A TIME COURSE METABOLISM COMPARISON AMONG *SACCHAROMYCES CEREVISIAE*, *S.*
UVARUM AND *S. KUDRIAVZEVII* SPECIES IN WINE FERMENTATION
Romain Minebois^a, Roberto Pérez-Torrado^a and Amparo Querol^{a*}

^aInstituto de Agroquímica y Tecnología de los Alimentos, IATA-CSIC. E-46980
Paterna, Spain.

*Corresponding author: aquerol@iata.csic.es

ABSTRACT

In this study, we presented the first metabolome time course analysis performed among a set of *S. uvarum*, *S. kudriavzevii* and *S. cerevisiae* strains under winemaking conditions. Extracellular and intracellular metabolites, as well as physiological parameters of yeast cells, were monitored along the process to find evidence of different metabolic strategies among species to perform alcoholic fermentation. A thorough inspection of time trends revealed several differences in utilization or accumulation of fermentation by-products. We confirmed the ability of *S. uvarum* and *S. kudriavzevii* strains to produce higher amounts of glycerol, succinate or some fusel alcohols and their corresponding esters. We also reported differences in the yields of less common fermentative by-products involved in redox homeostasis, namely 2,3 butanediol and erythritol. 2,3 butanediol yield was higher in must ferment with cryophilic strains and erythritol, a pentose phosphate pathway derivative, was particularly overproduced by *S. uvarum* strains. Contrary to *S. cerevisiae*, a singular production-consumption rate of acetate was also observed in *S. uvarum* and *S. kudriavzevii* fermentations. Since acetate is a precursor for acetyl-CoA production which is involved in the biosynthesis of membrane lipids, cryophilic strains might take advantage of extracellular acetate to remodel cell membrane as ethanol content increased during fermentation.

Keywords: *S. uvarum*, *S. kudriavzevii*, central carbon metabolism, nitrogen metabolism, redox balance, wine fermentation

1. INTRODUCTION

During wine fermentation, the majority of sugar content is metabolized by yeasts to produce ethanol and carbon dioxide. The remaining fraction of sugars is used for the synthesis of biomass and hundreds of additional metabolites, including organic acids (acetic acid, succinate), flavor-active secondary metabolites (esters, fusel alcohols, aldehydes, ketones, volatile sulfur compounds) or sugar alcohols such as glycerol, the most important by-product after carbon dioxide and ethanol. Therefore, there is a high diversity in the fermentative compounds originated from central carbon metabolism of yeasts, which determine the organoleptic properties (flavor and aroma) of wine. For this reason, the knowledge of their individual contribution to the global profile of the wine is a principal concern for oenologists who look for the most equilibrated beverages in terms of aromas, astringency, acidity and alcohol perception (Lambrechts and Pretorius, 2000). Today, with the increase of temperature during the growing season of grapes - as a result of climate change - the sugar level of grape musts tends to be higher at the cost of phenolic ripeness and acidity in most of the wine-producing regions (Cohen et al., 2008; Mira de Orduña, 2010). This high sugar content – subsequently converted to ethanol by yeasts – lead wines to be more alcoholic, increasing the perception of heat, altering the perception of wine aromas complexity and decreasing the color intensity and stability (Goldner et al., 2009; Pickering and Vanhanen, 1998). Thus, the production of wines with reduced ethanol concentration and enhanced aroma content without weakening product value has been the focus of extensive research over the past decades.

Viticulture techniques and dealcoholization treatments are some of the techniques currently employed to reduce alcohol concentration (Aguera et al., 2010; Catarino and Mendes, 2011; Kontoudakis et al., 2011; Pickering, 2000; Schmidtke et al., 2012). However, these approaches are difficult and expensive to implement. Alternative *Saccharomyces* species have attracted attention as a poor exploited resource of yeast biodiversity with interesting attributes not present in *S. cerevisiae*, the dominant species in alcoholic fermentation. Notably, the cryophilic species *S. uvarum* and *S. kudriavzevii* and their interspecific hybrids, have recently demonstrated interesting properties, especially at low fermentation temperatures. In particular, *S. uvarum* and *S. kudriavzevii* fermentation profiles differ from that of *S. cerevisiae* in terms of fermentative by-products yields and biomass production. Both cryophilic species generate higher levels of volatile fermentative compounds, particularly 2-phenylethanol and 2-phenylethyl acetate in *S. uvarum* (Gamero et al., 2013; Masneuf-Pomarède et al., 2010). These differences in aroma production have been correlated with differential gene regulations and enzymatic activities as compared to *S. cerevisiae* (Gamero et al., 2014; Stribny et al., 2016, 2015). Moreover, besides it produces lower levels of ethanol and acetic acid, *S. uvarum* also produces higher levels of glycerol and succinate (Castellari et al., 1994; Kishimoto, 1994; Rainieri et al., 1999). Focusing on *S. kudriavzevii*, it was observed that this species mainly directed carbon flux towards glycerol synthesis instead of ethanol at low pH, high sugar concentrations and low temperatures in comparison with *S. cerevisiae* (Arroyo-López et al., 2010). It has also been reported in chemostat cultures performed at 12°C that *S. kudriavzevii* produces more biomass and glycerol than *S. cerevisiae* and that this increase in the glycerol yield was due to a higher expression and enhanced enzymatic parameters of the glycerol-3-phosphate dehydrogenase 1 (Gpd1) protein (Oliveira et al., 2014). Finally, in a

metabolomics comparison carried out in steady-state among the three species, it was also pointed out the existence of another regulation of the central carbon metabolism involved in cold resistance strategies in cryophilic species (López-Malo et al., 2013). *S. uvarum* presented elevated shikimate pathway activity, while *S. kudriavzevii* displayed increased NAD⁺ synthesis.

Since *S. cerevisiae* is the most employed yeast in the field of food and fermented beverages, it has been the subject of many studies, and strong fundamental knowledge of its physiology, metabolism, and genetics are now available. On the contrary, there is still little information available on the metabolic basis that explains the fermentative characteristics of the cryophilic species *S. uvarum* and *S. kudriavzevii*. The aim of this study was to explore the dynamic alcoholic fermentation profile of a set of wine commercial (T73 and BMV58) and other isolates (CECT12600 and CR85) of the species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* in mimicked wine elaboration. During the fermentation process and using a metabolomics approach, the main intracellular and extracellular fermentative by-products, gases yields and physiological parameters were registered to understand the metabolic differences among the three species that could explain their oenological performance in those conditions.

2. MATERIAL AND METHODS

2.1 Yeast strains

A commercial *S. cerevisiae* wine strain (T73, Lallemant, Montreal), originally isolated from wine in Alicante, Spain (Querol et al., 1992) as well as two *S. uvarum* strains, respectively a wine commercial (Velluto BMV58) and a non-commercial strain isolated from a non-fermentative environment (CECT12600) and a wild (no commercial strains are available) *S. kudriavzevii* strain (CR85), previously identified and

differentiated in Lopes et al. (2010) were used in this work. The isolation source and geographical origin of the strains used herein are shown in **Table 1**.

Cryogenically preserved (-80°C) strains were cultured and maintained on GPY plates (2% glucose, 2% agar, 0,5% peptone, 0,5% yeast extract) and stored at 4°C. Bioreactors were inoculated at OD600 0.1 (approximately 1×10^6 cells/ml) from starter culture. Starter culture was prepared by growing strains overnight in an Erlenmeyer flask containing 25 ml of GPY liquid medium (2% glucose, 0.5% peptone, 0.5% yeast extract) at 25°C in an agitated incubator (Selecta, Barcelona, Spain) under aerobic conditions.

2.2 Microvinification experiments

The must obtained from the Merseguera white grapes variety, collected in Titaguas (Spain) winery and stored in several small frozen volumes (4 L, -20°C) was used for the microvinification assays. Before use, must was clarified by sedimentation for 24 h at 4°C and dimethyl dicarbonate (DMDC) at 1 mL^{-1} was added for sterilization. Before fermentation, the fermentable sugar level was corrected as performed in most European countries by adding chemically pure sucrose (AppliChem Panreac, Darmstadt, Germany) to reach a probable alcoholic grade of 12.5%. To avoid any stuck or sluggish fermentations, nitrogen content was adjusted by adding a nitrogen supplement, which consisted of 0.2 g/l of ammonium sulfate and 0.1 mg/l of thiamine hydrochloride (Sigma-Aldrich, Barcelona, Spain). Nitrogen composition of the grape juice is available in supplementary material (**Table S1**). All the fermentations were carried out in independent biological triplicates by using 470 ml of must in sterile 500 ml laboratory bioreactors (MiniBio, Applikon, the Netherlands). Data were integrated into the MyControl and BioExpert software (Applikon, The Netherlands). Dynamic evolution of the fermentation was controlled by using different probes and detectors

measuring temperature, dissolved O₂ (AppliSens, Applikon, The Netherlands) and effluent gas content (Multi-Gas Monitors INNOVA 1316, LumaSense Technologies). Fermentations were monitored by measuring the residual sugar content by HPLC until constant values were reached, considered to be the end of the fermentation.

