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Molecular and enological characterization of a natural *Saccharomyces uvarum* and *Saccharomyces cerevisiae* hybrid

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ABSTRACT

Saccharomyces cerevisiae plays a main role in the winemaking process, although other species, like *Saccharomyces uvarum* or *Saccharomyces paradoxus*, have been associated with must fermentations. It has been reported in recent years, that yeast hybrids of different *Saccharomyces* species might be responsible for wine productions. Although *S. cerevisiae* × *Saccharomyces kudriavzevii* hybrids have been well studied, very little attention has been paid to *S. cerevisiae* × *S. uvarum* hybrids. In this work we characterized the genomic composition of S6U, a widely used commercial *S. cerevisiae* × *S. uvarum* yeast hybrid isolated in wine fermentations containing one copy of the genome of each parental species, which suggests a relatively recent hybridization event. We also studied its performance under diverse enological conditions. The results show enhanced performance under low temperature enological conditions, increased glycerol production, lower acetic acid production and increased production of interesting aroma compounds. We also examined the transcriptomic response of the S6U hybrid strain compared with the reference species under enological conditions. The results show that although the hybrid strain transcriptome is more similar to *S. uvarum* than to *S. cerevisiae*, it presents specifically regulated genes involved in stress response, lipids and amino acid metabolism. The enological performance and aroma profile of this *S. cerevisiae* × *S. uvarum* hybrid makes it a good candidate for participating in winemaking, especially at low temperatures.

1. Introduction

Yeasts contribute positively to wine flavor through alcoholic fermentation by several mechanisms: (i) utilization of grape juice constituents; (ii) production of ethanol and other solvents that help extract flavor components from grape solids; (iii) production of enzymes that transform neutral grape compounds into flavor active compounds; (iv) generation of many hundreds of flavor-active secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes, ketones, volatile sulfur compounds); and (v) autolytic degradation of dead yeast cells, although this process can also negatively contribute to wine quality (Cole and Noble, 1997; Fleet, 2003; Lambrechts and Pretorius, 2000). Thus the conversion of grape sugars into alcohol and other end-products by specific yeast populations may yield wines with different organoleptic qualities. In particular, the characterization of *Saccharomyces cerevisiae* has revealed that, as well as producing ethanol, this yeast generates many important secondary metabolites for determining wine quality (Fleet and Heard, 1993 and Lambrechts and Pretorius, 2000). Ethanol and carbon dioxide, the major volatile products of yeast metabolism, make a relatively small contribution to wine flavor respect to other volatile compounds. Conversely, the higher alcohols and esters formed during alcoholic fermentation strongly influence the sensory properties of the resulting wine (Nykänen, 1986; Romano et al., 2003).

During natural wine fermentations, *S. cerevisiae* is the predominant yeast (Pretorius, 2000), but two other species belonging to this genus (*Saccharomyces bayanus* and *Saccharomyces paradoxus*) have also been described as playing a main role during wine fermentation (González et al., 2006; Pretorius, 2000). The *S. bayanus* is a taxon that includes genetically diverse lineages of pure and hybrid strains. It has been subdivided by some authors into two well-differentiated groups of strains: the molecularly and physiologically heterogeneous group of strains belonging to *S. bayanus* var. *bayanus*; the homogenous group of strains pertaining to *S. bayanus* var. *uvarum* (Pérez-Través et al., 2014; Vaughan-Martini and Martini, 2011). Libkind et al. (2011) recently discovered the species *Saccharomyces eubayanus* and proposed the use of *S. eubayanus* and *Saccharomyces uvarum* as descriptors of the *S. bayanus* species. *S. uvarum* strains are typically found in wine environments. It has been described that the interspecific hybrid strains between *Saccharomyces* species are related to wine fermentations. The *S. cerevisiae* × *Saccharomyces kudriavzevii* natural (González et al.,

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2006 and Lopandic et al., 2007) and commercial (Bradbury et al., 2006 and González et al., 2006) yeast hybrids have been associated with wine fermentations. These hybrids have been characterized from a molecular and enological point of view. *S. cerevisiae* × *S. uvarum* hybrids have been described (Masneuf et al., 1998; Le Jeune et al., 2007; Albertin et al., 2013), however, they have not been well studied from a genomic and enological point of view.

The fermentative power of *S. cerevisiae* has been used for food and alcoholic beverage production throughout human history, and industrial strains of this species are well-adapted to stress conditions, such as temperature, osmotic pressure and ethanol toxicity, conditions that are present during different fermentative processes. The *S. uvarum* fermentation profile in grape must differs from that of *S. cerevisiae* since it produces lower levels of ethanol and acetic acid, but more glycerol and succinic acid (Bertolini et al., 1996; Giudici et al., 1995; Kishimoto et al., 1993). *S. uvarum* also generates high levels of volatile fermentative compounds, such as phenylethanol and phenylacetate (Masneuf-Pomarède et al., 2010). Commercial *S. uvarum* strains are used to produce several types of wines and cider, usually at low temperatures (Almeida et al., 2014). The *S. cerevisiae* × *S. uvarum* hybrid strains are well-adapted to the stress conditions (low pH, high sugar and ethanol content) that are common to wine fermentations (Belloch et al., 2008), and in accordance with new winemaking trends, their aroma production profile in synthetic media reveals interesting properties (Gamero et al., 2011).

Given the interesting properties of these *S. cerevisiae* × *S. uvarum* hybrids, in the present work, we set out to compare genomic composition and wine fermentation performance of one of the most used commercial strain (S6U) in four distinct natural grape musts at four different temperatures, as well as wine composition and aroma. We also analyzed the global gene expression of *S. cerevisiae* × *S. uvarum* hybrid strain S6U in comparison to a representative *S. cerevisiae* strain (commercial wine strain T73) and a strain of *S. uvarum* (CECT 12930) under enological conditions (Macabeo juice fermentation at 18 °C).

2. Materials and methods

2.1. Yeast strains and media

Hybrid strain S6U (*S. cerevisiae* × *S. uvarum*) is a commercial active dry yeast from Lallemand (Montreal, Canada). It was first classified as *S. uvarum* and selected for its ability to ferment at very low temperatures in musts (Ciolfi, 1994). Originally isolated as an allotetrapoid (Naumov et al., 2000), we used a diploid industrial strain isolated from an LSA supplied by Lallemand. The *S. cerevisiae* strain T73, widely used as a wine yeast model (Gómez-Pastor et al., 2012; Pérez-Torrado et al., 2009) and *S. uvarum* strain CECT 12930 (Pérez-Través et al., 2014) were used as reference species to represent parental strains. Yeast precultures were carried out in YPD (glucose 2%, yeast extract 1% and peptone 2%).

