 ^b	8.42	±	0.10	75.52	±	8.01 ^{ab}
28	300	Sc+Se	1.12	±	0.06 ^{ab}	2.84	±	0.05 ^a	100.36	±	0.32 ^a	239.37	±	34.17 ^{ad}	8.94	±	0.21	119.41	±	18.60 ^{ab}
28	300	Sc+Su	1.79	±	0.02 ^a	2.79	±	0.04 ^a	97.69	±	2.38 ^a	204.47	±	15.88 ^{ac}	9.76	±	1.01	117.06	±	0.98 ^{ab}
28	300	ScxSe	2.75	±	0.08 ^c	3.80	±	0.19 ^a	68.20	±	4.71 ^c	233.09	±	11.83 ^a	11.78	±	0.84	136.24	±	19.60 ^b
28	300	ScxSu	2.82	±	0.00 ^c	2.56	±	0.47 ^{ab}	99.78	±	4.90 ^a	180.32	±	12.92 ^{abc}	11.29	±	0.86	95.21	±	3.24 ^{ab}

Supplementary Table 5. Concentration of volatile compounds in mg/L (different letters indicate significant differences, Tukey HSD test $p \leq 0.05$).

Temp (°C)	YAN (mg/L)	strain	ethyl acetate		isoamyl acetate		2-phenylethyl acetate		ethyl hexanoate		ethyl octanoate	
12	60	<i>Sc</i>	29.75	± 6.86 ^a	0.35	± 0.10 ^{ab}	0.00	± 0.00 ^d	0.23	± 0.01 ^b	0.27	± 0.02 ^d
12	60	<i>Se</i>	50.57	± 4.15 ^{ab}	0.63	± 0.03 ^c	1.18	± 0.05 ^c	0.21	± 0.01 ^b	0.06	± 0.01 ^c
12	60	<i>Su</i>	58.42	± 5.09 ^b	0.28	± 0.01 ^a	2.82	± 0.07 ^e	0.33	± 0.00 ^a	0.10	± 0.01 ^c
12	60	<i>Sc+Se</i>	37.23	± 7.22 ^{ab}	0.53	± 0.01 ^{bc}	0.75	± 0.00 ^b	0.32	± 0.04 ^a	0.16	± 0.00 ^{ab}
12	60	<i>Sc+Su</i>	38.58	± 1.94 ^{ab}	0.23	± 0.01 ^a	1.05	± 0.05 ^c	0.48	± 0.00 ^c	0.15	± 0.00 ^a
12	60	<i>ScxSe</i>	41.90	± 4.71 ^{ab}	0.33	± 0.03 ^a	0.36	± 0.02 ^a	0.36	± 0.00 ^a	0.22	± 0.01 ^{bd}
12	60	<i>ScxSu</i>	35.10	± 3.85 ^{ab}	0.26	± 0.00 ^a	0.56	± 0.04 ^{ab}	0.51	± 0.01 ^c	0.18	± 0.01 ^{ab}
12	300	<i>Sc</i>	56.21	± 0.10 ^{ab}	0.83	± 0.00 ^{ab}	0.21	± 0.01 ^a	0.74	± 0.03 ^e	0.90	± 0.05 ^c
12	300	<i>Se</i>	61.80	± 1.62 ^{ab}	1.18	± 0.10 ^b	0.69	± 0.01 ^c	0.32	± 0.01 ^a	0.42	± 0.03 ^a
12	300	<i>Su</i>	129.29	± 4.68 ^c	1.13	± 0.12 ^{ab}	1.11	± 0.05 ^d	0.59	± 0.07 ^{cde}	0.37	± 0.01 ^a
12	300	<i>Sc+Se</i>	92.57	± 4.92 ^b	0.87	± 0.04 ^{ab}	0.30	± 0.00 ^{ab}	0.40	± 0.02 ^{ab}	0.69	± 0.03 ^b
