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Physiological and genomic characterisation of *Saccharomyces cerevisiae* hybrids with improved fermentation performance and mannoprotein release capacity

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A B S T R A C T

Yeast mannoproteins contribute to several aspects of wine quality by protecting wine against protein haze, reducing astringency, retaining aroma compounds and stimulating lactic-acid bacteria growth. The selection of a yeast strain that simultaneously overproduces mannoproteins and presents good fermentative characteristics is a difficult task. In this work, a *Saccharomyces cerevisiae* × *S. cerevisiae* hybrid bearing the two oenologically relevant features was constructed. According to the genomic characterisation of the hybrids, different copy numbers of some genes probably related with these physiological features were detected. The hybrid shared not only a similar copy number of genes *SPR1*, *SWP1*, *MNN10* and *YPS7* related to cell wall integrity with parental Sc1, but also a similar copy number of some glycolytic genes with parental Sc2, such as *GPM1* and *HXK1*, as well as the genes involved in hexose transport, such as *HXT9*, *HXT11* and *HXT12*. This work demonstrates that hybridisation and stabilisation under winemaking conditions constitute an effective approach to obtain yeast strains with desirable physiological features, like mannoprotein overproducing capacity and improved fermentation performance, which genetically depend of the expression of numerous genes (multigenic characters).
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1. Introduction

Since the inoculation concept of wine fermentations with pure yeast starter cultures by Mueller–Thurgau from Geisenheim was introduced in 1890 and the subsequent development of active dry yeasts in winemaking, several *Saccharomyces cerevisiae* starter cultures with particular features of enological interest have been developed (Pretorius, 2000). The use of these starters ensures the production of consistent wines that have particular desirable organoleptic characteristics in successive vintages.

The selection of *S. cerevisiae* strains as starter cultures for wine fermentation has been based on different physiological features. These features include good fermentative vigour and fermentation rate, low production of SH_2 and acetic acid, low foam production, resistance to SO_2, and the production of balanced levels of volatile aromatic compounds such as higher alcohols and esters, among others (Schuller and Casal, 2005).

In the last 15 years, the capacity of yeast strains to release mannoproteins has also been included among the selection criteria applied for wine yeast selection. These highly glycosylated proteins, which are mostly present in the yeast cell wall, have been associated with positive quality and technological traits of wines, including protection against protein and tartaric instability, retention of aroma compounds, reduced astringency, increased body and mouthfeel, stimulation of lactic acid bacteria growth and foam quality improvement (Caridi, 2006).

Wine ageing with yeast lees and addition of enzymatic preparations that enhance the mannoproteins released to wine are two possible ways to increase the mannoprotein content of wines. However, these practices are subjected to normative limitations and require careful management to avoid off-flavours and wine spoilage. In this context, the use of selected yeasts that overproduce mannoproteins and show good fermentative features seems an interesting alternative.

Despite the selection pressure exerted by the millennia of winemaking on wine yeasts, the combination of desired interesting oenological traits that matches the actual requirements of starter cultures is not easy to find in a single strain. In particular, mannoprotein release is a difficult complex character to be used as a selection criterion, especially for screening large numbers of strains. For this reason, different strategies based on mutations of some specific genes or recombinant strains that have been improved for mannoprotein release have been developed (González-Ramos et al., 2008, 2009; González-Ramos and González, 2006; Quirós et al., 2010). However, the practical usefulness of some of these approaches is limited since the use of GMOs (Genetic Modified

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Organisms) in food applications – particularly in wine – is strictly regulated in most countries and often faces consumer rejection. In order to solve this limitation, other non-GMO-producing methodologies must be used to generate wine strains that offer good fermentative features and high production and release of mannoproteins.

Additionally, given the multigenic character of mannoprotein production and release by yeast cells – just the synthesis and organisation of the cell wall directly or indirectly involve about 1200 genes (Klis, 1994; Lesage et al., 2004) – and other oenologically relevant features like fermentative behaviour (Giudici et al., 2005; Marullo et al., 2004), wine strain improvement based on strategies such as the hybridisation of two genomes is one of the best methods to consider (Pérez-Través et al., 2012). Mating spores and rare-mating – based on the rare event of mating type switching in industrial yeasts – can be considered natural processes that can happen in nature without human intervention. Therefore, the obtained hybrid cells that make full use of these natural phenomena do not fall under GMO rules.

The objective of the present work is to improve the fermentation capability of a commercial strain of *S. cerevisiae* (Sc1) that has been selected as a good mannoprotein producer. We develop an intraspecific hybrid between the two commercial strains Sc1 and Sc2 by rare mating that gives rise to non-GMO strains. After the genomic stabilisation we obtain a strain that overproduces mannoprotein and shows excellent fermentation capacities. The potential relationship between the copy number of specific genes and the improved features was also evaluated by a CGH analysis of the parental and hybrid strains.

2. Materials and methods

2.1. Yeast strains and general culture conditions

Nineteen stable intraspecific hybrids, obtained in a previous work (Perez-Través et al., 2015), were used. 15 from the R (rare-mating) hybrids and 3 from the S (spore-to-spore) hybrids (Table 1).

The two parental strains, two *S. cerevisiae* industrial strains from Lallemand S.A.S., were used as a reference strains. According to producers Sc1 was selected for its capacity to release large amounts of mannoproteins during industrial winemaking (Sc1 improves mid-palate mouthfeel, softens tannins, and enhances the varietal characteristics of the fruit; shows a good compatibility with malolactic fermentation and is a moderate rate fermenter, and for not to be an excellent fermenting yeast; Lallemand personal communication). Sc2 was chosen for its excellent fermentative behaviour (Sc2 is resistant to difficult fermentation conditions, such as low turbidity, low temperature and low fatty acid content, presents a fast fermentation speed and low relative nitrogen needs; Lallemand personal communication).

Strains were maintained in GPY-agar medium (% w/v: yeast extract 0.5, peptone 0.5, glucose 2, agar-agar 2).

2.2. Fermentation experiments

2.2.1. Synthetic must fermentation

All the strains were used in synthetic must fermentations. Fermentations were carried out in 100-mL bottles containing 80 mL of synthetic must (Rossignol et al., 2003). The sugar concentration in the must (50% glucose + 50% fructose) was adjusted to 200 g/l. Must was inoculated independently with the different yeast strains to reach an initial population of $2 * 10^6$ CFU/mL and was maintained without aeration at 20 °C. The fermentation process was monitored by the quantification of the total sugar concentration. For this purpose, 1-mL aliquots of must were taken every 2 days and the sugar concentration was determined enzymatically (the glucose–fructose determination kit, Symta, Madrid, Spain). Fermentations were considered as stopped when the sugar amount was the same during 3 measures. Each fermentation experiment was done twice. The sugar consumption data obtained from each fermentation were fitted by the following exponential decay function: $Y = D + S * e^{(-K * t)}$ as previously used by Arroyo-López et al. (2009). In this function, "Y" is the total amount of sugar present in must, "t" is the time in days, "D" is the asymptotic value when $t \rightarrow \infty$, "S" is the estimated value of change, and "K" is the kinetic constant ($days^{-1}$) which defines the maximum fermentation rate. Equations were fitted by the linear and non-linear regression

Table 1
Main kinetic parameters of the fermentations carried out with both parental and hybrid strains on synthetic must at 20 °C and chemical analysis of the final fermented products.