2.2. Fermentations, enological determinations and volatile compounds analysis

All the fermentations were carried out in triplicate by using 450 ml of must in sterile 500 ml vessels. The Tempranillo and Bobal varieties were employed for microvinifications as red grape varieties, as were Macabeo and Parellada as white grape varieties. Assimilable nitrogen was 310.8, 274.4, 292.6 and 182 mg/l for the Tempranillo, Macabeo, Parellada and Bobal respectively. The initial sugar concentration for Tempranillo and Macabeo was 280 g/l whereas in Bobal and Parellada was 230 g/l. The initial pH was 3.3 ± 0.05 in Bobal and Parellada and 3.9 ± 0.05 in Tempranillo and Macabeo. The employed musts were supplemented with 0.2 g/l of diammonium phosphate (Panreac, Barcelona, Spain) and 0.1 mg/l of thiamine (Sigma, Steinheim, Germany). They were treated by adding 1 mg/l of dimethyl di-carbonate (Fluka, Buchs, Switzerland) to be sterilized and SO_2-free up to 20 ppm, and were allowed to settle overnight. Musts were inoculated with a final concentration of 10^6 cell/ml of pure yeast culture. The vinification process was conducted at four different temperatures, 14, 18, 22 and 32 °C, until alcoholic fermentation was completed. Samples were collected daily to assess fermentation by measuring reducing sugars and to enumerate yeast populations. Prior to sampling, flasks were stirred for homogeneity.

Total yeast cells were determined by counting under a light microscope (phase-contrast) using a Thoma chamber. Throughout the fermentation process, reducing sugars and concentrations of glycerol, acetic acid (volatile acidity) and malic acid in musts and wines were measured enzymatically in an Echo-Enosys analyzer (Tecnova, San Sebastián de los Reyes, Spain) following the supplier's instructions. The ethanol concentration in wines was quantified in an Alliance Infrascan (Alliance Instruments, Eragny-Sur-Oise, France). All measurements were taken in duplicate.

Higher alcohols and esters were analyzed by headspace solid-phase-microextraction sampling (SPME) using poly(dimethylsiloxane) (PDMS) fibers (Supelco, Sigma-Aldrich, Barcelona, Spain) following a previously described protocol (Rojas et al., 2001) and by gas chromatography–mass spectrometry (GC–MS). Gas chromatography was carried out in a Trace GC (ThermoFinnigan, San Jose, CA) gas chromatograph coupled to a Trace DSQ (ThermoFinnigan, San Jose, CA) mass spectrometer. A SolGel-WAX 0.25 (SGE, Austin, TX) 30 m × 0.25 mm ID capillary column coated with a 0.25 μm layer of cross-linked polyethylene glycol was used. The carrier gas was helium (1 ml/min) and the oven temperature program was as follows: 10 min at 40 °C, 2.5 °C/min to 150 °C, 20 °C/min to 250 °C and 4 min at 250 °C. The detector temperature was 250 °C and the injector temperature was 220 °C, splitless. The ionization voltage applied was 70 eV and the mass spectra were obtained within the 30–200 m/z scan range. A 20-μl volume of internal standard (2-heptanona at 0.05%) was added to each sample. Volatile compound concentrations were quantified by using the calibration graphs of the corresponding standard volatile compound (Fluka, Buchs, Switzerland), and are given as the average of three independent fermentations. The standard solution consisted of: ethyl acetate, isobutyl acetate, isobutanol, isoamyl acetate, isoamyl alcohol, ethyl caproate, hexyl acetate, 1-hexanol, ethyl caprylate, diethyl succinate, benzyl acetate, phenylethyl acetate, benzyl alcohol and 2-phenylethanol. For more details on calibration, see González et al. (2007).

The Statgraphics Plus v.4.0 package (Manugistics, Rockville, MD) was used to perform multiple range tests to compare sample means. The analyzed compound data were first studied by Cochran's test and Bartlett's test to verify that there was no statistically significant difference among the standard deviations at the 95.0% confidence level. After this preliminary study, one-way ANOVAs under each particular temperature and must condition were carried out using Tukey's test to determine the difference between means (statistical level of significance was set at $p \leq 0.05$).

2.3. Nuclear gene region characterization of Saccharomyces interspecific hybrid S6U by PCR amplification and restriction analysis of nuclear gene regions

The characterization of *Saccharomyces* interspecific hybrid S6U was performed by PCR amplification and a subsequent RFLP analysis of 35 protein-encoding genes randomly selected in the center and ends of each chromosome. The oligonucleotide primers designed for the symmetrical amplification of the protein-coding gene regions are described in González et al. (2008). Yeast DNA was isolated and PCR was performed according to standard procedures (Garre et al., 2009). PCR amplifications were carried out in Techgene or Touchgene thermocyclers (Techne, Cambridge, UK) as follows: initial denaturing at 95 °C for 5 min and then 40 PCR cycles of the following program: denaturing at

95 °C for 1 min, annealing at 56 °C (for most genes), and extension at 72 °C for 2 min with a final extension at 72 °C for 10 min. With genes *ATF1*, *DAL1*, *DAL5*, *EGT2*, *KIN82*, *MNT2*, *MRC1*, *RRI2*, and *UBP7*, annealing was performed at 50 °C. PCR products were run on 1.4% agarose (Pronadisa, Madrid, Spain) gels in 0.5× TBE (Tris–borate–EDTA) buffer. After electrophoresis, gels were stained with a 0.5 μg/ml ethidium bromide dilution (AppliChem, Darmstadt, Germany) and visualized under UV light. A 100-bp DNA ladder marker (Roche Molecular Biochemicals, Mannheim, Germany) served as a size standard.

Simple digestions with endonucleases were performed with 15 μl of amplified DNA to a final volume of 20 μl. Restriction endonucleases *Acc*I, *Cfo*I, *Eco*RI, *Hae*III, *Hinf*I, *Msp*I, *Pst*I, *Rsa*I, and *Scr*FI (Roche Molecular Biochemicals) were used according to the supplier's instructions. Restriction fragments were separated on 3% agarose (Pronadisa) gel in 0.5× TBE buffer. A combination of 50-bp and 100-bp DNA ladder markers (Roche Molecular Biochemicals) served as size standards. Restriction endonucleases were selected to yield species-specific patterns to differentiate the gene copies in the hybrids from each parent species.

2.4. Cell extract preparation and RNA extraction

Ten milliliters of each must fermentation culture was taken when 50% of sugars was consumed. Cells were harvested by centrifugation and resuspended in 0.5 ml of LETS buffer (200 mM LiCl, 20 mM EDTA, 20 mM Tris–HCl pH 8.0, 0.4% sodium dodecyl sulfate) and transferred to a screw-cap microcentrifuge tube containing 0.5 ml of phenol and 0.5 ml of glass beads (acid-washed beads, 0.4 mm diameter). The suspension was mixed vigorously 3 times for 1 min each time in a Mini Bead-Beater homogenizer (BioSpec). After centrifugation at 17,900 ×*g* for 10 min (at 4 °C), the upper phase was extracted successively with phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). These steps were repeated until the interface between the aqueous and organic layers became clear after centrifugation. Total nucleic acids were precipitated with two volumes of ice-cold 100% ethanol and a 0.1 volume of 3.0 M potassium acetate, left at −20 °C for 3 h, and then centrifuged at 21,100 ×*g* for 15 min at 4 °C. The pellet was washed with 70% ethanol, dried and resuspended in 50 μl of sterile diethyl pyrocarbonate-treated water. Total RNA was purified with an RNeasy mini column (QIAGEN) according to the manufacturer's instructions. The total RNA concentration was quantified by A_{260}, and the A_{260} to A_{280} ratio was used to estimate RNA purity. Nucleic acid contamination was also checked on a 1% agarose gel.