12	300	<i>Sc+Su</i>	195.67	± 18.45 ^d	0.70	± 0.16 ^a	0.36	± 0.05 ^{ab}	0.46	± 0.04 ^{abc}	0.36	± 0.05 ^a
12	300	<i>ScxSe</i>	67.17	± 7.32 ^{ab}	1.05	± 0.10 ^{ab}	0.44	± 0.05 ^b	0.67	± 0.00 ^{de}	0.80	± 0.02 ^{bc}
12	300	<i>ScxSu</i>	55.62	± 1.67 ^a	0.66	± 0.03 ^a	0.37	± 0.00 ^{ab}	0.53	± 0.01 ^{bcd}	0.43	± 0.01 ^a
28	60	<i>Sc</i>	127.72	± 24.53 ^a	2.22	± 0.23 ^b	0.55	± 0.02 ^b	0.81	± 0.04 ^a	0.58	± 0.02 ^a
28	60	<i>Se</i>	294.96	± 2.29 ^b	1.12	± 0.10 ^{cd}	0.96	± 0.03 ^a	0.14	± 0.01 ^d	0.37	± 0.01 ^b
28	60	<i>Su</i>	88.38	± 12.93 ^a	0.83	± 0.07 ^c	2.30	± 0.05 ^c	0.36	± 0.01 ^c	0.42	± 0.03 ^b
28	60	<i>Sc+Se</i>	116.83	± 0.35 ^a	1.84	± 0.19 ^{ab}	0.97	± 0.08 ^a	0.51	± 0.01 ^{bc}	0.52	± 0.05 ^{ab}
28	60	<i>Sc+Su</i>	124.25	± 15.36 ^a	1.48	± 0.17 ^{acd}	1.07	± 0.03 ^a	0.74	± 0.05 ^{ab}	0.65	± 0.03 ^a
28	60	<i>ScxSe</i>	88.69	± 4.22 ^a	1.83	± 0.06 ^{ab}	0.96	± 0.13 ^a	0.88	± 0.01 ^a	1.03	± 0.02 ^c
28	60	<i>ScxSu</i>	119.35	± 4.71 ^a	1.53	± 0.18 ^{abd}	1.06	± 0.01 ^a	0.73	± 0.10 ^{ab}	0.62	± 0.01 ^a
28	300	<i>Sc</i>	71.70	± 3.57 ^a	5.92	± 0.24 ^c	2.01	± 0.00 ^{bc}	1.03	± 0.07 ^b	1.01	± 0.10 ^{ab}
28	300	<i>Se</i>	92.52	± 0.52 ^b	0.63	± 0.02 ^b	0.93	± 0.09 ^a	0.23	± 0.00 ^c	0.71	± 0.04 ^{bc}
28	300	<i>Su</i>	68.02	± 1.44 ^a	0.97	± 0.18 ^b	1.01	± 0.12 ^a	0.22	± 0.02 ^c	0.31	± 0.05 ^d
28	300	<i>Sc+Se</i>	65.50	± 1.86 ^a	2.76	± 0.10 ^a	1.08	± 0.05 ^a	0.81	± 0.04 ^{ab}	0.65	± 0.07 ^{bd}
28	300	<i>Sc+Su</i>	60.68	± 1.19 ^a	2.98	± 0.02 ^a	1.45	± 0.08 ^{ab}	0.68	± 0.03 ^a	0.85	± 0.07 ^{abc}
28	300	<i>ScxSe</i>	76.96	± 3.62 ^{ab}	3.43	± 0.07 ^a	2.09	± 0.18 ^c	0.94	± 0.08 ^{ab}	1.18	± 0.02 ^a
28	300	<i>ScxSu</i>	72.19	± 5.54 ^a	3.16	± 0.01 ^a	1.40	± 0.07 ^a	0.80	± 0.08 ^{ab}	1.18	± 0.03 ^a

Supplementary Table 5. Concentration of volatile compounds in mg/L (continue) (different letters indicate significant differences, Tukey HSD test $p \leq 0.05$).