2.3 Sampling

Bioreactors were sampled in selected time points of the fermentation. In order not to perturb the fermentation conditions, the total sampling volume was limited to 10% (47 ml) of the initial working volume. Metabolic profiling was performed for extra and intracellular metabolites. Extracellular metabolites were determined from sample supernatants and included sugars, organic acids, glycerol, ethanol, 2,3 butanediol, erythritol, ammonia, amino acids, and volatile compounds. Intracellular metabolites including, sugars, intermediates of central carbon metabolism and amino acids, were extracted from quenched cell pellets following an adapted procedure from Villas-Bôas et al. (2005). Extracellular metabolites were determined in 10 sampling points along the fermentation, and in 5 of these points, intracellular metabolites were also measured. For intracellular metabolites determination approximately 20-30 OD₆₀₀ of cells were rapidly sampled from the bioreactor and quenched in a tube containing pure cold methanol (-40°C). The samples were carefully released into the center of the pure methanol solution to avoid freezing on the sides of the tubes. Cell separation was accomplished through 90 sec centrifugation of the sample tubes in an R5100 centrifuge (Eppendorf, Hamburg, Germany) at 4,000 rpm using a precooled rotor at minimum temperature (-9°C). The supernatant was discarded, cell pellet flashes frozen with nitrogen and tubes stored at -80°C until intracellular metabolites extraction. During all the procedure, sample tubes were handled at temperature below -20°C by using dry ice to prevent any reaction to proceed until complete protein denaturation during the extraction step.

2.4 Intracellular metabolites extraction and quantification

Intracellular metabolites were extracted by adapting the pure cold methanol extraction procedure used in Villas-Bôas et al. (2005). A 250 µl volume of cold (-40°C) absolute methanol was added to the cell pellet in 15 ml falcon tube. After rapid mixing, the tube was transferred into dry ice for 30 min. The sample was then thawed in an ice bath for 10 min and the cells were centrifuged in a pre-cooled Eppendorf centrifuge at maximum speed (4000 rpm) for 10 min at 0°C. The first supernatant was then transferred to a screw cap Eppendorf tube and to the extracted pellet, another 250 µl volume of pure cold methanol was added to extract any metabolites left after the first extraction. The first and second extracts were combined and stored at -80°C until quantification by GC-MS. Primary metabolite analysis was performed at the Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC, Valencia, Spain) Metabolomics Platform by a method modified from that described by Roessner et al. (2000). An extract volume corresponding to 0.5 mg of yeast fresh weight (2-5 µl) was mixed with 3 µl of internal standard (0.2 mg/ml ribitol in water) and reduced to dryness in a speed-vac. For derivatization, dry residues were redissolved in 40 µl of 20 mg/ml methoxyamine hydrochloride in pyridine and incubated for 90 min at 37°C, followed by addition of 60 µl MSTFA (N-methyl-N-[trimethylsilyl]trifluoroacetamide) and 6 µl of a retention time standard mixture (3.7% [w/v] mix of fatty acid methyl esters ranging from 8 to 24°C) and further incubation for 30 min at 37 °C. Sample volumes of 2 µl were injected in the split and splitless mode to increase metabolite detection range in a 6890 N gas chromatograph (Agilent Technologies Inc. Santa Clara, CA) coupled to a Pegasus 4D TOF mass spectrometer (LECO, St. Joseph, MI). Gas chromatography was performed on a BPX35 (30 m × 0.32 mm × 0.25 µm) column (SGE Analytical Science Pty Ltd., Australia) with helium as a carrier gas, constant flow 2 ml/min. The liner was

set at 230°C. Oven program was 85°C for 2 min, 8°C/min ramp until 360°C. Mass spectra were collected at 6.25 spectra s⁻¹ in the m/z range 35–900 and ionization energy of 70 eV. Chromatograms and mass spectra were evaluated using the CHROMATOF program (LECO, St. Joseph, MI). Peak areas were normalized with the internal standard area (ribitol) and with the average weight of yeast cells used for cold-methanol extraction. Finally, for each strain, the relative amount of each compound was computed in relation to the first sampling time point, excepting trehalose and citramalate for which last sampling time point was used.

2.5 Extracellular metabolites quantification

Sugars (glucose, fructose), fermentative by-products (glycerol, ethanol, 2,3 butanediol, and erythritol) and organic acids (acetate, succinate, tartrate, citrate and malate) were respectively determined by HPLC (Thermo Fisher Scientific, Waltham, MA) using a refraction index detector and UV/VIS (210nm) detector equipped with a HyperREZTM XP Carbohydrate H+ 8 mm column (Thermo Fisher Scientific, Waltham, MA) and HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific, Waltham, MA). The analysis conditions were: eluent, 1.5 mM of H₂SO₄; 0.6 ml.min⁻¹ flux and oven temperature of 50°C. For sucrose determination, the same HPLC was equipped with a Hi-Plex Pb, 300 x 7.7 mm column (Agilent Technologies, CA, USA) and peaks quantified by the RI detector. The analysis conditions were: eluent, Milli-Q water; 0.6 ml.min⁻¹ flux and oven temperature of 50°C. The retention times of the eluted peaks were compared to those of commercial analytical standards (Sigma-Aldrich, Madrid, Spain). Concentrations, in g/l, were quantified by the calibration graphs (R² value > 0.99) of the standards that were previously obtained from a linear curve fit of the peak areas using ten standards mixtures.

Determination of yeast assimilable nitrogen in the form of amino-acids and ammonia was carried following the same protocol than Su et al. (2020). A volume of supernatant was derivatised and amino acids and ammonia separated by UPLC (Dionex Ultimate 3000, Thermo Fisher Scientific, Waltham, MA) equipped with a Kinetex 2.6u C18 100A column (Phenomenex, Torrance, CA, USA) and Accucore C18 10 x 4.6 mm 2.6um Defender guards (Thermo Fisher Scientific, Waltham, MA). For derivatization, 400 µl of the sample were mixed with 430 µl borate buffer (1M, pH 10.2), 300 µl absolute methanol and 12 µl of diethyl ethoxymethylenemalonate (DEEMM), and ultra-sonicated for 30 min at 20°C. After ultra-sonicating, the sample was warmed up at 80°C for 2 hours to allow the complete degradation of excess DEEMM. Once the derivatization finished, the sample was filtered with 0.22 µm filter before injection. The target compounds in the sample were then identified and quantified according to the retention times, UV-vis spectral characteristics and calibration curves (R^2 value > 0.99) of the derivatives of the corresponding standards. Amino acid standard (ref AAS18), asparagine and glutamine purchased from Sigma-Aldrich were used for calibration.

2.6 Volatile compounds extraction and quantification

Volatile compounds extraction and gas chromatography were performed following the protocol of Stribny et al. (2015). 1.5 mL of the supernatant was transferred to 15-mL vials with 0.35 g of NaCl. 20 µl volume of 2-heptanone (0.005 %) was added as an internal standard. Higher alcohols and esters were analyzed by the headspace solid-phase microextraction (HS-SPME) technique with 100-µm polydimethylsiloxane (PDMS) fiber (Supelco, Sigma-Aldrich, Madrid, Spain). Solutions were maintained for 2 h at 20°C to establish the headspace-liquid equilibrium. The fiber was inserted into the headspace through a vial septum and was held for 7 min. The fiber was then inserted into the gas chromatograph inlet port for 4 min at 220°C

with helium flow (1 mL/min) to desorb analytes. A Thermo Science TRACE GC Ultra gas chromatograph 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, Valencia, Spain). The oven temperature program 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 was kept constant at 300°C. A chromatographic signal was recorded by the ChromQuest program. Volatile compounds were identified by the retention time for reference compounds. Quantification of the volatile compounds was determined using the calibration graphs of the corresponding standard volatile compounds.

2.7 Biomass and physiological parameters determination

Biomass and physiological parameters - OD600, dry weight (DW), colonies-forming unit (CFUs) and average cell diameter (ACD) - were determined in every sampling point providing that cell sample was sufficient to perform the corresponding measure. For DW determination, 2 ml of fresh sample placed in a pre-weighed Eppendorf tube were centrifuged at maximum speed (13,400 rpm) in a MiniSpin centrifuge (Eppendorf, Spain) for 5 minutes. The supernatant was carefully removed with a pipette, the pellet washed with 70 % (v/v) ethanol and centrifuged in the same conditions. After washing, the aqueous supernatant was carefully removed and tube stored at 65°C for 72h. DW was obtained by measuring the mass weight difference of tubes with a BP121S analytical balance (Sartorius, Goettingen, Germany). Due to the working volume limitation, DW determination was impossible at the beginning of the process. OD600 was measured at each sampling point using a diluted volume of sample and a Biophotometer spectrophotometer (Eppendorf, Hamburg, Germany). CFUs were determined using a 100-200 μl of a diluted volume of samples plated in GPY solid

medium and incubated two days at 25°C. After what, the resulting colonies were counted with a Comecta S.A Colony Counter. Plates with CFUs between 30 and 300 were used to calculate the CFUs of the original sample. For ACD determination, a volume of cell sample was diluted into phosphate-buffered saline solution and cell diameter measured using a Scepter Handled Automated Cell Counter equipped with a 40 µm sensor (Millipore, Billerica, USA).

2.8 Statistical analysis

The presented values are averages of biological triplicates with standard errors. Kinetics parameters were calculated by fitting OD₆₀₀ values to reparametrized Gompertz equation using the Statsoft Statistica 10 Package. The extracellular concentration over time of the main by-products (ethanol, glycerol, succinate, and 2,3 butanediol) were represented and fitted by nonlinear regression to allosteric sigmoidal, sigmoidal 4PL or asymmetric sigmoidal 5PL equations of the GraphPad Prism 6.0 Software package (La Jolla, CA, USA). Best-fit curves were selected following the recommendations of Motulsky and Christopoulos (2004). The first derivative of the fitted curve was subsequently calculated to determine the specific production rate (q) of each compound.