2.5. Synthesis of [^{33}P] dCTP-labeled cDNA

Ten micrograms of purified RNA, 1 μl of oligo$(dT)_{15}$ (Roche Molecular Biochemical, Mannheim, Germany) and 1 μl of RNA inhibitor (RNA Guard, Amersham Biosciences, Roosendaal, The Netherlands) were mixed with water to obtain a final volume of 10 μl, which was heated for 10 min at 70 °C and then chilled on ice. The following components were added: first strand buffer (Invitrogen, Carlsbad, Canada), 0.1 M dithiothreitol (Invitrogen, Carlsbad, Canada), 0.8 mM dATP, dGTP and dTTP, 200 U of SuperScript II reverse transcriptase, RNase H (Invitrogen, Carlsbad, Canada), 50 μCi [^{33}P] dCTP (Hartmann Analytic, Braunchweig; Germany) and water to give a final volume of 30 μl. The mixture was incubated at 43 °C for 1 h. cDNA was then purified with MicroSpin S300 HR columns (Amersham Biosciences, Roosendaal, The Netherlands) according to the manufacturer's instructions, and was quantified by a liquid scintillation counter.

2.6. Macroarray hybridization

The *S. cerevisiae* macrochip membranes, made by the DNA chip service of the Universitat de Valencia (Spain) (http://scsie.uv.es/chipsdna; Alberola et al., 2004), were washed for 30 min in 0.5% SDS at 80 °C. Membranes were prehybridized for 3 h with 5 ml of saline sodium citrate (SSC)-based hybridization solution (5× SSC, 5× Denhart's, 0.5% SDS, 50% deionized formamide and 100 μg herring sperm DNA/mL) at 42 °C in a roller oven. This temperature permits the heterologous hybridization of closely related species or hybrids using *S. cerevisiae* chips, calculated by taking into account *S. kudriavzevii* homology (Belloch et al., 2009). The purified cDNA probe was denatured for 5 min at 100 °C, cooled on ice and 3×10^6 dpm/ml was added to the prehybridization mixture. After overnight hybridization at 35 °C, filters were rinsed with 2× SSC–0.1% SDS at 65 °C for 20 min. Filters were then transferred to a plastic container and washed with 0.2× SSC–0.1% SDS at room temperature for 15 min. Filters were exposed to a high-resolution BAS-MP 2040S imaging plate (Fuji, Kyoto, Japan) for 48 h and scanned in a phosphor-imager (FLA-3000; Fuji).

To reduce quantification and reproducibility problems, all the used filters were taken from the same batch. Filters were stripped by pouring boiling stripping buffer (5.0 mM sodium phosphate, pH 7.5, 0.1% SDS) 2 or 3 times over the membrane. The first time, the stripping buffer was left at 65 °C for 20 min, while filters were left at room temperature after the second and third washes. To ensure radioactivity had been eliminated, filters were checked with a Geiger counter. Membranes were not dried at any time to avoid permanent radioactivity fixation.

Hybridization experiments were performed in triplicate. Replicates were made with RNA samples from three different bottles of parallel experiments performed at the same time to avoid cell growth and handling differences. In particular, some authors have noticed that cell density, even at different densities in the mid-log phase, had a significant effect on the expression level of a small number of genes (Wodicka et al., 1997).

2.7. Data analysis and spot validation

Spot intensities were quantified as artifact-removed-density (ARM), background and background-corrected ARM density (sARM) with the Arrays Vision Software (Imaging Research, Canada). Triplicate macroarray data were downloaded to Microsoft Excel files. To normalize the signal intensity of each replicate hybridization set, spot intensities were normalized against total spot intensity. To determine fold changes between pairs of yeast strains under the same hybridization conditions, average spots were normalized against highly conserved genes in the genus *Saccharomyces* as histones H2A and H2B (*HTA1-2* and *HTB1-2*) and translation elongation factor EF-1α (*TEF1–2*) (Kurtzman and Robnett, 2003). These genes were chosen because previous studies carried out in our laboratory have shown that all the strains used in this study (*Saccharomyces* species strains and hybrids) have the same number of copies from these genes and more than 98% nucleotide homology. Raw data are presented in Supplementary Table S1. Log (base 2) average values were used to calculate fold change. The SAM (Significance Analysis for Microarrays) analysis, implemented in the MeV 4.8 software (Saeed et al., 2003), was used to select significant genes with a false discovery rate of 1% to select significant data with Bonferroni's correction for false-positives. GO (Gene Ontology) terms were investigated by using the FunSpec online software. The functional groups with Bonferroni-corrected *p*-values below 0.05 were considered significant.

3. Results

3.1. Chromosomal composition in wine *S. cerevisiae* × *S. uvarum* hybrid

To understand the chromosomal and genetic structure of the *S. cerevisiae* × *S. uvarum* hybrid, we used a method based on PCR amplification and a restriction analysis of 35 gene regions (González et al., 2008). As shown in Fig. 1, 32 protein-coding genes were located near the ends of the 16 *S. cerevisiae* chromosomes, and three were in the central positions of large chromosomes II, IV, and X. The genome of the *S. uvarum* type strain was syntenic with that of *S. cerevisiae*; therefore, these genes were expected to occupy similar positions in the hybrid