Temp (°C)	YAN (mg/L)	strain	ethyl decanoate			diethyl succinate			isobutanol		isoamyl alcohol		benzyl alcohol		2-phenylethanol	
12	60	<i>Sc</i>	0.41	±	0.01 ^c	5.65	±	0.31 ^b	42.69	±	0.30 ^{ab}	205.15	±	17.62 ^a	nd*	78.94 ± 4.66 ^a
12	60	<i>Se</i>	0.09	±	0.01 ^b	3.30	±	0.44 ^a	54.92	±	0.06 ^{cd}	420.49	±	25.58 ^b	nd	113.29 ± 3.17 ^{ab}
12	60	<i>Su</i>	0.22	±	0.01 ^a	2.79	±	0.15 ^a	39.08	±	0.21 ^a	188.43	±	5.57 ^a	nd	108.81 ± 1.55 ^{ab}
12	60	<i>Sc+Se</i>	0.14	±	0.01 ^b	2.89	±	0.36 ^a	61.62	±	0.97 ^{de}	336.36	±	7.31 ^b	nd	131.68 ± 2.49 ^b
12	60	<i>Sc+Su</i>	0.27	±	0.03 ^a	2.06	±	0.45 ^a	47.47	±	2.29 ^{abc}	155.63	±	7.01 ^a	nd	73.00 ± 1.39 ^{ac}
12	60	<i>ScxSe</i>	0.41	±	0.00 ^c	6.10	±	0.01 ^b	67.39	±	1.97 ^e	334.89	±	31.75 ^b	nd	133.75 ± 21.46 ^b
12	60	<i>ScxSu</i>	0.24	±	0.02 ^a	2.72	±	0.16 ^a	49.83	±	3.74 ^{bc}	203.79	±	6.95 ^a	nd	63.11 ± 2.03 ^c
12	300	<i>Sc</i>	0.64	±	0.03 ^a	8.45	±	1.12 ^c	58.60	±	0.78 ^a	198.41	±	3.18 ^{bc}	10.11 ± 0.25	51.41 ± 2.69 ^a
12	300	<i>Se</i>	0.44	±	0.01 ^{bc}	4.16	±	0.50 ^a	60.67	±	1.14 ^a	253.39	±	2.30 ^{ac}	10.98 ± 0.59	63.66 ± 1.19 ^a
12	300	<i>Su</i>	0.83	±	0.05 ^a	2.88	±	0.22 ^a	39.82	±	1.86 ^b	295.98	±	15.84 ^a	9.74 ± 1.36	108.89 ± 8.36 ^b
12	300	<i>Sc+Se</i>	0.84	±	0.08 ^a	6.99	±	0.01 ^{bc}	58.33	±	1.80 ^a	205.99	±	7.63 ^{bc}	11.22 ± 0.54	41.54 ± 3.72 ^a
12	300	<i>Sc+Su</i>	0.41	±	0.02 ^b	4.48	±	0.42 ^{ab}	50.20	±	7.94 ^{ab}	179.55	±	14.48 ^b	11.59 ± 0.27	54.29 ± 8.79 ^a
12	300	<i>ScxSe</i>	0.82	±	0.03 ^a	6.68	±	0.54 ^{bc}	115.36	±	0.19 ^d	299.97	±	28.53 ^a	10.04 ± 0.75	70.67 ± 0.88 ^a
12	300	<i>ScxSu</i>	0.62	±	0.00 ^{abc}	3.03	±	0.25 ^a	81.56	±	0.54 ^c	286.25	±	9.41 ^a	7.75 ± 3.97	123.65 ± 5.08 ^b
28	60	<i>Sc</i>	0.30	±	0.00 ^a	6.29	±	0.11 ^a	154.84	±	2.74 ^a	703.89	±	9.93 ^a	nd	190.94 ± 0.44 ^c
28	60	<i>Se</i>	0.25	±	0.02 ^a	4.80	±	0.57 ^{ac}	45.40	±	1.14 ^e	391.24	±	3.47 ^b	nd	267.23 ± 10.04 ^a
28	60	<i>Su</i>	0.29	±	0.00 ^a	3.26	±	0.16 ^c	81.95	±	1.43 ^b	341.62	±	2.09 ^b	nd	353.34 ± 16.52 ^b
28	60	<i>Sc+Se</i>	0.36	±	0.04 ^a	9.26	±	0.25 ^b	149.94	±	7.45 ^a	687.72	±	69.92 ^a	nd	301.63 ± 18.82 ^{ab}
28	60	<i>Sc+Su</i>	0.34	±	0.00 ^a	9.19	±	0.05 ^b	142.55	±	1.25 ^a	599.33	±	57.28 ^a	nd	297.98 ± 14.35 ^{ab}
28	60	<i>ScxSe</i>	1.13	±	0.12 ^b	6.86	±	0.65 ^{ab}	116.50	±	7.89 ^{cd}	660.82	±	20.77 ^a	nd	277.43 ± 3.29 ^a
28	60	<i>ScxSu</i>	0.30	±	0.09 ^a	5.84	±	0.79 ^a	99.80	±	1.92 ^{bc}	634.59	±	15.85 ^a	nd	243.24 ± 7.46 ^{ac}
28	300	<i>Sc</i>	1.17	±	0.27 ^{ab}	2.45	±	0.50 ^{ab}	123.76	±	4.87 ^d	337.03	±	27.09 ^d	8.70 ± 2.19	63.50 ± 1.27 ^a
28	300	<i>Se</i>	1.64	±	0.29 ^a	7.18	±	1.23 ^c	53.45	±	1.05 ^{bc}	125.55	±	1.12 ^{bc}	10.42 ± 2.26	114.53 ± 14.56 ^{ab}
28	300	<i>Su</i>	0.68	±	0.12 ^b	0.00	±	0.00 ^b	44.33	±	2.85 ^b	103.10	±	1.10 ^b	8.42 ± 0.10	75.52 ± 8.01 ^{ab}
28	300	<i>Sc+Se</i>	1.12	±	0.06 ^{ab}	2.84	±	0.05 ^a	100.36	±	0.32 ^a	239.37	±	34.17 ^{ad}	8.94 ± 0.21	119.41 ± 18.60 ^{ab}
28	300	<i>Sc+Su</i>	1.79	±	0.02 ^a	2.79	±	0.04 ^a	97.69	±	2.38 ^a	204.47	±	15.88 ^{ac}	9.76 ± 1.01	117.06 ± 0.98 ^{ab}
28	300	<i>ScxSe</i>	2.75	±	0.08 ^c	3.80	±	0.19 ^a	68.20	±	4.71 ^c	233.09	±	11.83 ^a	11.78 ± 0.84	136.24 ± 19.60 ^b
28	300	<i>ScxSu</i>	2.82	±	0.00 ^c	2.56	±	0.47 ^{ab}	99.78	±	4.90 ^a	180.32	±	12.92 ^{abc}	11.29 ± 0.86	95.21 ± 3.24 ^{ab}