Strain[Ω]	Hybridization method[Ω]	Kinetic parameters[$]			Chemical parameters[$]			
		K ($days^{-1}$)[&]	t_{50} (days)[*]	t_2 (days)[#]	Glucose (g/L)[¥]	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
Sc1	**Parental**	**$0.105 \pm 0.004^{d-f}$**	**$6.76 \pm 0.05^{b-f}$**	**Stuck**	**0.38 ± 0.11^{a}**	**9.2 ± 0.78^{f}**	**$5.18 \pm 0.11^{a-d}$**	**$12.13 \pm 0.05^{d-f}$**
Sc2	**Parental**	**$0.082 \pm 0.004^{a-d}$**	**$7.15 \pm 0.08^{b-g}$**	**$22.58 \pm 0.69^{a,b}$**	**bdl**	**2.18 ± 0.25^{a}**	**$5.83 \pm 0.11^{d,e}$**	**12.48 ± 0.05^{f}**
R2 Io	Rare-mating	0.144 ± 0.003^{g}	4.93 ± 0.11^{a}	$23.48 \pm 0.15^{a-c}$	bdl	2.03 ± 0.32^{a}	$5.55 \pm 0.07^{c-e}$	$11.78 \pm 0.11^{b-f}$
R2 IIo	Rare-mating	$0.100 \pm 0.005^{b-f}$	$6.5 \pm 0.38^{a-e}$	$27.11 \pm 1.03^{b-e}$	bdl	2.40 ± 0.50^{a}	$5.45 \pm 0.07^{c-e}$	$12.29 \pm 0.08^{e,f}$
R2 IIIa	Rare-mating	$0.117 \pm 0.005^{e-g}$	$5.89 \pm 0.29^{a-c}$	Stuck	bdl	$5.18 \pm 0.39^{c,d}$	$5.55 \pm 0.07^{c-e}$	12.69 ± 0.02^{f}
R2 IIIo	Rare-mating	$0.102 \pm 0.020^{b-f}$	$6.25 \pm 0.51^{a-d}$	Stuck	bdl	7.00 ± 0.42^{e}	$5.20 \pm 0.00^{a-d}$	$12.03 \pm 0.06^{c-f}$
R2 IVo	**Rare-mating**	**$0.120 \pm 0.013^{f,g}$**	**$5.40 \pm 0.56^{a,b}$**	**16.85 ± 2.42^{a}**	**bdl**	**1.75 ± 0.21^{a}**	**$5.60 \pm 0.00^{c-e}$**	**$12.13 \pm 0.10^{d-f}$**
R2 VIo	Rare-mating	$0.104 \pm 0.006^{c-f}$	$6.81 \pm 0.19^{b-f}$	Stuck	0.3 ± 0.42^{a}	3.18 ± 0.47^{b}	4.65 ± 0.14^{a}	10.56 ± 0.24^{a}
R8 IIa	**Rare-mating**	**0.066 ± 0.009^{a}**	**$8.44 \pm 0.05^{f-h}$**	**$27.21 \pm 3.49^{b-e}$**	**0.31 ± 0.44^{a}**	**2.38 ± 0.30^{a}**	**$5.63 \pm 0.13^{c-e}$**	**$11.77 \pm 0.41^{b-f}$**
R8 IIo	Rare-mating	$0.080 \pm 0.008^{a-d}$	$8.06 \pm 0.59^{e-h}$	$32.89 \pm 2.42^{d,e}$	bdl	2.37 ± 0.68^{a}	$5.67 \pm 0.07^{c-e}$	12.36 ± 0.18^{f}
R8 IIIo	Rare-mating	$0.095 \pm 0.003^{a-f}$	$7.50 \pm 0.46^{c-h}$	Stuck	bdl	$4.58 \pm 0.46^{b,c}$	$5.76 \pm 0.14^{c-e}$	$12.23 \pm 0.20^{d-f}$
R8 IVo	Rare-mating	$0.082 \pm 0.016^{a-d}$	$7.84 \pm 0.99^{d-h}$	$30.23 \pm 2.05^{c-e}$	bdl	2.65 ± 0.48^{a}	$5.68 \pm 0.07^{c-e}$	12.33 ± 0.24^{f}
R8 Vo	Rare-mating	$0.072 \pm 0.003^{a-c}$	8.93 ± 0.53^{h}	$31.55 \pm 1.83^{d,e}$	bdl	2.48 ± 0.11^{a}	$5.13 \pm 0.13^{a-c}$	12.49 ± 0.01^{f}
R8 Vb	Rare-mating	$0.071 \pm 0.004^{a,b}$	$8.59 \pm 0.37^{g,h}$	$28.30 \pm 0.15^{b-e}$	bdl	1.71 ± 0.24^{a}	$5.70 \pm 0.00^{c-e}$	$11.03 \pm 0.02^{a-c}$
R8 VIo	Rare-mating	$0.070 \pm 0.003^{a,b}$	$8.51 \pm 0.07^{f-h}$	$30.84 \pm 1.03^{c-e}$	bdl	2.38 ± 0.01^{a}	$5.64 \pm 0.332^{c-e}$	$11.62 \pm 0.19^{a-f}$
R8 VIIo	Rare-mating	$0.071 \pm 0.006^{a,b}$	$8.33 \pm 0.39^{f-h}$	$30.22 \pm 1.04^{c-e}$	bdl	2.00 ± 0.11^{a}	6.09 ± 0.19^{e}	$12.23 \pm 0.16^{d-f}$
R8 VIIIo	Rare-mating	$0.086 \pm 0.001^{a-e}$	$7.55 \pm 0.16^{c-h}$	33.18 ± 3.89^{e}	bdl	2.58 ± 0.62^{a}	$5.36 \pm 0.24^{b-d}$	$11.19 \pm 0.29^{a-d}$
S2 Io	Spore to spore	$0.073 \pm 0.006^{a-d}$	$7.88 \pm 0.36^{d-h}$	$28.13 \pm 0.97^{b-e}$	bdl	2.05 ± 0.03^{a}	$5.20 \pm 0.17^{a-d}$	$11.24 \pm 0.36^{a-e}$
S2 IIo	Spore to spore	$0.070 \pm 0.007^{a,b}$	$8.13 \pm 0.24^{e-h}$	$28.81 \pm 1.83^{b-e}$	bdl	2.27 ± 0.52^{a}	$5.53 \pm 0.08^{c-e}$	$12.29 \pm 0.25^{e,f}$
S7	**Spore to spore**	**$0.091 \pm 0.008^{a-f}$**	**$6.87 \pm 0.31^{b-g}$**	**$25.35 \pm 0.38^{b-d}$**	**bdl**	**2.27 ± 0.06^{a}**	**$5.55 \pm 0.07^{c-e}$**	**$11.78 \pm 0.11^{a,b}$**