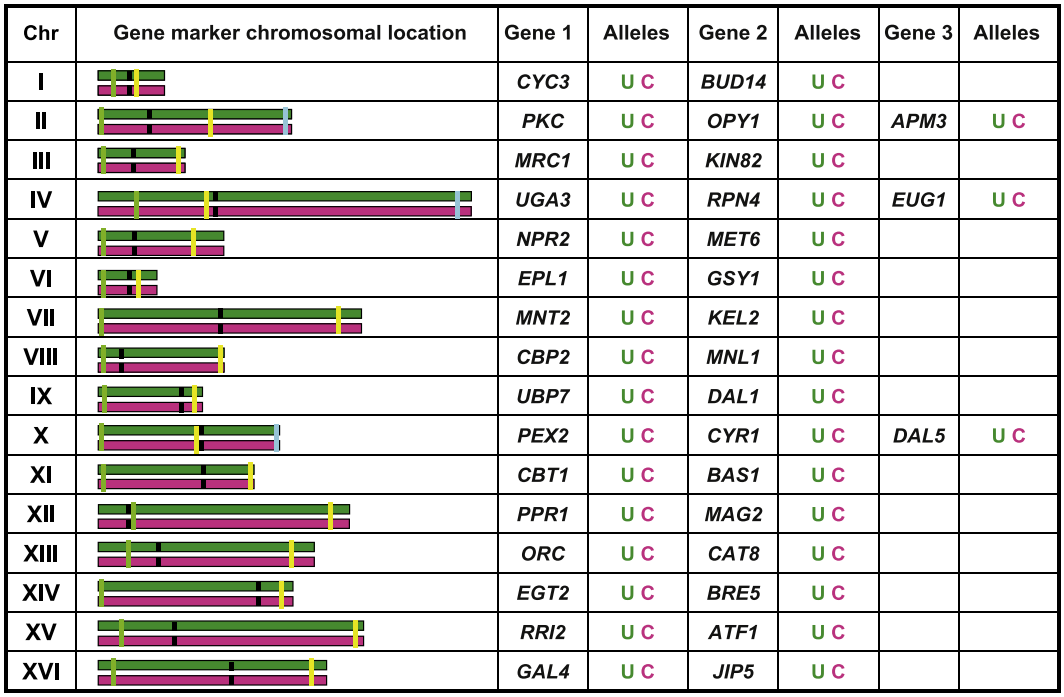

Fig. 1. Genotype of the *S. cerevisiae* × *S. uvarum* hybrid S6U. Green and purple bars represent the chromosomes of the *S. cerevisiae* and *S. uvarum* origin respectively. The presence or absence of *S. cerevisiae* (C) and *S. uvarum* (U) alleles from each parent species was determined by the restriction analysis done with the 35 gene regions amplified by PCR with general primers. Gene 1 chromosome location is represented with a light green vertical bar, gene 2 with a yellow bar and gene 3 with a cyan bar.

chromosomes from the *S. uvarum* parent. *Saccharomyces* general PCR primers were designed to amplify the genes of interest in the conserved nucleotide sequences that flanked variable regions, where the presence of variable restriction sites allowed species differentiation. The restriction endonucleases that yielded single or combined species-specific restriction patterns were selected for each gene region (González et al., 2008).

Fig. 1 summarizes the conformation of the S6U *S. cerevisiae* × *S. uvarum* hybrid genotype for each gene region according to the composite restriction patterns exhibited. The hybrid strain displayed a mixture of restriction patterns for all the gene regions due to the presence of two different alleles of each region, one exhibiting the typical restriction pattern of *S. cerevisiae* and the other displaying the same restriction pattern of *S. uvarum*, or a similar pattern. Thus we conclude that S6U is a 'perfect' hybrid that contains one copy of each species for all the genes.

3.2. Fermentation dynamics of the studied strains

Here we enologically characterized *S. cerevisiae* × *S. uvarum* hybrid strain S6U and two reference strains (*S. cerevisiae* T73 and *S. uvarum* CECT 12930) as being representative of parental species in four distinct natural musts, two red (Bobal and Tempranillo) and two white (Macabeo and Parellada), at four different temperatures (14, 18, 22 and 32 °C). Reducing sugars (glucose and fructose) were measured throughout fermentations to monitor the progress of the different studied strains (Fig. 2). As a general pattern, and as expected, we observed that the fermentation rate increased with temperature in all the grape varieties. However, the behavior of the strains varied from one natural must to another. In the fermentations performed with Tempranillo must, all the yeasts behaved similarly at all the temperatures assayed, except 32 °C where the *S. uvarum* strain was delayed by 1 day. A similar pattern was observed with Macabeo musts with the strains fermented at the same rate, except 32 °C where the S6U hybrid showed a 1-day delayed fermentation start. Greater variability was observed when the Bobal must was used, although the strains displayed similar fermentation performance at the lowest temperature. At 18 and 22 °C, the *S. uvarum* strain fermentation pattern was faster than the *S. cerevisiae* strain, and the hybrid strain was similar to *S. uvarum* at 22 °C and intermediate at 18 °C. At 32 °C, parental strains were faster than the hybrid, as observed for the Macabeo must. During the fermentations performed with Parellada, we observed the highest variability among strains, probably because this grape juice could have low levels of some components; e.g., amino acids or vitamins. At 14 °C, 22 °C, and especially at 18 °C, *S. uvarum* strain fermentation was faster than the *S. cerevisiae* strain. Once again, the hybrid strain showed variable behavior, which was slowest at 14 °C and similar to the *S. uvarum* strain at 18 and 22 °C. Unlike the other musts, all the strains completed fermentation with a similar pattern at 32 °C. In summary, *S. uvarum* was best suited to low and intermediate temperature conditions, like 14, 18 and 22 °C, if compared to the reference *S. cerevisiae* strain. Hybrid strain S6U showed variable fermentation performance and the best behavior at 18 °C.

3.3. Enological determinations

The main characteristics that influence enological properties of wine, such as alcohol production, sugar fermentation assimilation and the yield of some compounds, were studied at the end of all the fermentations. The main statistically significant differences were found in glycerol and acetic acid production (Table 1). At all the tested temperatures, reference strain *S. uvarum* (CECT 12930) produced more glycerol than commercial strain *S. cerevisiae* (T73), except at 32 °C where all the strains produced the same amount of glycerol. Commercial strain T73 usually produced the lowest amount of glycerol (with the exception mentioned at 32 °C) and the hybrid yeast generated an intermediate or similar quantity to the *S. uvarum* strain. Regarding acetic acid, it is interesting to note that the hybrid and the *S. uvarum* strain produced significantly less quantity than T73, except for the fermentation performed in Bobal must and the fermentations carried out at 32 °C, whose results

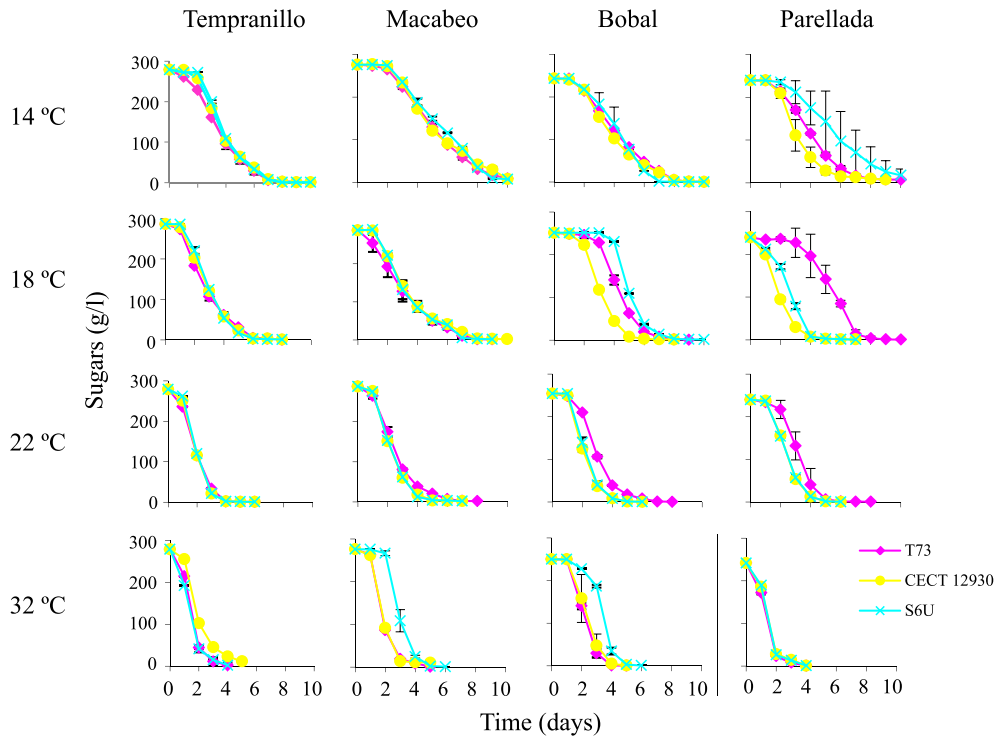

Fig. 2. Evolution of sugar content (glucose + fructose) during the fermentations of musts Tempranillo, Parellada, Bobal and Macabeo at 14, 18, 22 and 32 °C with yeasts *S. cerevisiae* T73 (◆, purple), *S. uvarum* CECT 12930 (●, yellow) and *S. cerevisiae* × *S. uvarum* hybrid S6U (x, light blue).

were reversed. This scenario indicates that the hybrid and *S. uvarum* strain are not well-adapted at higher temperatures as they behave worse than at lower and intermediate temperatures when they produce more glycerol than *S. cerevisiae*.