nd*: not detected

Supplementary Table 6. Factorial ANOVA of volatile compounds.

	Strain	Temperature	Nitrogen	S*T	S*N	T*N	S*T*N
glycerol	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
ethanol	0.000*	0.000*	0.000*	0.000*	0.002*	0.000*	0.000*
citric acid	0.000*	0.000*	0.000*	0.040*	0.000*	0.000*	0.000*
tartaric acid	0.000*	0.913	0.001*	0.010*	0.056	0.168	0.318
malic acid	0.000*	0.898	0.000*	0.000*	0.000*	0.000*	0.523
succinic acid	0.000*	0.000*	0.000*	0.000*	0.165	0.000*	0.098
lactic acid	0.005*	0.232	0.009*	0.006*	0.049*	0.000*	0.494
acetic acid	0.000*	0.000*	0.000*	0.000*	0.003*	0.000*	0.000*
ethyl acetate	0.000*	0.000*	0.031*	0.000*	0.000*	0.000*	0.000*
isoamyl acetate	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
2-phenylethyl acetate	0.000*	0.000*	0.003*	0.000*	0.000*	0.000*	0.000*
ethyl hexanoate	0.000*	0.000*	0.000*	0.000*	0.000*	0.002*	0.000*
ethyl octanoate	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
ethyl decanoate	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
diethyl succinate	0.000*	0.042*	0.000*	0.000*	0.000*	0.000*	0.000*
isobutanol	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
isoamyl alcohol	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
benzyl alcohol	0.905	0.747	0.000*	0.591	0.905	0.747	0.591
2-phenylethanol	0.000*	0.000*	0.000*	0.003*	0.000*	0.000*	0.000*

S=strain, T=temperature, N=nitrogen.

* $p \leq 0.05$ indicates a significant difference.