Indicated in bold are those strains chosen to be used in the following selection steps.
[Ω]Extracted from Pérez-Través et al. (2015).
[$]Values expressed as mean ± standard deviation. Values not sharing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, $\alpha = 0.05$, n = 2).
[&]K: kinetic constant.
[*]t50: time necessary to consume 50% w/v of the total sugars.
[#]t2g/L: time necessary to reach 2 g/L of residual sugars.
[¥]bdl: value below detection limit (0,05 g/L).

procedures with the Statistica 7.0 software package (StatSoft, Tulsa, OK, USA), and by minimizing the sum of the squares of the difference between the experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R^2) in relation to the experimental data. The obtained equations were used to calculate the time required to consume 50% of the initial sugar content present in must (t_{50}) and the time needed to consume almost all the amount of sugars leaving a residual amount of 2 g/L (t_2). t_2 wasn't obtained in the stuck fermentations.

2.2.2. Natural must fermentations

Sauvignon Blanc must was used to perform the stabilisation tests and Verdejo must was used to perform mannoprotein determination. Grape berries were pressed and 1 mL/L of dimethyl dicarbamate (DMDC) was added in order to obtain microbiological stability. Before the fermentation, Verdejo must was supplemented with Lalvin nutritive supplements (0.3 g/L). Fermentations were done with parental and selected hybrid strains (R2 IVo, R8 IIa and S7 in Sauvignon Blanc fermentation and R2 IVo in Verdejo fermentation), at 20 °C in 250-mL flasks containing 175 mL of must and were inoculated with an initial population of $2 * 10^6$ CFU/mL. Flasks were closed with Müller valves and were monitored by weight loss until reaching a constant weight. Immediately after fermentations ended, yeast cells were removed by centrifugation and supernatants were stored at 4 °C until use. All the fermentations were analysed by HPLC in order to determine the amounts of residual sugars, glycerol, and ethanol as is described in a previous section. Each fermentation experiment in Sauvignon Blanc must was done twice (due to problems of availability of natural must) as a better must variety to perform the stabilisation tests and each fermentation experiment in Verdejo must was done three times (is the most similar musts to Sauvignon Blanc).

Before curve fitting, weight loss data were corrected to % of consumed sugar according to the following formula:

$$C = ((m * [S-R])/(mf * S)) * 100$$

where C is the % of sugar consumed at each sample time, m is the weight loss value at this sampling time, S is the sugar concentration in the must at the beginning of experiment (g/L), R is the final sugar concentration in the fermented must (residual sugar, g/L) and mf is the total weight loss value at the end of the fermentation (g).

Curve fitting was carried out using the reparametized Gompertz equation proposed by Zwietering et al. (1990):

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

where y is the % of consumed sugar; D is the maximum sugar consumption value reached (the asymptotic maximum, %), μmax is the maximum sugar consumption rate (h − 1), and λ the lag phase period which sugar consumption was not observed (h). Data were fitted using the nonlinear regression module of Statistica 7.0 software package (StatSoft, Tulsa, OK, USA), minimizing the sum of squares of the difference between experimental data and the fitted model. Fit adequacy was checked by the proportion of variance explained by the model (R2) respect to experimental data.

2.3. HPLC analysis of wines

The supernatants of the fermentation end points were analysed by HPLC in order to determine the amounts of residual sugars (glucose and fructose), glycerol, and ethanol. A Thermo chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector was used. The column employed was a HyperREZ™ XP Carbohydrate H+ 8 μm (Thermo Fisher Scientific) and it was protected by a HyperREZ™ XP Carbohydrate Guard (Thermo Fisher Scientific). The conditions used in the analysis were as follows: eluent, 1.5 mM H_2SO_4; flux, 0.6 mL/min; and oven temperature, 50 °C. Samples were diluted 5-fold, filtered through a 0.22-μm nylon filter (Symta, Madrid, Spain) and injected in duplicate.

2.4. Analysis of polysaccharides and mannoproteins

2.4.1. Synthetic must

Once fermentation is finished, wines were centrifuged to remove yeast cells and monosaccharides were removed from the cultures' supernatants by one gel filtration in Econo-Pac columns (Bio-Rad, Alcobendas, Spain) following the manufacturer's recommendations.

The concentration of the total mannoproteins and polysaccharides in the eluted fraction was determined against a standard curve of commercial mannan (Sigma, Tres Cantos, Spain) according to the phenol-sulphuric acid method as described by Segarra et al. (1995). Five replicates were performed for each determination. Standard curve of commercial mannan was:

$$\text{mannan(mg/L)} = (\text{A490nm}-0.0473)/0.0106.$$

For the specific detection of mannoproteins, supernatants were resolved by SDS-PAGE (Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane using the Mini Protean transfer system (Bio-Rad) following the manufacturer's directions. The mannoproteins present in the membrane were detected by the use of peroxidase-conjugated concanavaline A (Sigma) as described by Klis et al.: incubate the membrane during 1 h in blocking solution (BSA 3% prepared in PBS-Tween20); wash, during 5 min, two times, with PBS-Tween20 (NaH_2PO_4 100 mM, NaCl 100 mM, Tween20 0.1% v/v, pH 6.8, adjusted with NaOH); incubate 1 h with hybridization solution (2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 μg/mL Concanavaline A solved in blocking solution); wash, during 5 min, two times, with PBS-Tween20; wash, during 10 min, one time, with PBS-Tween20; remove all the PBS-Tween20 solution and incubate during 1 min with 1 mL/8 cm^2 of ECL reactive (Amersham); expose and reveal the membrane. This method isn't a quantitative method, but allows us to establish differences in mannoprotein production. The analysis complements the polysaccharide quantification.

2.4.2. Natural must

For mannoprotein analysis in Verdejo must, the methodology proposed by Quirós et al.(2012) was followed with few modifications. Wines were centrifuged to remove yeast cells. Samples were gel filtered twice through 30 × 10 mm Econo-Pac® 10 DG disposable chromatography columns (Bio-Rad Laboratories, Hercules, CA). Two aliquots of 1.9 mL of the macromolecular fraction were concentrated in 2 mL screw-capped microtubes until complete evaporation. The dried material was carefully suspended in 100 μL of 1 M H_2SO_4. Tubes were tightly capped and incubated in a water bath at 100 °C for 5 h 30 min to undergo acid hydrolysis. After this treatment, tubes were briefly spun down, and 10-fold diluted with MilliQ water. Sulphuric acid was removed by solid-phase extraction (SPE) with a Strata NH_2 500 mg/3 mL column (Phenomenex, Torrance, CA, USA). After SPE, samples were filtered through 0.22 μm pore size nylon filters (Membrane Solutions) and analysed in duplicate in a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector (Surveyor RI Plus Detector). The column employed was a 300 × 7.7 mm PL Hi-Plex Pb 8 μm (Varian, Inc., Shropshire, UK). MilliQ water was used as the mobile phase at a flux of 0.6 mL/min and a column temperature of 70 °C.