3. RESULTS

3.1 Fermentative and kinetic parameters

To determine the metabolic differences among different species of *Saccharomyces* in wine fermentation, we performed fermentation in 0.5 L vessel bioreactors at 25 °C using natural white must with *S. cerevisiae* T73 strain, BMV58 and CECT12600 *S. uvarum* strains and CR85 *S. kudriavzevii* strain. Monitoring and evaluation of sugar consumption, OD600, dissolved oxygen and carbon dioxide released were used as an indicator of yeast growth and fermentative activity while YAN

(Yeast Assimilable Nitrogen) content was used as a limiting factor. These parameters are presented in **Figure 1**.

Kinetic parameters were determined by adjusting OD600 measures to the reparametrized Gompertz equations and are presented in **Table 2**. The T73 wine strain was the quickest initiating fermentation with the lowest lag phase estimated at 2.14 hours according to Gompertz parametrization (**Table 2**). Both *S. uvarum* strains showed a slightly higher lag phase –approximately 2 folds higher– compared to the commercial *S. cerevisiae* strain. However, BMV58 strain was the strain with the highest maximum growth rate among the four strains estimated at 0.34 h^{-1} consistent with previously described anaerobic growth values. There were no significant differences between the *S. cerevisiae* wine strain and both *S. uvarum* strains in terms of cell population reached during stationary phase. Non-surprisingly, at 25°C, the natural and cryophilic CR85 strain was the slowest in performing the alcoholic fermentation. CR85 strain showed a significantly larger lag phase, respectively 28-folds, 14-folds and 11-folds higher than T73, CECT12600 and BMV58 strains. Results obtained from the end of exponential growth phase showed that at 25°C, the T73 wine strain produced more biomass than BMV58, CECT12600 and CR85 strains. Regarding the CR85 strain, it had the lowest DW values as well as the lowest cell population in stationary phase (**Table 2; Figure 2A**). Moreover, it did not consume the totality of the fermentable sugars presented in the must (**Figure 1**), which pointed out its regular fermentative ability at 25°C.

To obtain additional physiological information, the average cell diameter (ACD) was measured along the fermentation process in T73, CECT12600 and BMV58, the three strains that showed good fermentative behavior. Along the entire fermentation process, ACD of the *S. cerevisiae* wine strain was higher than both *S. uvarum* strains (**Figure 2B**). Nearly after 8 hours of fermentation, maximum ACD values of $6.19\text{ }\mu\text{m}$,

5.58 μm , and 5.45 μm were reached by T73, CECT12600 and BMV58 strains respectively. In addition, we observed that the three strains globally shared the same pattern of ACD. Very interestingly, the comparison of ACD with the rest of metabolic data led us to confirm that slope changes observed in **Figure 2B** were concomitant with metabolic transitions during cell growth due to modifications in the fermentation conditions.

Based on this observation and the timing of the fermentative activity parameters (CO_2 released, dissolved oxygen, sugar consumption, and YAN content), the cultivation could be divided into four phases, namely a transitory aerobic phase (I), an exponential growth phase delimited by the nitrogen content (II), a stationary phase (III) and the end of fermentation (IV), as indicated on the bottom of **Figure 1**.

3.2 Phase I - a transitory aerobic phase

Gas probes registered a rapid depletion of the dissolved oxygen initially present inside the bioreactors. There was no more dissolved oxygen present in the medium nearly after 8 hours in T73, BMV58 and CECT12600 fermentations (**Figure 1**). In CR85, oxygen depletion occurred after 70 hours, which was in accordance with its longer lag phase (**Figure 1; Table 2**). Molecular oxygen exhaustion represented the end of phase I and the transition from aerobic to self-generated anaerobic conditions. Interestingly, molecular oxygen depletion concurred with the mentioned peak of ACD observed after 8 hours in *S. cerevisiae* and *S. uvarum* fermentations (**Figure 2B**). Moreover, the extracellular levels of acetate and proline, relative to their initial concentration at the time of inoculation, were calculated and are presented in **Figure 3**. We observed that the relative extracellular level of acetate decreased at the beginning of the fermentation in the four strains (**Figure 3A**). The same occurred with the proline

level in BMV58, CECT12600 and CR85 strains, while in T73 fermentation no decrease was observed (**Figure 3B**).

3.3 Phase II - an exponential growth phase delimited by the nitrogen content

Nitrogen availability is a critical factor for yeasts during alcoholic fermentation because it affects both fermentation kinetics and the formation of metabolites. In the present work, extracellular ammonium and free amino acids concentrations in the medium were determined by UPLC (**Figure S1**) and served for the calculation of the yeast assimilable nitrogen (YAN) presented in **Figure 1**. Moreover, for each strain, the extracellular concentration across time of each nitrogen source was presented in the form of a heatmap in **Figure 4**. The timing of nitrogen uptake and cell growth was coherent with growth arrest triggered by total (T73, BMV58, and CECT12600) or partial depletion (CR85) of YAN corresponding with the end of phase II (**Figure 1; Figure 4**). Monitoring of YAN showed that the assimilable nitrogen was completely consumed after the exponential growth phase finished in *S. cerevisiae* and *S. uvarum* fermentations. YAN decreased below 2% of its initial content after 27 and 44 hours in *S. cerevisiae* and *S. uvarum* fermentations respectively. Focussing on arginine, the predominant amino-acid in the natural white must (**Table S1**), 25% of its initial content was still available after 27 hours in BMV58 and CECT12600 fermentations while it was already depleted at this time in T73 fermentation (**Figure 4; Figure S1**). In CR85 strain, YAN content decreased until 22% after 115 hours of fermentation and coincided with the end of its exponential growth phase (**Figure 1**). Consistent with its lower biomass yield and its poor fermentability at 25°C, we observed that CR85 strain consumed only a half of the arginine content initially present in the grape must (**Figure 4; Figure S1**).

The level of intracellular amino-acids was determined by GC-MS for the four strains (**Figure 5A; Figure S2**) and in the five intracellular sampling points. Except for

leucine, valine, and proline, the rest of amino-acids reached their minimum intracellular level after 27 hours (Glutamine, GABA) or 44 hours (others) in T73 strain fermentation, coinciding with the end of the exponential growth phase. From then on, an increase in alanine, asparagine and glutamate content inside T73 cells was reported while levels of other amino-acids remained stable or null. In BMV58 strain, most of the amino-acids reached their minimal value after 44 hours of fermentation excepting asparagine, serine, glutamine, pyroglutamic acid and proline. In CECT12600 strain, tryptophan depletion was observed earlier (27 h) and isoleucine and aspartate seemed to be totally catabolized after 69 hours. On the other hand, minimum intracellular nitrogen values were mostly reported after 44 hours (**Figure 5A; Figure S2**). In CR85 strain, the maximum intracellular amino-acid levels were observed after 115 hours of fermentation, corresponding to the end of phase II (**Figure 5A; Figure S2**). From that time on, most amino acid levels dropped to reach their minimum value after 144 hours. To sum up, despite some individual variations between the four strains, results showed that a general decrease in the intracellular amino-acids levels occurred with the entry into stationary phase at the end of phase II.

The extracellular concentration of the main by-products and sugars during the fermentations were determined by HPLC (**Figure S3**). For ethanol, glycerol, succinate and 2,3 butanediol, their extracellular concentration over time were fitted to distinct mathematical models and the derived function subsequently calculated to obtain the specific production rate (q_{Eth} , q_{Gly} , q_{Succ} and q_{But}) of each compound (**Figure 6**). During the whole growth phase, q_{Eth} and q_{Gly} increased in parallel in the four fermentations (**Figure 6**), pointing out the importance of glycerol as redox valve during biomass synthesis. Moreover, q_{Eth} and q_{Gly} peaks were concomitant with both CO_2 exhaustion (**Figure 1**) and intracellular glycerol peaks at the end of phase II (**Figure 5B**;

Figure S4). In concordance with their higher glycerol yields, *S. uvarum* strains BMV58 and CECT12600 and *S. kudriavzevii* CR85 strains showed the highest values for qGly (**Figure 6**). Interestingly, the natural isolate CECT12600 was the strain with the highest qEth at the end of phase II but it was not able to ferment all the sugars (**Figure 1**), pointing out a certain sensitivity to ethanol. A clear production of acetate by the four strains was observed during the exponential growth phase, and CR85 strain had the highest acetate level at the end of phase II (**Figure 3**). During phase II, we also observed the decrease of ACD in T73, BMV58 and CECT12600 fermentations (**Figure 2B**), which most likely accounted for the accumulation of smaller daughter cells during exponential growth.

3.4 Phase III – distinct metabolic responses among species following the onset of nitrogen starvation

When yeast cells enter into nitrogen starvation, the production of the main carbon products of the fermentation, ethanol, and glycerol, decreased (**Figure 6**), and also the level of carbon dioxide reached 0 (**Figure 1**). Only 20-25% of total sugars were exhausted when the four strains entered into stationary phase (**Figure 1**). Interestingly we reported a variety of metabolic responses among the four strains to sustain fermentation following the onset of nitrogen starvation. Among others, significant differences were observed in the yields of organic molecules that participate in redox homeostasis, and in the evolution of the extracellular acetate content.