3.4. Production of volatile compounds after must microvinifications

The concentrations of the major volatile compounds produced during the fermentations described above are shown in Table 2. One interesting result was that the hybrid strain was generally the major or intermediate producer of isobutanol and isoamyl alcohol, except for the fermentations at 32 °C. The S6U hybrid produced more quantity of 1-Hexanal in Tempranillo must at 18, 22 and 32 °C, and in Macabeo at 14 and 32 °C. Likewise, this hybrid produced a larger quantity of ethyl caprylate during the Parellada and Macabeo fermentations performed at 14 °C and for Parellada at 18 and 22 °C. S6U was also the best producer of diethyl succinate at 18 °C with all the assayed musts, and at 18 °C with musts Tempranillo and Bobal. Finally, T73 was a good producer of ethyl caprylate, especially at 22 and 32 °C. It is also possible that evaporation could affect absolute values of some compounds at high temperatures respect to low temperatures.

To gain an overview of the aroma production ability of the different strains, we compared the sum of total aroma compounds or esters or higher alcohols produced during alcoholic fermentation by all the strains under each temperature and must (Table 3). A one-way ANOVA for each temperature condition and must was done with all

Table 1
Comparison of the mean glycerol and acetic acid production at the end of the fermentations by hybrid *S. cerevisiae* × *S. uvarum* (S6U) with the reference strains of *S. cerevisiae* (T73) and *S. uvarum* (CECT 12930) under each must and temperature condition assayed.

Temperature (°C)	Must	Glycerol (g/l)			Acetic acid (g/l)		
		T73	CECT 12930	S6U	T73	CECT 12930	S6U
14	Bobal	0.59 a	0.83 c	0.70 b	0.58 ab	0.45 a	0.47 a
	Tempranillo	0.63 a	1.12 e	0.81 c	0.59 b	0.45 a	0.43 a
	Parellada	0.54 a	0.70 bc	0.60 abc	0.52 b	0.39 a	0.42 ab
	Macabeo	0.63 a	1.00 d	0.86 c	0.67 c	0.43 a	0.44 a
18	Bobal	0.69 a	0.85 a	0.80 a	0.35 a	0.45 a	0.37 a
	Tempranillo	0.76 a	1.12 d	1.10 d	0.43 c	0.38 abc	0.35 ab
	Parellada	0.56 ab	0.73 c	0.61 b	0.63 d	0.44 ab	0.48 ab
	Macabeo	0.66 a	1.06 c	0.94 bc	0.52 a	0.40 a	0.41 a
22	Bobal	0.62 a	0.77 a	0.74 a	0.41 a	0.48 a	0.43 a
	Tempranillo	0.73 a	0.09 d	0.11 d	0.44 b	0.40 a	0.39 a
	Parellada	0.59 ab	0.72 c	0.64 bc	0.48 ab	0.52 b	0.45 a
	Macabeo	0.71 a	1.00 c	0.88 b	0.54 a	0.46 a	0.47 a
32	Bobal	0.68 a	0.77 a	0.71 a	0.55 a	1.04 a	0.66 a
	Tempranillo	0.90 abc	0.98 bc	1.02 c	0.62 a	0.83 a	0.91 a
	Parellada	0.61 d	0.62 d	0.62 d	0.63 ab	0.60 a	0.83 b
	Macabeo	0.83 b	0.81 b	0.75 b	0.63 a	0.84 c	0.76 bc

The results are the mean value of three replicates. Standard errors were always lower than 20% of the mean values. The means with the same letters do not differ significantly in Tukey's test ($p < 0.05$) within the same row of each compound.

Table 2
Mean major volatile compounds production in the musts fermented by hybrid *S. cerevisiae* × *S. uvarum* (S6U) with the reference strains of *S. cerevisiae* (T73) and *S. uvarum* (CECT 12930) at different temperatures.