Mannoprotein amount was determined against a standard curve of commercial mannan (Sigma, Tres Cantos, Spain) processed in the same conditions.

$$\text{mannan(mg/L)} = (\text{mannose(mg/L)} + 0.9296)/0.7205.$$

2.5. Protein haze analysis (heat test)

For the bentonite fining assays, bentonite was previously suspended and hydrated in distilled water at 50 g/L. Different amounts of the homogenised suspension were added to 25 mL of wine to reach 0, 12, 24 36, 48, or 60 g/hL. Closed tubes were incubated at room temperature in a rocking shaker for 30 min. Wines were then clarified by centrifugation, 5 min at 3000 *g*, and were filtered through a 0.45 μm PVDF filter. The stability of the bentonite-treated wines was assayed by incubating 5-mL aliquots (5 aliquots of 5 mL were measured for each sample) at 85 °C for 30 min and cooling on ice. The turbidity of wines was determined in a nephelometer (Hach, Loveland, CO, USA).

2.6. Statistical analyses

The kinetic parameters, HPLC and polysaccharide data were analysed using the Statistica 7.0 software package (StatSoft, Tulsa, OK, USA) by one-way ANOVA and a Tukey test for the means comparison.

2.7. Comparative genomic hybridisation analysis (aCGH)

2.7.1. DNA labelling and microarray competitive genome hybridisation

Parental and R2IVo cells were grown overnight (o/n) in 5 mL of GPY medium at 25 °C. DNA was extracted following the methodology proposed by Querol et al. (1992), was resuspended in 50 μL of deionised water and was digested with endonuclease *Hinf* I (Roche Applied Science, Germany) according to the manufacturer's instructions to fragments of an average length of 250 bp to 8 kb. Each sample was purified using the High Pure PCR Product Purification Kit (Roche Applied Science) and 2 μg was labelled in the BioPrime Array CGH Genomic Labelling System (Invitrogen, Carlsbad, CA, USA). The unincorporated label was removed using the MinElute PCR Purification Kit (Qiagen, Germany). Equal amounts of labelled DNA from the corresponding strains were used as probes for microarray hybridisation.

Array competitive genomic hybridisation (CGH) was performed as described in Peris et al. (2012). Experiments were carried out in duplicate and the Cy5-dCTP and Cy3-dCTP dye-swap assays were performed to reduce the dye-specific bias.

2.7.2. Microarray scanning and data normalisation

Microarray scanning was done in a GenePix Personal 4100A scanner (Axon Instruments/Molecular Devices Corp., USA). Microarray images and raw data were produced with the GenePix Pro 6.1 software (Axon Instruments/Molecular Devices Corp.) and the background was subtracted by applying the local feature background median option. M–A plots (M = Log2 ratios; A = log2 of the product of the intensities) were represented to evaluate if the ratio data were intensity-dependent. The normalisation process and filtering were done using Acuity 4.0 (Axon Instruments/Molecular Devices Corp.). Raw data were normalised by the ratio-based option. Features with artefacts or those flagged as bad were removed from the analysis. Replicates were averaged after filtering. The data from this study are available from GEO (http://www.ncbi.nlm.nih.gov/geo/); the accession number is GSE48117.

2.7.3. Gene ontology (GO) analysis of overrepresented genes

GO Term finder (available in the Saccharomyces Genome Database, SGD) was used to perform three different gene ontology (GO) analyses of the genes overrepresented in each particular strain based on the results obtained from the CGH analyses: i) Sc1 vs. Sc2, ii) R2 IVo vs. Sc1 and iii) R2 IVo vs. Sc2. In all cases, statistically significant GO term enrichments were shown by computing a *p*-value using the hypergeometric distribution (the background set of genes was 6241, the number of ORFs measured in the microarray experiments). GO terms showing significant values (z-score > 2 and p-value < 0.05) were sorted according to their corresponding GO category.

2.8. qRT-PCR analysis

PCR primers for interesting genes (*MNN*10, *YPS*7, *HXT*9, *HXT*11 and *HXK*1) were designed according to the available genome sequences of *S. cerevisiae* (laboratory and wine) strains, using PrimerBlast software from NCBI web site. Specificity, efficiency, and accuracy of the primers were tested and optimized by standard PCRs. Primers showing specific amplification (*MNN*10, *YPS*7 and *HXK*1) were used in the subsequent quantitative real-time PCR (qRT-PCR) analysis. Primer sequences are listed in Suppl. Mat. Table 1.

2.8.1. Gene copy number estimation

Parental and R2IVo cells were grown overnight (o/n) in 5 mL of GPY medium at 25 °C. For every strain, DNA was extracted, in duplicate, from 10^6 CFU according to Querol et al. (1992). DNA was purified using phenol. qRT-PCR was performed with gene-specific primers (200 nM) in a 10-μL reaction mixture, using the LightCycler 480 SYBR Green I Master (Roche Applied Science, Germany) in a LightCycler 480 System (Roche Applied Science, Germany) device. All samples were processed for melting curve analysis, amplification efficiency, and DNA concentration determination using the LightCycler 480 1.5.0 software. For every strain, DNA extracted from 10^6 CFU and serial dilutions (10^{-1} to 10^{-5}) were used for a standard curve. The copy number for each gene was estimated by comparing the DNA concentration for S288c (haploid *S. cerevisiae* strain).

2.8.2. Expression analysis

Expression of selected genes was studied along a fermentation in synthetic must. Fermentations were carried out as in Section 2.2.2 and samples were taken at 24 h (end latency-beginning of the exponential sugar consumption phase), 55 h (middle of the exponential sugar consumption phase) and 120 h (end of the exponential sugar consumption phase-beginning of the stationary consumption phase). When collected, samples were washed with cold DEPC water and frozen immediately until their use.