3.4.1 Succinate and 2,3 butanediol, redox sinks in cryophilic strains

Beside ethanol and glycerol, other organic molecules can serve as electron acceptors from reduced forms of redox cofactors. For instance, succinate and 2,3 butanediol can also contribute to NADH reoxidation during alcoholic fermentation. A

summary of the organic molecules that balance ratios of nicotinamide and flavin adenine redox cofactors during alcoholic fermentation and their places in the central carbon metabolism is presented in **Figure 7**. For each compound, the yield (g of compound produced/ g of sugar consumed) at the end of the fermentation was calculated and compared between the four strains. Our results suggested that *S. uvarum* and *S. kudriavzevii* strains had other balancing strategies in comparison to the *S. cerevisiae* wine strain. BMV58 (0,033 g/g), CECT12600 (0,034 g/g) and CR85 (0,038 g/g) strains had significantly higher glycerol yields than T73 (0,023 g/g) strain (**Figure 7**). Also CR85 strain had the highest 2,3 butanediol yield ($6,6 \cdot 10^{-3}$ g/g), followed by CECT12600 ($5,6 \cdot 10^{-3}$ g/g) and BMV58 ($3,1 \cdot 10^{-3}$ g/g) strains. On the contrary, almost no 2,3 butanediol ($4 \cdot 10^{-4}$ g/g) was yielded by the *S. cerevisiae* wine strain (**Figure 7**). It was concordant with the horizontal linear fitting of 2,3 butanediol concentration across time obtained for T73 in **Figure 6**. Regarding succinate, the BMV58 (0,038 g/g) strain had the highest yield, far from CECT12600 ($6,4 \cdot 10^{-3}$ g/g), CR85 ($3,6 \cdot 10^{-3}$ g/g) and T73 ($2,3 \cdot 10^{-3}$ g/g) strains. In the BMV58 fermentation, the increase in q_{2,3But} rate concurred with the decreases of ethanol and glycerol yields at the end of phase II, while, interestingly, the increase in q_{Succ} rate seemed to occurred only a few hours later during phase III (**Figure 6**). Moreover, q_{Succ} and q_{2,3But} peaked approximately at the same time in CECT12600 and BMV58 strains. Nevertheless, CECT12600 and CR85 strains had significantly higher 2,3 butanediol yields than BMV58 (**Figure 7**). This last observation suggested that CECT12600 and CR85 may preferably shuttled carbon flux from pyruvate to 2,3 butanediol synthesis to control NADH/NAD⁺ ratio. On the contrary, the BMV58 strain might preferably used the succinate sink. Finally, both *S. uvarum* strains had significantly higher D-Lactate yields compared with the T73 and CR85 strains (**Figure 7**).

3.4.2 Differences in acetate metabolism

Following the onset of nitrogen starvation, we reported interesting fluctuations in the extracellular acetate content in BMV58, CECT12600 and CR85 fermentations. In T73 fermentation, the extracellular acetate concentration increased and remained constant from the entry into stationary phase (**Figure 3**). On the contrary, for BMV58, CECT12600 and CR85 strains, the extracellular acetate level decreased when they entered into stationary phase. This decrease was particularly pronounced in CECT12600 strain in which no acetate was detected when the fermentation concluded (**Figure 3; Figure 7**).

In addition, among the organic acids intracellularly detected by GC-MS (**Figure 5C; Figure S5**), citramalate (produced from acetate) showed a different accumulation pattern in T73, CECT12600 and CR85 strains (no data was available for BMV58 strain). Its accumulation occurred almost with the entry into nitrogen limited conditions in T73 and CR85 strains while it seemed to occur later in CECT12600 (**Figure 5C, Figure S5**).

3.4.3 Differences in erythritol synthesis

In this study, the quantification of yeast intracellular compounds by GC-MS led us to detect the production of erythritol by our set of *Saccharomyces* strains (**Figure 5B**). After what, the extracellular erythritol accumulation was also determined by HPLC. We observed that *S. uvarum* strains BMV58 ($2,1 \cdot 10^{-3}$ g/g) and CECT12600 ($1,6 \cdot 10^{-3}$ g/g) had the highest erythritol yields in our experimental conditions (**Figure 7; Table S2**). These results were notably in accordance with the higher intracellular accumulation of erythritol observed in CECT12600 strain (**Figure 5B**). In addition, the increase of extracellular and intracellular levels of erythritol seemed to concur with the

entry into stationary phase in CECT12600 fermentation (**Figure 5B; Figure S4**). On the contrary, intracellular level of erythritol increased during exponential growth phase (phase II) in T73 and CR85 strains, and then decreased in phase III when cells entered into nitrogen starved conditions (**Figure 5B; Figure S4**).

3.4.4 Trehalose accumulation

Trehalose is an important reserve carbohydrate and a stress protectant in yeasts that was intracellularly measured by GC-MS (**Figure 5B; Figure S4**). We observed that trehalose accumulation started simultaneously with the end of the exponential phase and respectively ended after in the four strains (**Figure 5B; Figure S4**). It started to be consumed after 44h of fermentation in T73, BMV58 and CECT12600 strains while after 115h in CR85 strain and concurred with the timing of YAN depletion. Interestingly, CECT12600 and CR85 strains seemed to accumulate higher intracellular amounts of trehalose if compared with the T73 and BMV58 wine strains (**Figure 5B; Figure S4**). This high trehalose accumulation suggest a higher stress response in CECT12600 and CR85 natural isolate strains in comparison with both wine strains, maybe to cope with nutrient starvation and to face to ethanol stress.

3.5 Phase IV – end of fermentation

The end of the fermentation process was marked by the end of carbon dioxide released in the four fermentations and the following increase in molecular oxygen concentration in the medium (**Figure 3**). In parallel, we reported an increase in the extracellular content of various amino acids like lysine, glycine or glutamate, above all in the CR85 strain (**Figure 4**). Moreover, for T73 and BMV58 strains which showed good fermentative behavior, we reported a decrease in cell viability from 90-100 hours of fermentations (**Figure S6**). Thus, some autolysis processes were likely to occur at the

end of fermentation due to nutrient depletion and ethanol accumulation. Interestingly, the decrease in cell viability was steeper in BMV58 strain than in T73 (**Figure S6**), pointing out a higher ethanol resistance of cells of the *S. cerevisiae* wine strain.

In this study, we also evaluated the final concentration of a set of fusel alcohols, acetate esters and ethyl esters due to their relevance in oenological production processes as well as a mechanism to compensate the redox balance. The timing of aroma synthesis is presented in the form of a heat map including the four phases of the fermentation process and suggested that aromas compounds are principally being synthesized at the end of phase II (**Figure 8; Figure S7**). In addition, a PCA analysis was used to integrate the various aroma traits of the four strains. In the resulting PCA plot, the Factor 1 axis (42.83% of the variance) mostly corresponds to 2-phenylethanol, 2-phenylethyl acetate, and total fusels variables, on the left, 1-hexanol, benzyl alcohol and benzyl acetate on the right. The Factor 2 axis (30,52% of the variance) mostly corresponds to the total esters and ethyl acetate variables, on the top, and isobutanol, isoamyl alcohol and ethyl caprylate on the bottom; these two groups of variables being negatively correlated. Consistent with previous studies of our group (Gamero et al., 2013), BMV58 and CECT12600 strains produced higher amounts of 2-phenylethanol and 2-phenylethyl acetate (**Figure 8; Table S2**). CECT12600 was also the strain with the highest total esters yield, mainly on account of its high ethyl acetate production. 2-phenylethanol represented approximately 56% and 48% of the total fusel alcohols yield in BMV58 and CECT12600, while only 21% and 26% in T73 and CR85 strains respectively. Interestingly, benzyl alcohol and benzyl acetate were more produced by T73, CECT12600 and CR85 strains. Finally, isoamyl alcohol, its acetate ester, and several medium chain fatty acid ethyl esters (caproate, caprylate) were presents in a significantly higher amount in T73 fermentation.

4. DISCUSSION

The results presented above illustrate that yeast strains of species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* show distinct responses with respect to the occurrence of alcoholic fermentation under mimicked winemaking conditions. In this study, we used a dynamic approach to explore the time course metabolome of four strains isolated from distinct sources. The yeasts *S. cerevisiae* T73 and *S. uvarum* BMV58 are commercial wine strains currently used in the winemaking process, while *S. uvarum* CECT12600 and *S. kudriavzevii* CR85 strains are not used in industrial fermentations. The monitoring of fermentation was performed in conditions representative of white wine elaboration. To mimic winemaking conditions, we used a natural grape must rather than synthetic media. Indeed, frozen grape juices conserved their fermentation properties and can be kept for long periods (Da Silva et al., 2015). Throughout the complex ripening process of grapes, a wide range of compounds like sugars, organic acids, phenolic compounds, fatty acids, amino acids, vitamins or nucleic acids are synthesized and enter in must composition which is further fermented by yeasts. Consequently, because it is very difficult to reproduce such a complex matrix, synthetic media might be less pertinent than a natural must for assessing quantitative traits due to their incomplete composition (Ambroset et al., 2011; Marullo et al., 2007; Peltier et al., 2018). Recently, most of the studies that focused on comparing phenotypic or metabolomics traits of *S. uvarum* and *S. kudriavzevii* species with *S. cerevisiae* were performed in model synthetic medium, using steady-state culture and in aerobic or strict anaerobic conditions (López-Malo et al., 2013; Masneuf-Pomarède et al., 2010). Thus they give us little information about how these species could metabolically respond to self-generated anaerobic conditions that are generated in wineries, notably during white wine elaboration. In our study, the consumption of the initial dissolved oxygen present in

grape must and the subsequent release of carbon dioxide by yeasts lead to self-generated anaerobic conditions inside the fermenters.