Tmp (°C)	Must	Strain	Ethylacetate (mg/l)	Isobutylacetate (mg/l)	Isobutanol (mg/l)	Isoamylacetate (mg/l)	Isoamyl alcohol (mg/l)	Ethylcaproate (mg/l)	Hexylacetate (mg/l)	1-Hexanol (mg/l)	Ethylcaprylate (mg/l)	Di-ethyl succinate (mg/l)	Phenylethylacetate (mg/l)	2-Phenyl ethanol (mg/l)
14	B	T73	26.95	n.d.	9.01	1.62	94.76	0.43	n.d.	0.72	1.01	0.22	n.d.	13.63
		CECT 12930	26.11	n.d.	23.2	1.24	143.38	0.14	n.d.	0.8	0.86	0.42	1.4	100.67
		S6U	32.8	n.d.	10.2	3.38	144.8	0.51	0.03	0.7	1.3	0.31	0.75	38.73
	T	T73	36.73	n.d.	12.07	4.28	111.77	1.48	0.12	0.66	3.06	0.15	n.d.	8.98
		CECT 12930	56.23	0.16	40.48	5.75	220.23	0.79	0.07	0.8	1.41	0.82	4.57	128.35
		S6U	63.03	0.22	16.54	8.86	145.71	1.49	0.18	0.47	1.27	0.52	1.21	22.89
	P	T73	16.6	n.d.	9.64	2.41	80.06	0.8	0.18	1.78	1.3	0.21	0.29	15.64
		CECT 12930	14.91	n.d.	23	1.1	123.16	0.23	n.d.	1.64	0.84	0.41	0.98	51.72
		S6U	27	n.d.	12.65	3.91	106.69	1.1	0.58	1.64	1.73	0.3	0.96	23.43
	M	T73	43.53	n.d.	12.42	3.77	124.98	0.89	0.26	1.19	1.1	0.18	0.02$^{n.c.}$	13.27
		CECT 12930	39.53	n.d.	25.75	2.65	187.1	0.24	n.d.	1.09	0.02	0.62	2.52	134.05
		S6U	44.66	n.d.	21.69	5.58	208.06	1.35	0.18	1.33	2.07	0.62	1.27	63.69
18	B	T73	25.13	n.d.	12.5	2.96	151.1	0.36	0.01	0.65	1.2	0.38	0.15	27.64
		CECT 12930	30.02	n.d.	29.88	3.91	171.89	0.52	0.01	0.58	1.93	0.49	3.29	123.4
		S6U	39.56	n.d.	25.78	7.02	184.49	0.6	0.04	0.5	1.54	0.38	1.96	59.92
	T	T73	56.65	n.d.	17.4	11.17	161.57	1.34	0.2	0.59	3.01	0.34	0.32	12.32
		CECT 12930	56.54	n.d.	17	5.57	176.74	0.57	0.08	0.85	1.56	0.93	3.01	94.37
		S6U	63.45	0.14	39.22	8.43	255.36	1.18	0.06	1.13	1.87	1.04	0.89	44.99
	P	T73	26.55	n.d.	9.56	2.82	103.54	1.25	0.35	1.58	1.95	0.21	0.42	16.29
		CECT 12930	24.18	n.d.	21.87	1.63	117.87	0.48	0.12	1.92	1.44	0.34	0.84	51.9
		S6U	44.08	n.d.	8.36	6.87	135.4	1.76	0.76	1.63	3.04	0.51	1.3	37.04
	M	T73	59.89	n.d.	25.9	5.59	243.52	1.25	0.3	1.23	2.62	0.44	1.53	57.67
		CECT 12930	38.48	n.d.	29.16	4.03	178.38	0.49	0.03	0.77	1.39	0.48	3.41	121.5
		S6U	56.96	0.07$^{n.c.}$	36.14	7.02	274.02	1.03	0.31	1.16	2.33	0.5	2.03	83
22	B	T73	40.36	0.10$^{n.c.}$	16.49	5.01	166.28	0.66	0.06	0.66	2.56	0.37	0.34	20.01
		CECT 12930	30.97	0.13	43.18	2.4	105.2	0.13	n.d.	0.6	1.21	0.24	1.59	59.56
		S6U	39.44	0.24	46.61	5.41	159.41	0.65	0.06	0.62	2.11	0.38	1.17	43.88
	T	T73	36.15	n.d.	17.11	6.94	141.18	1.03	0.12	0.69	2.7	0.48	0.21	17.69
		CECT 12930	30.94	n.d.	46.51	1.9	180.71	n.d.	n.d.	1.38	1.24	0.61	0.42	44.54
		S6U	29.34	n.d.	46.61	1.2	212.05	0.17	n.d.	1.79	1.27	0.7	n.d.	36.41
	P	T73	34.53	n.d.	19.83	4.02	143.32	0.91	0.52	2.03	2.53	0.54	0.59	26.84
		CECT 12930	36.27	n.d.	24.34	4.36	122.91	0.35	0.52	1.81	1.08	0.38	2.75	68.35
		S6U	45.29	0.15	29.01	6.23	138.07	1.29	0.71	1.74	3.03	0.53	1.17	43.69
	M	T73	54.27	0.03$^{n.c.}$	25.35	9.19	218.32	1.13	0.32	0.96	2.98	0.34	0.59	34.57
		CECT 12930	53.66	n.d.	33.73	5.06	189.71	0.44	0.17	1.12	1.1	0.65	4.99	197.18
		S6U	52.75	0.03$^{n.c.}$	44.8	6.62	217.78	0.97	0.25	0.95	2.67	0.64	1.76	69.01
32	B	T73	39.22	0.07$^{n.c.}$	36.4	4.17	139.42	0.04	n.d.	0.48	1.37	0.41	0.28	16.79
		CECT 12930	20.25	n.d.	13.91	0.64	57.23	n.d.	n.d.	0.71	0.04	n.d.	0.02$^{n.c.}$	19.48
		S6U	32.66	n.d.	27.93	2.58	130.3	0.1	n.d.	0.67	1.14	0.31	0.22	18.31
	T	T73	64.61	n.d.	24.02	7.18	181.71	0.25	0.01	0.51	2.16	0.55	0.49	18.88
		CECT 12930	19.11	n.d.	15.46	0.46	57.05	n.d.	n.d.	0.66	0.07	n.d.	n.d.	12.13
		S6U	45.19	n.d.	22.25	0.99	91.43	n.d.	n.d.	0.89	0.93	0.22	n.d.	16.24
	P	T73	39.29	0.08$^{n.c.}$	20.07	3.62	129.88	0.21	0.32	1.81	1.37	0.34	0.79	22.73
		CECT 12930	17.02	n.d.	10.51	0.79	64.37	n.d.	0.08	1.71	n.d.	n.d.	0.07	13.5
		S6U	25.7	n.d.	10.6	1.17	62.42	n.d.	0.18	1.68	0.17	0.12$^{n.c.}$	0.11	12.32
	M	T73	56.6	0.09$^{n.c.}$	20.62	7.07	197.37	0.22	0.09	0.84	1.59	0.48	0.66	28.39
		CECT 12930	17.31	n.d.	7.22	0.39	43.92	n.d.	n.d.	0.87	n.d.	n.d.	n.d.	10.88
		S6U	33.52	n.d.	18.43	1.33	98.19	n.d.	n.d.	0.91	0.45	0.22	0.03$^{n.c.}$	14.01

The results are the mean value of three replicates. Standard errors were always lower than 20% of the mean values. n.d.: not detected; n.c.: under quantification limit. Tmp, temperature; T, Tempranillo grape variety; M, Macabeo grape variety; B, Bobal grape variety; P, Parellada grape variety.

Table 3
Comparison of the mean production of total esters and total higher alcohols by hybrid *S. cerevisiae* × *S. uvarum* (S6U) with the reference strains of *S. cerevisiae* (T73) and *S. uvarum* (CECT 12930) under each must and temperature condition assayed.

Tmp (°C)	Must	Total aroma (mg/l)			Esters (mg/l)			Higher alcohols (mg/l)		
		T73	CECT 12930	S6U	T73	CECT 12930	S6U	T73	CECT 12930	S6U
14	B	148.35 a	298.22 c	233.49 bc	3.28 a	4.06 a	6.28 a	118.12 a	268.04 c	194.42 b
	T	179.26 a	459.58 c	262.39 b	9.04 ab	13.49 c	13.76 c	133.49 a	389.86 c	185.60 ab
	P	128.92 a	217.82 a	179.99 a	5.19 a	3.56 a	8.58 a	107.12 a	199.52 a	144.41 a
	M	201.60 a	393.62 c	350.49 c	6.21 ab	6.10 ab	11.07 b	151.85 a	347.99 e	294.76 de
18	B	222.07 a	365.93 a	321.78 a	5.05 a	10.16 ab	11.53 b	191.89 a	325.75 a	270.68 a
	T	264.91 a	357.23 a	417.77 a	16.38 a	11.73 a	13.64 a	191.88 a	288.96 a	340.69 a
	P	164.53 a	222.59 ab	240.75 b	7.00 b	4.85 ab	14.14 d	130.97 a	193.56 b	182.42 ab
	M	399.94 bc	378.13 abc	464.50 c	11.73 a	9.83 a	13.22 a	328.32 cd	329.81 cd	394.32 d
22	B	252.86 a	245.21 a	299.98 a	9.07 a	5.70 a	10.02 a	203.43 a	208.54 a	250.52 a
	T	224.28 a	308.37 ab	329.54 ab	11.46 b	4.28 a	3.34 a	176.67 a	273.15 abc	296.86 abc
	P	235.67 a	263.12 a	216.33 a	9.12 a	9.44 a	13.12 a	192.02 a	217.40 a	170.72 a
	M	348.02 b	487.82 c	398.20 bc	14.55 a	12.41 a	12.91 a	279.21 b	421.74 c	332.54 bc
32	B	238.64 a	112.28 a	214.26 a	6.34 ab	0.70 a	4.38 ab	193.08 a	91.32 a	177.22 a
	T	300.36 b	104.94 a	178.13 ab	10.63 c	0.52 a	2.13 ab	225.11 b	85.30 a	130.81 ab
	P	220.43 b	108.06 a	114.34 a	6.64 d	0.95 a	1.62 ab	174.50 b	90.08 a	87.01 a
	M	313.99 d	80.59 a	167.06 b	10.17 d	0.39 a	2.00 ab	247.22 de	62.88 a	131.55 bc