Frozen cells were lysed with zymolyase (Seikagaku Corporation) and total RNA was extracted using the High Pure RNA Isolation Kit (Roche Applied Science, Germany). RNA was reversed transcripted to cDNA with Reverse Transcriptase Core kit (EuroGentec) following instructions from the manufacturer: 200 ng of RNA is used as template and oligo $d(T)_{15}$VN at 2.5 μM as final concentration in a reaction volume of 10 μL. The reverse transcription reaction was setup in a TECHN E PCR System: 10 min at 25 °C, 45 min at 48 °C and 5 min at 95 °C. mRNA level of the three genes, in different strains and conditions, was quantified by qRT-PCR with gene-specific primers (200 nM) in a 10 μL reaction, using the LightCycler 480 SYBR Green I Master (Roche Applied Science, Germany) in a LightCycler® 480 System (Roche Applied Science, Germany) device. All samples were processed for melting curve analysis, amplification efficiency and DNA concentration determination using LightCycler® 480 1.5.0 software. A mix of all samples and serial dilutions (10^{-1} to 10^{-5}) were used as standard curve. The mean of gene expression from constitutive genes *ACT*1 and *RDN*18 was used to normalise the amount of mRNA and absolute values are represented.

3. Results

3.1. Fermentation performance in synthetic must

As a first selection step, all the stable hybrids along with the two parental strains were evaluated for fermentative features (see Table 1 and Suppl. Mat. F1). Fermentations were carried out at 20 °C and were monitored by measuring the sugar content until constant values were reached for 3 consecutive days. Table 1 shows the fermentation parameters calculated for all the evaluated strains, including the maximum fermentation rate (K), the time required to consume 50% w/v of the

total sugars (t_{50}) and the time needed to reach 2% w/v of the residual sugars (t_2), as described in the Materials and methods section.

Although no differences between both parental strains were detected in both the K and t_{50} parameters, Sc1 parental was unable to complete fermentation and showed an estimated t_2 that was more than twice as high as Sc2 (Table 1).

As a general trend, no differences in the fermentation parameters were observed between the hybrids obtained by rare-mating and those obtained by spore-to-spore mating (Table 1). Strain R2 Io obtained the highest K value among the hybrids, higher than the values obtained for both parental strains. Hybrids R2 IIIa and R2 IVo gave a higher K value than parental Sc2, but no differences with parental Sc1 were seen (Table 1). The same three hybrid strains (R2 Io, R2 IIIa and R2 IVo) achieved the lowest values for t_{50}, although only hybrid R2 Io exhibited significant differences for this value as compared to both parental strains (Table 1). Finally, strains R2 Io and R2 IVo also showed the lowest t_2 values.

Strains R2 IIIo and R8 IIIo displayed the same behaviour as Sc1, were unable to complete fermentation, and their estimated t_2 values were higher than 42 days (Table 1), according to these data these strains suffered a stuck fermentation as was indicated in Table 1.

By the end of fermentation, the concentration of some relevant metabolites (glucose, fructose, ethanol and glycerol) was analysed (Table 1). Even though all the hybrids and the two parental strains were able to consume almost all the glucose present in the medium, the amount of fructose remaining at the end of fermentations was variable. The fermentations carried out with strains R2 IIIa, R2 IIIo, R2 VIo, R8IIIo and parental strain Sc1 showed significantly higher residual fructose values than the rest, including those fermentations carried out with parental strain Sc2 (Table 1).

Regarding glycerol and ethanol production, no significant differences were observed among the fermentations conducted by the two parental strains and most hybrids. In particular, hybrids R2 VIo, R8 Vb and S7 produced significantly lower levels of ethanol than both the Sc1 and Sc2 parental strains.

Based on their fermentation performance (long t_2 and fructose amount higher than 2 g/L, which indicates a stuck fermentation), hybrid strains R2 IIIa, R2 IIIo, R2 VIo and R8IIIo were not included in the second selection step (release polysaccharides and mannoproteins).

3.2. Release of total polysaccharides and mannoproteins in synthetic must

The release of total polysaccharides for all the parental and hybrid strains showing good fermentative performance is shown in Fig. 1. The aim of this selection step was to compare the production of mannoproteins (because the only polysaccharides present in synthetic must are mannoproteins) by yeast strains under fermentation conditions at 20 °C using a synthetic must that mimicked real grape must. Under these assay conditions, parental strain Sc2 produced a significantly larger amount of total polysaccharides (67.1 mg/L) than strain Sc1 (56.8 mg/L), the last one selected in this work for its mannoprotein release capacity. Moreover, 12 of the 14 analysed hybrid strains released significantly bigger amounts of polysaccharides than both the parental strains (Fig. 1). The remaining two hybrid strains, R8 VIo and R8 VIIo, released a similar amount of polysaccharides to parental Sc2. The maximum polysaccharide content was detected in the medium inoculated with hybrid R2 IVo (100 mg/L). This value represents an increase of around 1.5 times as compared to the values obtained with parental Sc2, and of around 2 times if compared to parental Sc1.

To confirm that the total amount of polysaccharides was in accordance with the presence of the mannoproteins in the medium, we carried out the specific detection of mannoproteins in fermented synthetic musts using peroxidase-conjugated concavalin A. As a general rule, the results obtained with this methodology confirmed those obtained by the quantification of total polysaccharides. Even though this is a qualitative detection method, our results clearly demonstrate that most hybrids released a larger amount of mannoproteins than the parental strains.

Comparing in each gel the intensity of the bands of the hybrids versus the parental strains, the fermentations carried out by hybrids R8IIa and S7 gave the largest amount of mannoproteins, followed by those obtained with hybrids R2 Io, R2 IIo and R2 IVo (Fig. 2). Hybrids R2 Io and R2 IIo produced slightly different mannoprotein band patterns from those produced by the parental strains and the remaining hybrids (Fig. 2). Finally, the amount of mannoproteins released by hybrid strains R8 VIo and R8 VIIo was similar to that released by the other hybrids, which evidences similar mannoprotein profiles (Fig. 2). Nonetheless, these two hybrids produced a smaller amount of total polysaccharides than the other hybrid strains (Fig. 1).

Based on the results obtained from the total polysaccharides and mannoprotein release, we selected hybrid strains R2 IVo, R8 IIa and S7 to evaluate their capacity to increase the stabilisation of a white wine against protein haze.

3.3. Protein haze stability of the wines fermented by the higher mannoprotein producer hybrids

Fermentations of Sauvignon Blanc grape must were carried out with the three higher mannoprotein producer hybrids and the two parental strains to evaluate the effect on wine stability of the mannoproteins

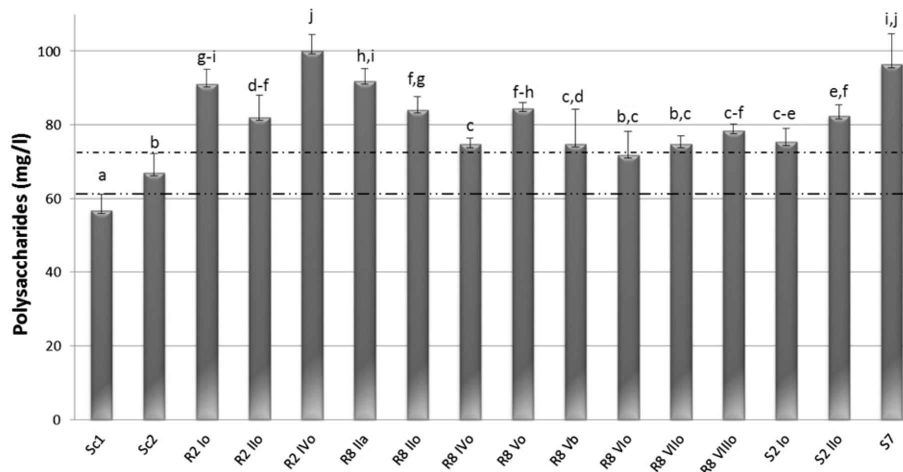

Fig. 1. Final concentrations of the polysaccharides released by hybrids and parental strains in synthetic must. Bars not sharing the same letter were significantly different according to one way ANOVA and Tukey test (α = 0.05). Dotted lines shown the parental polysaccharide value.