In this work, starter cultures were prepared in GPY liquid medium at 25°C and in aerobic conditions. According to Salmon et al. (1998), in such conditions yeast cells exhibited normal mitochondria, high cytochrome content as well as high oxygen uptake rate at the time of inoculation. On the other hand, *Saccharomyces* yeasts are Crabtree-positive and ferment when sugar concentrations are high, even if oxygen is present for more efficient aerobic respiration (Piškur et al., 2006). Here, following the inoculation, we observed that the four strains behaved quite similarly while dissolved oxygen was present at the beginning of the alcoholic fermentation. In accordance with the duration of their lag phase and their fermentative ability, the level of dissolved oxygen in the grape juice rapidly decreased for the four strains. Dissolved oxygen depletion occurred between 6 and 8 hours in T73, BMV58 and CECT12600 fermentations while it happened slower (70 hours) in CR85 fermentation. Thus, the previous observations together with the aerobic pre-culture preparation suggested that yeast cells could have been aerobically active at the beginning of the fermentation, and that existed a transitory respire-fermentative phase during step I, while dissolved oxygen was available. Consistent with this hypothetic transitory oxidative activity, we reported that extracellular acetate was consumed by the four strains in the early hours of phase I. After acetate uptake by yeasts, cytosolic acetate could be activated to acetyl CoA by the cytosolic acetyl CoA synthetase ACS1 before being shuttled through the mitochondria membrane by acetyl-carnitine shuttle and subsequently metabolized through the TCA cycle as a carbon energy source (Krivoruchko et al., 2015).

In the early stages of the fermentation, we also observed differences in the metabolism of nitrogen sources between *S. cerevisiae* and cryophilic species *S. uvarum*

and *S. kudriavzevii*, notably proline and arginine. We noticed that BMV58, CECT12600 and CR85 strains picked up proline from the medium when dissolved oxygen was present while the wine strain T73 did not. Regarding arginine, its removal was carried out slower by BMV58 and CECT12600 strains, or even only partially by the CR85 strain while in T73 it was removed rapidly. These results suggested differences in the regulation of nitrogen metabolism. Proline catabolism is capable of a high-energy output and may provide amino nitrogen and reducing power to cells. Each molecule of proline, when oxidized, can yield 30 ATP equivalents (Hare and Cress, 1997). Nevertheless, despite its interesting energetic properties, its catabolism by yeasts under oenological conditions is extremely limited because the presence of oxygen is a requirement for proline oxidases PUT1 catalytic activity which is responsible for the first step in proline ring catabolism into the mitochondria (Duteurtre et al., 1971). In addition, nitrogen sources are generally classified on the basis of the growth rate reached when present as the unique source, and on their impact on the transcriptional regulation of the pathways involved in the utilization of other nitrogen sources (Magasanik and Kaiser, 2002). According to both criteria, proline is widely considered a poor source of nitrogen among *Saccharomyces* and non-*Saccharomyces* yeasts while arginine as a good one. The difference observed in proline uptake may, therefore, account for a higher Crabtree effect repression on the respiration pathway or for stronger nitrogen-catabolite repression of proline permeases in the *S. cerevisiae* wine strain (T73). On the contrary, in BMV58, CECT12600 and CR85 strains, the early proline uptake was concordant with an hypothetic transient oxidative activity during phase I. Nevertheless, further studies are needed to deeply explore that difference in proline assimilation between cryophilic species and *S. cerevisiae* under aerobic conditions at the beginning of fermentation.

We also noticed important differences in the metabolism of acetate among the three species. The CR85 strain showed important shortcomings in growth and produced the highest level of acetate. In T73 fermentation, the extracellular acetate level remained almost constant after the entry into stationary phase while we observed a singular production-consumption profile in CR58, BMV58 and CECT12600 strains. This consumption was particularly pronounced in CECT12600 strain in which no acetate was detected when the fermentation concluded. During growth on glucose, the assimilation of ammonium and the excretion of oxidized low-molecular metabolites by yeast cells result in a net production of reduced equivalent of NADH (Bakker et al., 2001). Consequently, biomass formation has a determinant impact on redox metabolism. It is responsible for NADPH consumption and for the largest part of NADH produced during the growth phase. Regarding NADPH, the oxidative branch of the pentose phosphate pathway supplies most of the NADPH requested together with various precursors for the biosynthesis of nucleic acids and amino acids. But besides the pentose phosphate pathway, the conversion of acetaldehyde into acetate by acetaldehyde dehydrogenase (ACDH) can also generate reducing equivalents in the form of NADPH (Saint-Prix et al., 2004).

As an activated form of acetate, acetyl-CoA serves as a crucial intermediate metabolite in the metabolic network of yeasts and is involved in metabolism in multiple subcellular compartments like cytosol, mitochondrion, peroxisome and nucleus (Krivoruchko et al., 2015). Among others, acetyl-CoA is required for the synthesis of fatty acids, sterols, glutathione, amino acids (e.g. leucine, arginine, cysteine, and methionine) and it is the substrate for protein acetylation, which plays a role in the regulation of enzyme function and DNA transcription (Galdieri et al., 2014). Except in mitochondria where acetyl-CoA can be generated from pyruvate by the actions of the

pyruvate dehydrogenase complex (PDH), acetate is the precursor used to meet acetyl-CoA requirements via the action of acetyl-CoA synthetase (ACS) in the rest of subcellular compartments. The singular profile of extracellular acetate concentration observed in BMV58, CECT12600 and CR85 fermentations following nitrogen starvation suggested higher requirements in acetyl-CoA in cryophilic strains at this stage of the process that could be related with lipid synthesis or transcription control mechanisms. Tronchoni et al. (2012) showed in synthetic media and aerobic conditions that *S. kudriavzevii* strains, including the CR85 strain, had different lipid composition compared with the *S. cerevisiae* T73 strain, notably higher percentages of medium-chain fatty acids and squalene and shorter chain lengths regardless of the growth temperature. Moreover, we detected an earlier intracellular accumulation of citramalate by T73 and CR85 strains between the end of the growth phase (phase II) and the entry into stationary phase (phase III), in comparison with the *S. uvarum* CECT12600 strain. Yeasts condense acetic acid (in the form of acetyl-CoA) and pyruvate to produce citramalate (Ribéreau-Gayon et al., 2006). The entry into the stationary phase is an important step of alcoholic fermentation that is associated with major changes in the physiological state of yeast. Cell size and cell membrane composition are influenced by the stage of the cell cycle and the environmental conditions, like ethanol stress or anaerobic conditions (Turner et al., 2012). Here we notably showed that ACD could be used as an indicator of the stage of the fermentation process since the time trends of ACD coincided with major changes in the medium conditions. The management of cell size and the remodeling of the membrane between the beginning and the end of fermentation are key factors for the cell to balance metabolism expenditure or to acquire a higher ethanol tolerance. Thus, it is possible that the evolutionary adaptation of T73 cells to fermentation conditions enable their higher ethanol resistance and might not

require such important membrane remodeling. On the other hand, both strains of *S. uvarum* (BMV58 and CECT12600) and to a lesser extent, the *S. kudriavzevii* strain (CR85), might partially take advantage of the extracellular acetate they produced during the growth phase to fulfill their acetyl-CoA requirement for membrane remodeling. Interestingly, this acetate uptake seemed to be triggered by nitrogen depletion in BMV58, CECT12600 and CR85 strains. Also, the earlier citramalate production by T73 and CR85 strains might be aimed at balancing the intracellular levels of acetyl-CoA and pyruvate from the end of the exponential growth phase.

The dynamic approach used in our study also enables us to obtain the timing of esters and fusel alcohols production. In accordance with Mouret et al. (2014), we confirm that their synthesis mainly started with the beginning of the stationary phase. Notably, the higher production of 2-phenyl ethanol and 2-phenylethyl acetate produced by BMV58 and CECT12600 strains was accompanied by an erythritol overproduction concomitant with the entry into nitrogen-limited conditions. On the contrary, erythritol production was detected later and to a lesser extent in and CR85 strains. In accordance with the phenotypic traits – low acetate yield and high phenyl ethanol yield – described by Cadière et al. (2011) in the evolved strain ECA5, our results suggested a higher activity of the pentose phosphate pathway in BMV58 and CECT12600 strains when entering into stationary phase. Erythritol, phenylethanol and phenylethyl acetate share the same precursor, erythrose-4-phosphate, an intermediate of the non-oxidative branch of the pentose phosphate pathway. In yeasts, erythritol could serve as an osmolyte and can be synthesized from erythrose-4-phosphate after dephosphorylation and a reduction reaction catalyzed by an erythrose reductase, an NAD(P)H-dependant aldose reductase, resulting in the net regeneration of NAD(P)⁺ from NAD(P)H (Moon et al., 2010). Nevertheless, to the best of our knowledge, there is no bibliography that reported the

synthesis of such metabolite during alcoholic fermentation within the *Saccharomyces* genus. Thus, only high carbon fluxes through both the pentose phosphate pathway and glycolysis generate sufficient reduction capacity to lead to the overflow of erythritol we observed in BMV58 and CECT12600 strains. Finally, concordant with the production consumption profile of acetate in *S. uvarum* and *S. kudriavzevii* strains and with the hypothesis of membrane remodelling, flux analysis obtained from ^{13}C label experiments with the ECA5 strain by Cadière et al. (2011) suggested that the lower level of acetate excretion by the ECA5 strain might result from an increase in carbon flux from acetate towards acetyl-CoA for lipid synthesis.