The results are the mean value of three replicates. Standard errors were always lower than 20% of the mean values. The means with the same letters do not differ significantly in Tukey's test ($p < 0.05$) within the same row of each group of compounds. Tmp, temperature; T, Tempranillo grape variety; M, Macabeo grape variety; B, Bobal grape variety; P, Parellada grape variety.

the strains, and Tukey's test was used to determine the difference between means (statistical level of significance was set at $p \leq 0.05$). Ethyl acetate was excluded from the total sum of esters because of its distinctive contribution to wine aroma (Cabrera et al., 1998; Lema et al., 1996). As seen in Table 3, hybrid strain S6U produced the highest levels of the total aroma compounds in musts Tempranillo and Parellada at 18 °C. Interestingly, S6U generated the highest levels of esters in Tempranillo at 14 °C and in Tempranillo and Parellada at 18 °C. The reference strain of *S. cerevisiae* (T73) produced the lowest levels of aromatic compounds, mainly higher alcohols, at the low and intermediate temperatures, whereas it was the highest producer at 32 °C. The aroma analysis confirmed that the *S. cerevisiae* strain is better adapted to ferment at high temperatures as it produces more amounts of some compounds than *S. uvarum* and the hybrid strain.

3.5. Global gene expression analysis of hybrid S6U and the Saccharomyces reference species

We monitored the global gene expression in hybrid wine strain S6U compared with its reference species *S. cerevisiae* (T73) and *S. uvarum* (CECT 12930). For the transcriptome analysis, we selected Macabeo juice alcoholic fermentation at 18 °C since all the strains presented similar fermentation performances and biases as different physiological situations can be avoided. Cells were harvested at the end of the logarithmic phase, immediately before entry in the stationary phase, when 50% of sugar was consumed. Transcriptomic values were normalized and significant data were selected for further analyses. The expression comparison made between the cells of hybrid S6U and both reference species (T73 and CECT 12930) under oenological conditions identified a relatively small number of genes (Fig. 3). The comparison made between S6U and the reference strains revealed that the hybrid strain increased the expression of 196 genes if compared to *S. cerevisiae* (T73), and also the expression of 42 genes if compared to *S. uvarum* (CECT 12930), of which 22 were common in both reference strains. These 22 genes were similarly expressed between the reference strains. A smaller number of genes were down-regulated in hybrid strain S6U when compared with the reference species, 36 for *S. uvarum* (CECT 12930) and 46 for *S. cerevisiae* (T73), of which 26 were common. A few genes were up- (14) or down- (39) regulated between the parental *S. cerevisiae* (T73) and the *S. uvarum* (CECT 12930) strain. These changes were not common for the S6U differentially regulated genes, except for four up-regulated genes for the *S. uvarum* (CECT 12930) strain.

The GO term analysis is an interesting tool for finding significantly over-represented functional groups in a gene set. This functional analysis was done with the up- and down-regulated genes in S6U vs. each reference species (Table 4). The down-regulated genes in hybrid S6U, compared to the *S. uvarum* CECT 12930 strain, performed functions

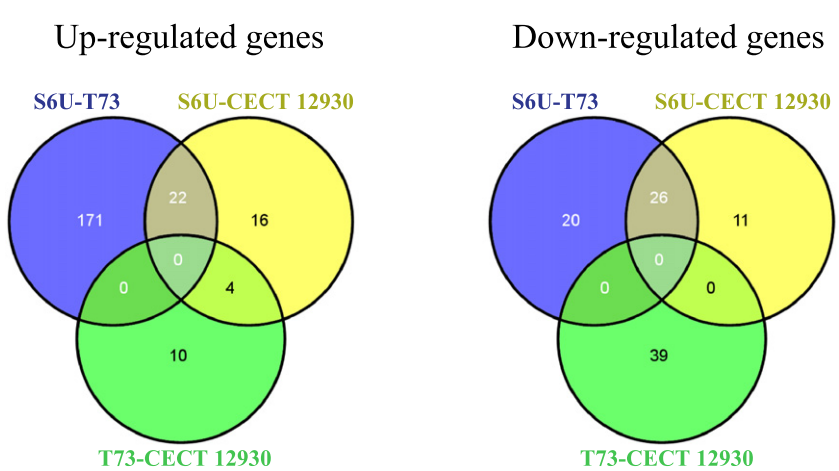

Fig. 3. Differentially expressed genes in *S. cerevisiae* T73, *S. uvarum* CECT 12930, and *S. cerevisiae* × *S. uvarum* hybrid S6U. Venn diagrams showing the number of up- or down-regulated genes after 50% sugar consumption at 18 °C Macabeo must fermentation.

Table 4
Functional analysis of the transcriptomic comparison between hybrid *S. cerevisiae* × *S. uvarum* (S6U) with the reference strains of *S. cerevisiae* (T73) and *S. uvarum* (CECT 12930) during the Macabeo must fermentation at 18 °C.

Comparison		Category	*p*-Value	Genes	No. of genes in the category	Total no. of genes in the category
S6U vs T73	Up-	–	–	–	–	–
	Down-	Response to stress [GO:0006950]	6.22E−05	PAU2 PAU5 PAU1 PAU17 PAU18 PAU23 PAU6 ZEO1 PAU20 PAU21	10	152
S6U vs CECT 12930	Up-	–	–	–	–	–
	Down-	Structural constituent of ribosome [GO:0003735]	2.10E−02	MRPL37 RPS18A RPL29 RPL34B RPL13B RPS7B RPS30B RPL20B	8	218
		Response to stress [GO:0006950]	6.35E−06	PAU2 PAU1 PAU17 PAU18 PAU23 PAU4 PAU6 ZEO1 PAU20 PAU21	10	152
T73 vs CECT 12930	Up-	Ion transport [20.01.01]	8.70E−05	POR1 FIT3	2	7
	Down-	Electron transport and membrane-associated energy conservation [02.11]	9.19E−04	ATP1 COX9 STF1 COX20 QCR7 QCR10	6	58
		Aerobic respiration [02.13.03]	7.99E−05	COX9 DLD1 COX20 QCR7 QCR10	5	77
		Mitochondrial inner membrane [755.05]	2.00E−03	ATP1 SHM1 COX9 STF1 DLD1 COX20 QCR7 QCR10	8	150

relating to response to stress and to the genes related to the structural ribosome constituent. Interestingly, the comparison of S6U with the *S. cerevisiae* T73 strain also revealed the down-regulation of the functional groups related to response to stress. When we analyzed the 26 commonly regulated genes in the S6U hybrid, compared to both parents, the GO response to stress was also significant (3.21E−05). The genes up-regulated in the S6U hybrid, compared to both reference species, did not show any significantly overrepresented functional group. When we compared *S. cerevisiae* T73 with the *S. uvarum* CECT 12930 strain, we observed that T73 had up-regulated ion transport genes, whereas *S. uvarum* had overexpressed the genes related to electron transport and membrane-associated energy conservation, aerobic respiration and the mitochondrial inner membrane.