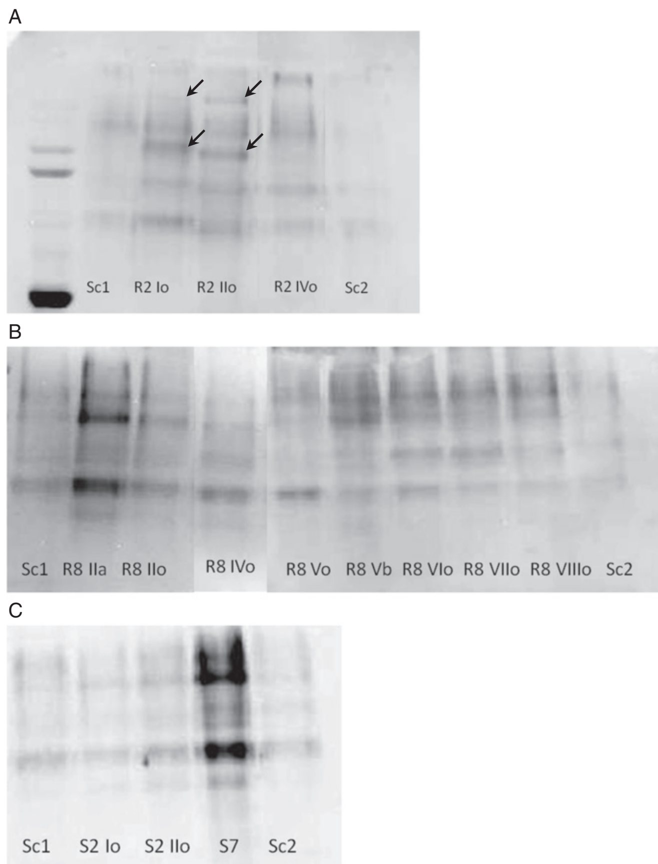

Fig. 2. Mannoproteins released during fermentation of a synthetic must by the hybrid strains compared to their parental. The identities of the strains are indicated in each panel. A and B: hybrids obtained by rare-mating methodology; C: hybrids obtained by spore to spore mating. Arrows in A indicate mannoprotein bands present in hybrids and not observed in parental.

produced by each different strain. Chemical analyses of the wines evidenced that parental Sc1 and hybrid S7 were unable to consume all the fructose that was originally present in the must, and they left as much as 6.4 and 5.6 g/L of residual fructose, respectively (data not shown). After fermentation, wines were subjected to the heat test for protein stability before and after bentonite fining, as described in the Materials and methods. Turbidity values close to 75 nefelos (nephelometric turbidity units) were obtained for the wines fermented with Sc1, Sc2 and R2 IVo without the addition of bentonite, whilst higher values were observed for hybrids R8 IIa and S7 (Fig. 3). R2 IVo and the R8 IIa hybrid strains showed the best stabilisation profiles, with R2 IVo seemed to require slightly less bentonite for complete stabilisation. The results for R8 IIa and Sc1 reveal lack of correlation between protein instability before bentonite stabilisation and the response of the corresponding wine to bentonite fining. Although no clear differences were obtained with this approach, we can conclude that R2 IVo was the best in this test and was also the strain that produces maximum levels of polysaccharides, for this reason this hybrid was selected for further analysis.

3.4. Measuring of the mannoprotein production in Verdejo fermentations

To ensure that R2 IVo hybrid produces higher amounts of mannoproteins than its parental strains, we performed fermentation in Verdejo must. Fermentations were carried out at 20 °C and were monitored by measuring the sugar content until constant values were reached for 3 consecutive days. Table 2 shows the fermentation parameters calculated for all the evaluated strains, including the maximum fermentation rate (K), the latency (l) and the time required to consume 95% w/v of the total sugars (t_{95}), as well as the main chemical parameters (glucose, fructose, glycerol and ethanol). The three strains finished the fermentation. Although Sc2 was the strain that showed the higher Vmax and Sc1 and R2 IVo showed similar value of this parameter, the hybrid R2 IVo finished the process earlier than parental Sc1, indicating an improvement of the fermentative capability.

As the natural must contains other polysaccharides different to mannoproteins and the phenol sulphuric method detects polysaccharides in general, we used the methodology described by Quirós et al. (2012) in order to analyse the amount of mannoproteins released by the selected strains. The results are shown in Fig. 4. Sc2 was the strain that lowers the amount of mannoproteins produced (~123 mg/L), followed by Sc1. The hybrid R2 IVo produced, statistically, more mannoproteins than both of its parental strains (~157 mg/L).

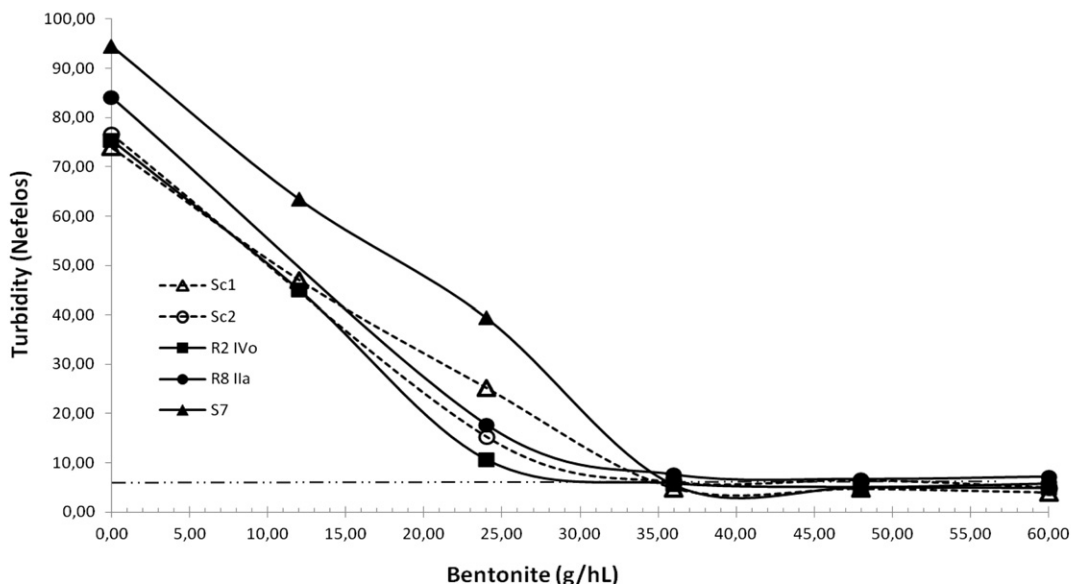

Fig. 3. Effect of bentonite finning on the heat-test results of Sauvignon Blanc wines fermented with selected hybrids compared to their parental strains. Horizontal dotted line indicates the asymptotic turbidity level representing wine stability. Error bars are included.