The cellular machinery also involves the rapid synthesis of protective and storage carbohydrate molecules to cope with stress conditions. Intracellular data revealed that both natural isolate strains, CECT12600 and CR85, accumulated higher amount of trehalose among the four strains used, the timing of trehalose accumulation being concurrent with nitrogen depletion. Trehalose is present in yeast cells as a reserve carbohydrate and as a stress protectant but, according to recent studies, the functional role of trehalose seemed to be as a stress protectant rather than as an energy store (Wang et al., 2014). Its accumulation is highly correlated with ethanol production and could be responsible for sustaining cell viability in nitrogen-poor musts independent of the initial assimilable nitrogen content (Varela et al., 2004). Moreover, the trehalose pathway appears to be required to balance ATP consumption and production with phosphate homeostasis when the upper glycolytic flux suddenly increases (van Heerden et al., 2014). Thus, on the one hand the higher trehalose synthesis by CECT12600 and CR85 natural strains seemed to be an evidence of their lower adaptability to fermentative conditions, notably to face to the increasing ethanol content. On the other hand, it might

be a mechanism for regulating phosphate homeostasis when they entered into stationary phase

Finally, monitoring of fermentation also revealed that the four strains differed one from another in their redox balancing strategies. Following the onset of nitrogen starvation, we observed that strains of *S. uvarum* and *S. kudriavzevii* produced higher levels of a number of by-products responsible for the maintenance of redox homeostasis in comparison to the *S. cerevisiae* strain. We observed that the CR85 strain yielded the highest levels of glycerol and 2,3 butanediol. BMV58 and CECT12600 strains yielded highest amount of D-lactate among the four strains, and also more 2,3 butanediol and succinate than the *S. cerevisiae* T73 strain. In addition, within the set of *S. uvarum* strains, the non-wine isolate CECT1600 produced higher levels of 2,3 butanediol and less succinate than the BMV58 wine strain. In yeasts, metabolic networks have evolved to have tight regulation to maintain redox homeostasis which is principally manifested by the relative levels of cofactors pairs NADH/NAD^+ and NADPH/NADP^+ which are involved as electron carriers in about 200 reactions in *Saccharomyces cerevisiae* (Nielsen, 2017). In anaerobic conditions, NADH cannot be reoxidized by NADH dehydrogenases and none of the wine-related yeasts that were investigated contained transhydrogenase activity able to transfer excess reducing equivalents in the form of NADH to NADP^+ . Thus balancing the NADH/NAD^+ ratio can be achieved by the formation of organic molecules which serve as the final acceptors of reducing equivalents from NADH. After acetaldehyde, the glycolytic intermediate dihydroxyacetone phosphate is the second most important acceptor of reducing equivalents from NADH within fermenting yeasts. Nevertheless, besides ethanol and glycerol formation, the production of 2,3 butanediol, succinate or D-lactate, which are all originated from pyruvate, can act as useful redox valves during alcoholic

fermentation (Camarasa, 2007; Camarasa et al., 2003; de Klerk, 2010; Ribéreau-Gayon et al., 2005). Both succinate and 2,3 butanediol are derivatives from pyruvate. However, even under anaerobic conditions, most of the reactions leading to succinate formation via the reductive or oxidative branch of the truncated TCA occurred inside the mitochondrion, requiring then its incorporation by a mitochondrial pyruvate carrier. On the contrary, reactions related to 2,3 butanediol occurred in the cytosol. Prevalence in CECT12600 and CR85 fermentations (both non-wine strains), for 2,3 butanediol production might be linked to compartmentation reasons or might account for the different affinity of the enzymes acting around the pyruvate node.

5. CONCLUSION

This study advances our understanding of the metabolic response within strains of the species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* to conditions representative of the winemaking industry at the metabolic level. The differences observed in the management of the nitrogen substrates, in the production of polyols and organic molecules responsible for the maintenance redox balance including glycerol, erythritol, 2,3 butanediol and succinate, in the acetate metabolism and in the production of fusel alcohols and esters, indicate that it is possible to segregate the metabolism of the *S. cerevisiae* wine strain (T73) from cryophilic strains of *S. uvarum* (BMV58, CECT12600) and *S. kudriavzevii* (CR85). The *S. kudriavzevii* CR85 strain had important shortcomings in growth at 25°C but still produced higher glycerol level than T73 strain. On the other hand, the good fermentative ability of the *S. uvarum* strains and their interesting oenological skills at 25°C, notably to reduce extracellular acetate level or to produce more glycerol or succinate point out their biotechnological interest. The selection of strains of the *S. uvarum* species could help to reduce volatile acidity of

wines, as well as to enhance their acidity and their mouth-feel properties by the mean of higher succinate and glycerol content.

ACKNOWLEDGMENTS

RM was supported by a FPI grant from the Ministerio de Economía y Competitividad (ref. BES-2016-078202). This work was supported by the Spanish government projects AGL2015-67504-C3-1-R and RTI2018-093744-B-C31 awarded to AQ

REFERENCES

- Aguera, E., Bes, M., Roy, A., Camarasa, C., Sablayrolles, J.M., 2010. Partial removal of ethanol during fermentation to obtain reduced-alcohol wines. *Am. J. Enol. Vitic.* 61, 53–60.
- Ambroset, C., Petit, M., Brion, C., Sanchez, I., Delobel, P., Guérin, C., Chiapello, H., Nicolas, P., Bigey, F., Dequin, S., Blondin, B., 2011. Deciphering the Molecular Basis of Wine Yeast Fermentation Traits Using a Combined Genetic and Genomic Approach. *G3: Genes|Genomes|Genetics* 1, 263–281.
<https://doi.org/10.1534/g3.111.000422>
- Arroyo-López, F.N., Pérez-Torrado, R., Querol, A., Barrio, E., 2010. Modulation of the glycerol and ethanol syntheses in the yeast *Saccharomyces kudriavzevii* differs from that exhibited by *Saccharomyces cerevisiae* and their hybrid. *Food Microbiol.* 27, 628–637. <https://doi.org/10.1016/j.fm.2010.02.001>
- Bakker, B.M., Overkamp, K.M., Van Maris, A.J.A., Kötter, P., Luttik, M.A.H., Van Dijken, J.P., Pronk, J.T., 2001. Stoichiometry and compartmentation of NADH

739 metabolism in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 25, 15–37.
 740 [https://doi.org/10.1016/S0168-6445\(00\)00039-5](https://doi.org/10.1016/S0168-6445(00)00039-5)

741 Cadière, A., Ortiz-Julien, A., Camarasa, C., Dequin, S., 2011. Evolutionary engineered
 742 *Saccharomyces cerevisiae* wine yeast strains with increased in vivo flux through
 743 the pentose phosphate pathway. Metab. Eng. 13, 263–271.
 744 <https://doi.org/10.1016/J.YMBEN.2011.01.008>

745 Camarasa, C., 2007. Role in anaerobiosis of the isoenzymes for *Saccharomyces*
 746 *cerevisiae* fumarate reductase encoded by OSM1 and FRDS1. Yeast 26, 545–551.
 747 <https://doi.org/10.1002/yea>

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

752 Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P., Zambonelli, C.,
 753 1994. Unbalanced wine fermentation by cryotolerant vs. non-cryotolerant
 754 *Saccharomyces* strains. Vitis 33, 49–52.

755 Catarino, M., Mendes, A., 2011. Dealcoholizing wine by membrane separation
 756 processes. Innov. Food Sci. Emerg. Technol. 12, 330–337.
 757 <https://doi.org/10.1016/J.IFSET.2011.03.006>

758 Cohen, S.D., Tarara, J.M., Kennedy, J.A., 2008. Assessing the impact of temperature on
 759 grape phenolic metabolism. Anal. Chim. Acta 621, 57–67.
 760 <https://doi.org/10.1016/j.aca.2007.11.029>

761 Da Silva, T., Albertin, W., Dillmann, C., Bely, M., La Guerche, S., Giraud, C., Huet, S.,

762 Sicard, D., Masneuf-Pomarede, I., De Vienne, D., Marullo, P., 2015. Hybridization
 763 within *Saccharomyces* genus results in homeostasis and phenotypic novelty in
 764 winemaking conditions. *PLoS One* 10, e0123834.
 765 <https://doi.org/10.1371/journal.pone.0123834>

766 de Klerk, J.-L., 2010. Succinic acid production by wine yeasts. Notes. Stellenbosch :
 767 University of Stellenbosch.