The observation made of the regulation of several specific genes can help us to understand the phenotypic differences observed between hybrid and reference species when focusing on groups of genes. Among the S6U hybrid up-regulated genes, if compared to both parents, some genes were related to signal transduction (*STE18*, *CLA4*, *MSG5*, *RLM1*, *CRZ1*), amino acid metabolism (*ARG4*, *SHM1*) and glycolysis (*ERR1*, *ERR2*). Of the 26 genes commonly down-regulated in the S6U hybrid, if compared to both parents, it is worth highlighting the presence of several genes related to vitamins (*THI74*, *SPE2*), inositol (*OPI10*) and sterol metabolism (*ARE2*, *ERG25*, *ERG28*), the last two groups related to cold adaptation. Some other genes (*DAN2*, *PAU1*, *PAU2*, *PAU4*, *PAU6*, *PAU17*, *PAU18*, *PAU23*, *PAU20*, *PAU21*) belonged to the *PAU*, *DAN*/*TIR* families, which are also linked to cold shock adaptation (Table 4).

4. Discussion

Since the discovery of the participation of *Saccharomyces* hybrids in enological fermentations, several studies have focused on their characterization, mainly for hybrid strains *S. cerevisiae* × *S. kudriavzevii* (Combina et al. 2012; González et al., 2006, 2007, 2008). In this work, we describe the genomic composition of a widely used commercial *S. cerevisiae* × *S. uvarum* hybrid, its performance under enological conditions, the composition of the produced wine and its transcriptional regulation.

Regarding hybrids *S. cerevisiae* × *S. kudriavzevii*, it has been shown that certain chromosomes from the *S. kudriavzevii* parent are also completely absent in hybrids *S. cerevisiae* × *S. kudriavzevii* (González et al., 2008; Peris et al., 2012). In these yeast hybrids, a trend of maintaining the *S. cerevisiae* genome and of reducing the non *S. cerevisiae* (*S. kudriavzevii*-like) fraction was maintained. However, lager *Saccharomyces pastorianus* strains exhibited the opposite trend, that of preserving the non-*S. cerevisiae* (*S. eubayanus*-like) genome and reducing the *S. cerevisiae* fraction. In contrast, both types of natural hybrids contain the non *S. cerevisiae* mitochondrial genomes (de Barros Lopes et al., 2002; Petersen et al., 2002). In the *S. cerevisiae* × *S. uvarum* S6U hybrid, we observed that it equally maintained the genomes from both parents in accordance with a similar ploidy to a diploid, as previously observed (González et al., 2006). These data suggest that this commercial hybrid strain may be of relatively recent generation and it could present similar characteristics of hybrids newly formed by artificial methods. In fact it has been shown that new artificially generated *Saccharomyces* hybrids tend to maintain one copy of the genome of each parental (Pérez-Través et al., 2012; Solieri et al., 2008).

In the present study we have seen that the *S. cerevisiae* × *S. uvarum* S6U hybrid display in glycerol produced an intermediate behavior if compared to their parental reference stains (*S. cerevisiae*, strain T73 and *S. uvarum*, strain CECT 12930), as well as a smaller quantity of acetic acid. In aromatic compound production, they are better producers of higher alcohols and esters. From both assays, we conclude that the hybrid strains between *S. cerevisiae* and *S. uvarum* are better-adapted to lower and intermediate temperatures and they produce larger amounts of aromatic compounds than their reference strains. As other authors have pointed out (Gangl et al., 2009), these data suggest that hybrid phenotypes are not just an intermediate or average from parents, but new specific abilities can arise after the hybridization event as can been seen also in artificial hybrids between strains of *S. cerevisiae* (Pérez-Través, personal communication). This fact is even more intriguing when it happens in a hybrid with an equal genomic contribution of parental species, such as the S6U hybrid.

The global gene expression analysis indicates that, under oenological conditions, the S6U hybrid has a new transcriptional profile, which differs significantly from the expression patterns of the reference species. We also observed a down-regulation of the genes involved in cold adaptation and stress response. We propose that the interaction between both parental genomes which occurred after hybrid formation leads to the appearance of this new transcriptional pattern to adapt to fermentative conditions. In fact several genes related to signal transduction and regulation showed a differential regulation compared to both parents. It is interesting to observe that during natural Macabeo must fermentations, the S6U hybrid was the best producer of isobutanol, isoamyl alcohol, isoamyl acetate and phenylethyl acetate. This can correlate with the higher expression in the S6U hybrid compared to both reference parental strains of the genes related to amino acid biosynthesis, which are the precursors of aroma compounds via the Ehrlich pathway (Hazelwood et al., 2008). On the contrary, when we observed the total aroma profile variation and the fermentation kinetics in the S6U hybrid under all the conditions, we concluded that it was similar to the *S. uvarum* parental strain than to the *S. cerevisiae* strain. This situation was also reflected in the transcriptomic profile since the S6U hybrid strain showed 3-fold more differentially regulated genes compared to *S. cerevisiae* than

S. uvarum. After taking into account the equal genomic composition, an open question arises to explain which molecular mechanism influenced strain adaptation to the enological conditions to favor *S. uvarum*'s influence on strain physiology if compared with *S. cerevisiae*. Possibly, the environmental conditions where the S6U hybrid strain had adapted, e.g., low temperatures, benefitted the imposition of some *S. uvarum* alleles. Thus the molecular mechanism involved in the adaptative equilibrium among the parental genomes in the S6U hybrid merits further research.

In summary, *S. cerevisiae* × *S. uvarum* hybrid strain S6U seems better-adapted to low and intermediate temperature fermentative conditions, and has an aromatic compound profile that differs from its reference strains. Both genome composition and S6U structure seem to be constituted by one genomic copy of each parental species, which suggests that *S. cerevisiae* × *S. uvarum* hybrid strains tend to be a genetic mixture of *S. cerevisiae* and *S. uvarum*. However, S6U hybrid behavior under enological conditions is not seen as being intermediate between both parentals and tends to come closer to *S. uvarum* in fermentation kinetics and wine composition. Our results support the idea that the construction of laboratory hybrids using selected reference *Saccharomyces* strains of interesting species is a promising method to genetically improve wine yeasts.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2015.03.012.

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