Table 2
Main kinetic parameters of the fermentations carried out on Verdejo must at 20 °C and chemical analysis of the final fermented products.

Strain	Kinetic parameters$			Chemical parameters$			
	K (hours^{-1})&	l(hours)*	t_{95} (hours)#	Glucose (g/L)¥	Fructose (g/L)	Glycerol (g/L)	Ethanol (% v/v)
Sc1	1.24 ± 0.01[a]	21.50 ± 0.22[a]	164.57 ± 2.69[c]	bdl	1.01 ± 0.08[a]	5.90 ± 0.10[c]	13.27 ± 0.11[a]
Sc2	1.40 ± 0.02[b]	22.61 ± 0.41[a]	134.36 ± 1.15[a]	bdl	bdl	5.75 ± 0.05[b,c]	13.25 ± 0.11[a]
R2 IVo	1.20 ± 0.00[a]	21.97 ± 0.12[a]	152.44 ± 0.44[b]	bdl	bdl	5.45 ± 0.06[a]	13.16 ± 0.06[a]

$Values expressed as mean ± standard deviation. Values not sharing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test. $\alpha = 0.05$. n = 2).
&K: kinetic constant.
*l: latency.
#t_{95}: time necessary to consume 95% of residual sugars.
¥bdl: value below detection limit (0.05 g/L).

As a resume, hybrid strain R2 IVo exhibited good fermentative behaviour in both synthetic and natural grape musts (Tables 1 and 2; Suppl. Mat. F1), and released large amounts of mannoproteins and polysaccharides that seem related with protection of wine against protein haze (Figs. 1, 2, 3 and 4). This strain seems to have inherited the positive physiological features from each parental strain. In order to characterise the potential genomic changes that may have occurred during hybrid generation and stabilisation, and which could be related with the improved physiological features of this strain, we performed array-comparative genomic hybridisation (CGH).

3.5. Comparative genomic hybridisation analysis of hybrid R2 IVo and the parental strains

For the CGH analysis, genomic DNA from hybrid strain R2 IVo was competitively hybridised with genomic DNA from each parental strain. The DNA from the two parental strains was also competitively hybridised against each other to evaluate the genomic differences between them by following the methodology described in the Materials and methods.

Of the 6000+ gene probes contained in the DNA microarray, only a few hundred showed a significant copy number variation among the three strains analysed (the hybrid and the two parental strains). An analysis of the data derived from the comparative hybridisation of the parental strains (Sc1 vs. Sc2) revealed significant differences in the copy number of some interesting genes. Ninety-four ORFs showed a significantly higher copy number in strain Sc2 and 41 ORFs had higher copy numbers in Sc1 (Fig. 5 and Suppl. Mat. Table 2). A considerable number of these variable genes were located in the telomeric or subtelomeric regions, but only a few of them corresponded to the genes with an annotated function. Big groups of variable subtelomeric ORFs were identified as transposons and they were particularly overrepresented in parental Sc2. Another group of genes overrepresented in Sc2 corresponded to those belonging to the *HXT* family (Fig. 5 and Suppl. Mat. Table 2). Interestingly, genes *GPM1* and *HXK1*, which codify for a phosphoglycerate mutase and hexokinase isoenzyme 1, respectively, seemed to be also overrepresented in parental Sc2 and displayed good fermentation performance.

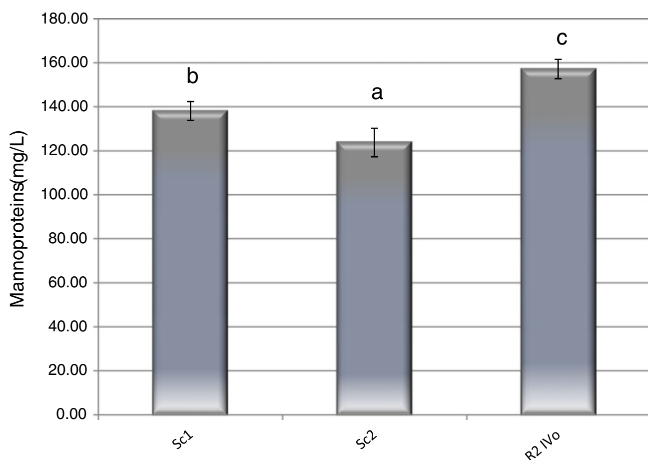

Fig. 4. Final concentrations of released mannoproteins by hybrid, parental and control strains in verdejo must. Bars not sharing the same letter were significantly different according to one way ANOVA and Tukey test ($\alpha = 0.05$).

Parental Sc1 was characterised by an overrepresentation of the genes typically found in wine yeast strains (Carreto et al., 2008), such as *MAL11*, *MAL13*, *CUP1*-1 and *CUP1*-2 (Fig. 5 and Suppl. Mat. Table 2). This parental strain, characterised by its ability to produce and release mannoproteins also displayed an overrepresentation of some of the genes involved in oligosaccharide metabolism and processing (e.g., *SPR1*), which codify for a glucan 1,3-beta glycosidase), *SWP1* (dolichyl-diphosphooligosaccharide protein glycotransferase) and *IMA1* (a α-1,6-glucosidase).

The gene ontology (GO) analysis was carried out with the overrepresented genes detected in each particular parental strain and the significant GO terms obtained were sorted according to their corresponding GO categories (Suppl. Mat. Table 3). According to that analysis, the terms related to disaccharide and oligosaccharide metabolism were significantly overrepresented in parental strain Sc1, whilst terms related to transposition were associated with parental Sc2 (Suppl. Mat. Table 3).