768 Duteurtre, B., Bourgeois, C., Chollot, B., 1971. Study of the assimilation of proline by
 769 brewing yeasts. *J. Inst. Brew.* 77, 28–35. [https://doi.org/10.1002/j.2050-](https://doi.org/10.1002/j.2050-0416.1971.tb03350.x)
 770 [0416.1971.tb03350.x](https://doi.org/10.1002/j.2050-0416.1971.tb03350.x)

771 Galdieri, L., Zhang, T., Rogerson, D., Lleshi, R., Vancura, A., 2014. Protein
 772 Acetylation and Acetyl Coenzyme A Metabolism in Budding Yeast. *Eukaryot. Cell*
 773 13, 1472–1483. <https://doi.org/10.1128/ec.00189-14>

774 Gamero, A., Belloch, C., Ibáñez, C., Querol, A., 2014. Molecular analysis of the genes
 775 involved in aroma synthesis in the species *S. cerevisiae*, *S. kudriavzevii* and *S.*
 776 *bayanus* var. *uvarum* in winemaking conditions. *PLoS One* 9, 1–10.
 777 <https://doi.org/10.1371/journal.pone.0097626>

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

782 Goldner, M.C., Zamora, M.C., Lira, P.D.L., Gianninoto, H., Bandoni, A., 2009. Effect
 783 of ethanol level in the perception of aroma attributes and the detection of volatile
 784 compounds in red wine. *J. Sens. Stud.* 24, 243–257. [https://doi.org/10.1111/j.1745-](https://doi.org/10.1111/j.1745-459X.2009.00208.x)
 785 [459X.2009.00208.x](https://doi.org/10.1111/j.1745-459X.2009.00208.x)

786 Hare, P.D., Cress, W.A., 1997. Metabolic implications of stress-induced proline
787 accumulation in plants. *Plant Growth Regul.* 21, 79–102.
788 <https://doi.org/10.1023/A:1005703923347>

789 Kishimoto, M., 1994. Fermentation characteristics of hybrids between the cryophilic
790 wine yeast *Saccharomyces bayanus* and the mesophilic wine yeast *Saccharomyces*
791 *cerevisiae*. *J. Ferment. Bioeng.* 77, 432–435. [https://doi.org/10.1016/0922-](https://doi.org/10.1016/0922-338X(94)90019-1)
792 [338X\(94\)90019-1](https://doi.org/10.1016/0922-338X(94)90019-1)

793 Kontoudakis, N., Esteruelas, M., Fort, F., Canals, J.M., Zamora, F., 2011. Use of unripe
794 grapes harvested during cluster thinning as a method for reducing alcohol content
795 and pH of wine. *Aust. J. Grape Wine Res.* 17, 230–238.
796 <https://doi.org/10.1111/j.1755-0238.2011.00142.x>

797 Krivoruchko, A., Zhang, Y., Siewers, V., Chen, Y., Nielsen, J., 2015. Microbial acetyl-
798 CoA metabolism and metabolic engineering. *Metab. Eng.*
799 <https://doi.org/10.1016/j.ymben.2014.11.009>

800 Lambrechts, M.G., Pretorius, I.S., 2000. Yeast and its Importance to Wine Aroma - A
801 Review. *South African J. Enol. Vitic.* 21, 97–129.

802 Lopes, C.A., Barrio, E., Querol, A., 2010. Natural hybrids of *S. cerevisiae* × *S.*
803 *kudriavzevii* share alleles with European wild populations of *Saccharomyces*
804 *kudriavzevii*. *FEMS Yeast Res.* 10, 412–421. [https://doi.org/10.1111/j.1567-](https://doi.org/10.1111/j.1567-1364.2010.00614.x)
805 [1364.2010.00614.x](https://doi.org/10.1111/j.1567-1364.2010.00614.x)

806 López-Malo, M., Querol, A., Guillamon, J.M., 2013. Metabolomic Comparison of
807 *Saccharomyces cerevisiae* and the Cryotolerant Species *S. bayanus* var. *uvarum*
808 and *S. kudriavzevii* during Wine Fermentation at Low Temperature. *PLoS One* 8.
809 <https://doi.org/10.1371/journal.pone.0060135>

810 Magasanik, B., Kaiser, C.A., 2002. Nitrogen regulation in *Saccharomyces cerevisiae*.
811 Gene. [https://doi.org/10.1016/S0378-1119\(02\)00558-9](https://doi.org/10.1016/S0378-1119(02)00558-9)

812 Marullo, P., Aigle, M., Bely, M., Masneuf-Pomarède, I., Durrens, P., Dubourdieu, D.,
813 Yvert, G., 2007. Single QTL mapping and nucleotide-level resolution of a
814 physiologic trait in wine *Saccharomyces cerevisiae* strains. *FEMS Yeast Res.* 7,
815 941–952. <https://doi.org/10.1111/j.1567-1364.2007.00252.x>

816 Masneuf-Pomarède, I., Bely, M., Marullo, P., Lonvaud-Funel, A., Dubourdieu, D.,
817 2010. Reassessment of phenotypic traits for *Saccharomyces bayanus* var. *uvarum*
818 wine yeast strains. *Int. J. Food Microbiol.* 139, 79–86.
819 <https://doi.org/10.1016/j.ijfoodmicro.2010.01.038>

820 Mira de Orduña, R., 2010. Climate change associated effects on grape and wine quality
821 and production. *Food Res. Int.* 43, 1844–1855.
822 <https://doi.org/10.1016/j.foodres.2010.05.001>

823 Moon, H.-J., Jeya, M., Kim, I.-W., Lee, J.-K., 2010. Biotechnological production of
824 erythritol and its applications. *Appl. Microbiol. Biotechnol.* 86, 1017–1025.
825 <https://doi.org/10.1007/s00253-010-2496-4>

826 Motulsky, H., Christopoulos, A., 2004. Fitting Models to Biological Data Using Linear
827 and Nonlinear Regression. A practical guide to curve fitting, in: *Fitting Models to*
828 *Biological Data Using Linear and Nonlinear Regression*. pp. 160–165.
829 <https://doi.org/10.1002/pst.167>

830 Mouret, J.R., Cadiere, A., Aguera, E., Rollero, S., Ortiz-Julien, A., Sablayrolles, J.M.,
831 Dequin, S., 2014. Dynamics and quantitative analysis of the synthesis of
832 fermentative aromas by an evolved wine strain of *Saccharomyces cerevisiae*. *Yeast*
833 32, 257–269. <https://doi.org/10.1002/yea.3028>

834 Nielsen, J., 2017. Systems Biology of Metabolism: A Driver for Developing
835 Personalized and Precision Medicine. *Cell Metab.*
836 <https://doi.org/10.1016/j.cmet.2017.02.002>

837 Oliveira, B.M., Barrio, E., Querol, A., Pérez-Torrado, R., 2014. Enhanced enzymatic
838 activity of glycerol-3-phosphate dehydrogenase from the cryophilic
839 *Saccharomyces kudriavzevii*. *PLoS One* 9.
840 <https://doi.org/10.1371/journal.pone.0087290>

841 Peltier, E., Bernard, M., Trujillo, M., Prodhomme, D., Barbe, J.C., Gibon, Y., Marullo,
842 P., 2018. Wine yeast phenomics: A standardized fermentation method for assessing
843 quantitative traits of *Saccharomyces cerevisiae* strains in enological conditions.
844 *PLoS One* 13, e0190094. <https://doi.org/10.1371/journal.pone.0190094>

845 Pickering, G.J., 2000. Low- and Reduced-alcohol Wine: A Review. *J. Wine Res.* 11,
846 129–144. <https://doi.org/10.1080/09571260020001575>

847 Pickering, G.J., Vanhanen, L., 1998. The effect of ethanol concentration on the temporal
848 perception of viscosity and density in white wine. *Am. J. Enol. Vitic.* 49, 306–318.

849 Piškur, J., Rozpedowska, E., Polakova, S., Merico, A., Compagno, C., 2006. How did
850 *Saccharomyces* evolve to become a good brewer? *Trends Genet.* 22, 183–186.
851 <https://doi.org/10.1016/j.tig.2006.02.002>

852 Querol, A., Huerta, T., Barrio, E., Ramon, D., 1992. Dry Yeast Strain For Use in
853 Fermentation of Alicante Wines: Selection and DNA Patterns. *J. Food Sci.* 57,
854 183–185. <https://doi.org/10.1111/j.1365-2621.1992.tb05451.x>

855 Rainieri, S., Zambonelli, C., Hallsworth, J.E., Pulvirenti, A., Giudici, P., 1999.
856 *Saccharomyces uvarum* , a distinct group within *Saccharomyces sensu stricto*.

857 FEMS Microbiol. Lett. 177, 177–185. <https://doi.org/10.1111/j.1574->
858 6968.1999.tb13729.x

859 Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2006. Handbook of
860 Enology: Volume 1, The Microbiology of Wine and Vinifications. Handb. Enol. 1,
861 1–441. <https://doi.org/10.1002/0470010398>

862 Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., Lonvaud, A., 2005. Biochemistry of
863 Alcoholic Fermentation and Metabolic Pathways of Wine Yeasts, in: Handbook of
864 Enology. John Wiley & Sons, Ltd, Chichester, UK, pp. 53–77.
865 <https://doi.org/10.1002/0470010363.ch2>

866 Roessner, U., Wagner, C., Kopka, J., Trethewey, R.N., Willmitzer, L., 2000.
867 Simultaneous analysis of metabolites in potato tuber by gas chromatography-mass
868 spectrometry. Plant J. 23, 131–142. <https://doi.org/10.1046/j.1365->
869 313x.2000.00774.x

870 Saint-Prix, F., Bönquist, L., Dequin, S., 2004. Functional analysis of the ALD gene
871 family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the
872 NADP⁺-dependent Ald6p and Ald5p isoforms play a major role in acetate
873 formation. Microbiology 150, 2209–2220. <https://doi.org/10.1099/mic.0.26999-0>

874 Salmon, J.M., Fornairon, C., Barre, P., 1998. Determination of oxygen utilization
875 pathways in an industrial strain of *Saccharomyces cerevisiae* during enological
876 fermentation. J. Ferment. Bioeng. 86, 154–163. <https://doi.org/10.1016/S0922->
877 338X(98)80054-8

878 Schmidtke, L.M., Blackman, J.W., Agboola, S.O., 2012. Production technologies for
879 reduced alcoholic wines. J. Food Sci. <https://doi.org/10.1111/j.1750->
880 3841.2011.02448.x

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

886 Stribny, J., Romagnoli, G., Pérez-Torrado, R., Daran, J.M., Querol, A., 2016.
887 Characterisation of the broad substrate specificity 2-keto acid decarboxylase
888 Aro10p of *Saccharomyces kudriavzevii* and its implication in aroma development.
889 *Microb. Cell Fact.* 15, 1–12. <https://doi.org/10.1186/s12934-016-0449-z>

890 Su, Y., Seguinot, P., Sanchez, I., Ortiz-Julien, A., Heras, J.M., Querol, A., Camarasa,
891 C., Guillamón, J.M., 2020. Nitrogen sources preferences of non-*Saccharomyces*
892 yeasts to sustain growth and fermentation under winemaking conditions. *Food*
893 *Microbiol.* 85. <https://doi.org/10.1016/j.fm.2019.103287>

894 Tronchoni, J., Rozès, N., Querol, A., Guillamón, J.M., 2012. Lipid composition of wine
895 strains of *Saccharomyces kudriavzevii* and *Saccharomyces cerevisiae* grown at low
896 temperature. *Int. J. Food Microbiol.* 155, 191–198.
897 <https://doi.org/10.1016/j.ijfoodmicro.2012.02.004>

898 Turner, J.J., Ewald, J.C., Skotheim, J.M., 2012. Cell size control in yeast. *Curr. Biol.* 22,
899 R350-9. <https://doi.org/10.1016/j.cub.2012.02.041>

900 van Heerden, J.H., Wortel, M.T., Bruggeman, F.J., Heijnen, J.J., Bollen, Y.J.M.,
901 Planque, R., Hulshof, J., O’Toole, T.G., Wahl, S.A., Teusink, B., 2014. Lost in
902 Transition: Start-Up of Glycolysis Yields Subpopulations of Nongrowing Cells.
903 *Science* (80-.). 343, 1245114–1245114. <https://doi.org/10.1126/science.1245114>

904 Varela, C., Pizarro, F., Agosin, E., 2004. Biomass content governs fermentation rate in

905 nitrogen-deficient wine musts. *Appl. Environ. Microbiol.* 70, 3392–400.
906 <https://doi.org/10.1128/AEM.70.6.3392-3400.2004>

907 Villas-Bôas, S.G., Højer-Pedersen, J., Åkesson, M., Smedsgaard, J., Nielsen, J., 2005.
908 Global metabolite analysis of yeast: Evaluation of sample preparation methods.
909 *Yeast* 22, 1155–1169. <https://doi.org/10.1002/yea.1308>

910 Wang, P.-M., Zheng, D.-Q., Chi, X.-Q., Li, O., Qian, C.-D., Liu, T.-Z., Zhang, X.-Y.,
911 Du, F.-G., Sun, P.-Y., Qu, A.-M., Wu, X.-C., 2014. Relationship of trehalose
912 accumulation with ethanol fermentation in industrial *Saccharomyces cerevisiae*
913 yeast strains. *Bioresour. Technol.* 152, 371–376.
914 <https://doi.org/10.1016/J.BIORTECH.2013.11.033>

915

916

Figure legends.

Figure 1. Progress of total sugar consumption expressed as percentage (purple solid line), YAN content expressed as percentage (orange dashed line), dissolved oxygen expressed as percentage (solid blue line), CO₂ exhaust concentration as percentage (black dashed line) and cell population expressed as $\ln[\text{OD600 (t)} / \text{OD600 (t}_0)]$ (green solid line) with t₀ the time of inoculation, during natural grape must fermentation in bioreactors at 25 °C. Metabolic phases (I, II, III and IV) are indicated below the x-axis of time expressed in hours. The values are displayed as mean and standard deviations from triplicate experiments.

Figure 2. A) Dry weight amount (g/l) and B) average cell diameter (μm) along the fermentation process. For average cell diameter, the metabolic phases (I, II, III and IV) are indicated below the x-axis of time expressed in hours. The values are displayed as mean and standard deviations from triplicate experiments

Figure 3. Relative extracellular acetate (A) and proline (B) concentrations (%) during natural grape must fermentation in bioreactors at 25 °C. The relative concentrations were calculated as the difference between the extracellular concentration of the metabolite and its initial concentration in the must, and divided by the initial metabolite concentration. Metabolic phases (I, II, III and IV) are indicated below the x-axis of time expressed in hours. The values are displayed as mean and standard deviations from triplicate experiments.

Figure 4. Heat map of the extracellular concentration across time of amino acids, ammonia and yeast assimilable nitrogen (YAN). Metabolic phases (I, II, III and IV) are

indicated below the x-axis of time expressed in hours. Hierarchical clustering (average linkage with Euclidean distance measurement) was used to group the compounds with the online tool Heatmapper.

Figure 5. Heat maps of the relative intracellular content of amino acids (A), organic acids (B) and carbohydrates (C), determined in five sampling time points in our set of *Saccharomyces* strains. Metabolic phase (II, III and IV) are indicated below the horizontal axis of time expressed in hours. Hierarchical clustering (average linkage with Euclidean distance measurement) was used to group the compounds with the online tool Heatmapper. Missing values corresponding to compounds not detected by GC-MS are shown in light grey.

Figure 6. Fit curves of the main by-products responsible for NADH reoxidation during alcoholic fermentation according to Klerk (2010). Best-fit curves were determined as reported by Motulsky and Christopoulos (2004) and the first derivative of the fitted curves subsequently calculated to determine the correspondent specific production rates. Ethanol (black solid line), glycerol (grey solid line), 2,3 butanediol (orange solid line) and succinate (blue solid line) with their corresponding derivatives qEth (black dashed line), qGly (grey dashed line), qBut (red dashed line) and qSuc (blue dashed line). Metabolic phases (I, II, III and IV) are indicated below the x-axis of time expressed in hours.

Figure 7. Role of nicotinamide adenine dinucleotide and flavin adenine dinucleotide cofactors in central carbon metabolism under anaerobic conditions in *S. cerevisiae*.

Only the main representative redox balance reactions are represented. For each strain, the yields of the main fermentative by-products, computed as g of compound produced/g of sugar consumed, were represented. The values are displayed as mean and standard deviations from triplicate experiments. Superscript letters indicate the significant homogeneous groups obtained by one-way ANOVA analysis (Tukey test, $n = 3$, p -value < 0.05). **G-6-P**: glucose-6-phosphate; **F-6-P**: fructose-6-phosphate; **DHAP**: dihydroxyacetone phosphate; **GA-3-P**: glyceraldehyde-3-phosphate; **PEP**: phosphoenolpyruvate; **Pyr**: pyruvate; **AcCoA**: acetyl coenzyme A; **OxA**: oxaloacetate; **Ribul-5-P**: ribulose-5-phosphate; **DAHP**: 3-deoxy-D-arabino-heptulosonate-7-phosphate; **CHR**: chorismate.

Figure 8. Heat map of the complete aroma dataset obtained in this work for each strain (A) and PCA plots of the aroma compounds quantified at the end of the fermentations (B). We included five fusel alcohols, nine esters and their respective total content, namely: isobutanol, isoamyl alcohol, 1-hexanol, benzyl alcohol, 2-phenylethanol, ethyl acetate, isoamyl acetate, isobutyl acetate, ethyl caprylate, ethyl caproate, ethyl caprate, hexyl acetate, benzyl acetate, 2-phenylethyl acetate, total fusels and total esters levels. For heat map, hierarchical clustering (average linkage with Spearman's rank correlation for distance measurements) was used to group the compounds with the online tool Heatmapper.

989 **Table 1.** List of the *Saccharomyces* strains used in this study

Strain [†]	Species	Commercial name	Source	Isolation region
T73	<i>S. cerevisiae</i>	Lalvin T73 [‡]	Wine	Alicante, Spain
BMV58	<i>S. uvarum</i>	Velluto BMV58 YSEO (Lallemand) [‡]	Wine	Alicante, Spain
CECT12600	<i>S. uvarum</i>	---	Non fermented liquor (Mistela)	Alicante, Spain
CR85	<i>S. kudriavzevii</i>	---	<i>Quercus ilex</i> bark	Ciudad Real, Spain

990 [†] Reference yeast used in the present work.
991 [‡]Yeast strains currently commercialized as active dry yeasts.

992
993

994

995

996

997

998

999

1000

1001

1002

1003

1004

Table 2. Growth curve parameters and biomass levels during natural grape must fermentations in bioreactors at 25°C

Strain	Species	$\mu_{\max}$	λ	A	Max DW
T73	<i>S. cerevisiae</i>	$0,33 \pm 0,01^{b,c}$	$2,14 \pm 0,1^a$	$5,19 \pm 0,05^b$	$5,63 \pm 0,72^b$
BMV58	<i>S. uvarum</i>	$0,34 \pm 0,03^c$	$5,49 \pm 0,98^a$	$5,32 \pm 0,18^b$	$4,40 \pm 1,57_{a,b}$
CECT12600	<i>S. uvarum</i>	$0,28 \pm 0,01^b$	$4,22 \pm 0,75^a$	$5,08 \pm 0,05^b$	$4,70 \pm 0,30_{a,b}$
CR85	<i>S. kudriavzevii</i>	$0,23 \pm 0,01^a$	$60,2 \pm 2,99^b$	$4,8 \pm 0,03^a$	$3,30 \pm 0,20^a$

$\mu_{\max}$ is the maximum growth rate (h^{-1}), λ is the lag phase period (h), A is the maximum cell population value and Max DW is the maximum dry weight value. Superscript letters indicate the significant homogeneous groups obtained by one-way ANOVA analysis (Tukey test, $n = 3$, p -value < 0.05).

Figure 1

A

B

Figure 2

A

B

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8