The comparative analysis, which derived from the competitive hybridisation of hybrid R2 IVo versus each parental strain, evidenced that the hybrid maintained the copy number of one parental strain or the other for several genes. The hybrid did not show genes significantly overrepresented in relation to the two parental strains. However we observed significantly overrepresented in the hybrid with regard to Sc1 (genes in red in R2-IVo in Fig. 5) that do not show differences in copy numbers with Sc2, indicating that probably Sc2 has an intermediate copy number between Sc1 and R2-IVo for these genes. Then, the hybrid possesses more copies of these genes than the two parentals. The same explanation could be associated with genes in blue in R2-IVo in Fig. 5, with genes significantly overrepresented in the hybrid with regard to Sc2. According to the data shown in Fig. 5, the hybrid R2-IVo presented 25 overrepresented ORFs against Sc1 and 65 different genes overrepresented against Sc2 (Fig. 5 and Suppl. Mat. Table 4). Both strains Sc1 and the hybrid shared nine overrepresented ORFs, which included five annotated genes (*CUP1*-1 and 2, *RMD6*, *HXT15* and *SEO1*). However, 18 ORFs, including six annotated subtelomeric genes (*HXT9*, *HXT11*, two ORFs of *HXT12*, *FSP2*, *REE1* and *BSC3*) and eight genes corresponding to transposons, were commonly overrepresented in both the hybrid and parental strains Sc2 (Fig. 5 and Suppl. Mat. Tables 2 and 4).

Apart from the overrepresented ORFs shared between the hybrid and parental strains, the hybrid exhibited 7 and 56 genes in significantly higher copy numbers than Sc1 and Sc2, respectively (Fig. 5 and Suppl. Mat. Table 4). In particular, those genes involved in cell wall organisation and maintenance, like the endopeptidase coding gene YPS7 and the gene coding for α-1,6-mannosyltransferase *MNN10*, had significantly higher copy numbers in the hybrid than in parental Sc2. No differences between hybrid and Sc1 were observed for these ORFs, indicating a similar copy number between these two mannoprotein higher producer strains.

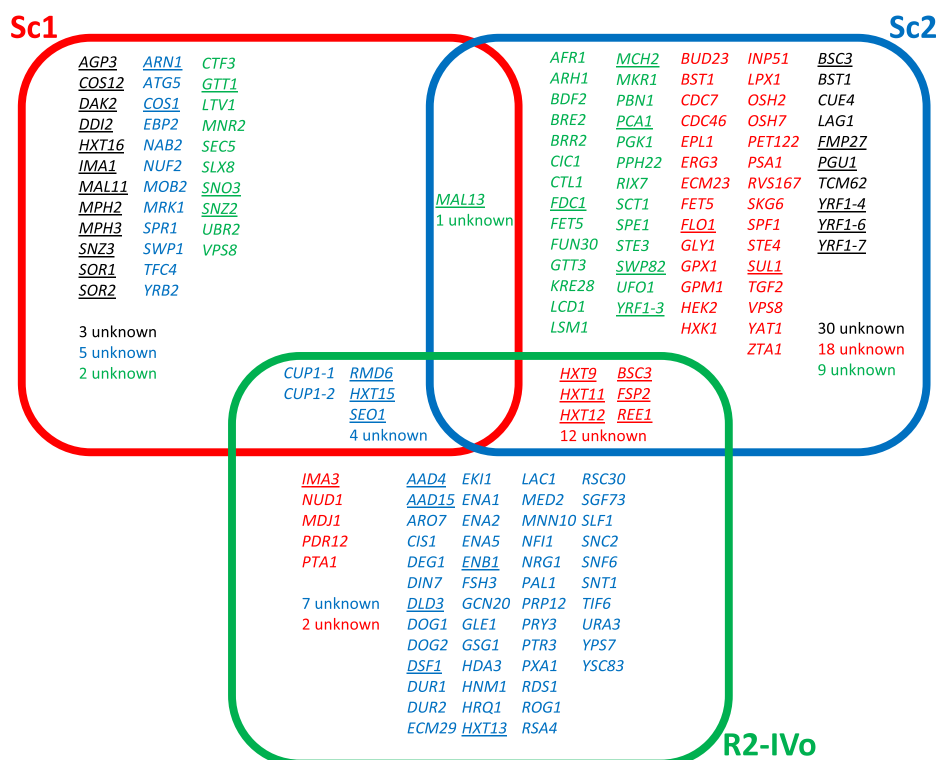

Fig. 5. Schematic grouping of genes significantly overrepresented in each strain under study. Underlined: subtelomeric genes. In red: genes significantly overrepresented in Sc1. In blue: genes significantly overrepresented in Sc2. In green: genes significantly overrepresented in R2-Ivo. In black: genes significantly overrepresented in the two remaining strains. Genes in the intersections are overrepresented genes in two strains with respect to the remaining one. Genes significantly overrepresented in the hybrid with respect to Sc1 (in red in R2-Ivo) that did not show copy number differences with Sc2, likely indicate that Sc2 has an intermediate copy number between Sc1 and R2-Ivo for these genes. Therefore, the hybrid should possess more copies of these genes than the two parentals. The same explanation applies to genes in blue in R2-Ivo, corresponding to genes significantly overrepresented in the hybrid with regard to Sc2.

In this case, the GO analysis was separately performed with the ratio data obtained from the hybridisation of the hybrid versus parental Sc1 and parental Sc2 (Suppl. Mat. Table 3). According to this analysis, the terms related to transposition were also significantly overrepresented in the hybrid as compared to parental Sc1, as were some other terms related to carbohydrate metabolism and glycosidase activity (Suppl. Mat. Table 3). The GO analysis done with the over/underrepresented genes between the hybrid and Sc2 evidenced an overrepresentation of the terms related to detoxification in the hybrid genome.

If we consider its better fermentation performance, its greater mannoprotein release, and its effects on protein haze protection, the R2 IVo hybrid strain proved to be the most suitable strain for industrial purposes. These physiological properties may be related with the genes of the HXT family (HXT9, HXT11, HXT12), which showed significantly higher copy numbers in the hybrid and the strain Sc2. In addition, the genes associated with cell wall organisation were overrepresented in the hybrid genome and in parental Sc1, and may be responsible for the increase in polysaccharides produced by these two strains.

3.6. Validation of comparative genomic hybridisation analysis

To validate the results observed in the CGH analysis, we perform qRT-PCR of several of the genes indicated above, as *MNN*10, *YPS*7, *HXT*9, *HXT*11 and *HXK*1, in order to confirm the gene copy number. As *HXT* genes are quite similar, were removed from the analysis. According the rest of the genes the hybrid R2 IVo should have more copies of *MNN*10 and *YPS*7 than Sc2 and should have more copies of *HXK*1 than Sc1, but less than Sc2. Using this approach the copy number differences were no conclusive (data not shown).

For this reason we decided to study the expression of these three genes during fermentation. Results are shown in Fig. 6 and Table S5.

Comparing the relative expression of *MNN*10 gene, of the same strain at different time points (Suppl. Mat. Table 5B) the hybrid R2 IVo maintained a high relative expression value at 24 h and 55 h, diminishing at 120 h; Sc1 diminished its expression at 55 h and Sc2 maintained similar lower expression values at all fermentation points.

For *YPS*7 gene relative expression values of the same strain at different time points (Suppl. Mat. Table 5B), showed that the R2 IVo increased its expression values at 55 h, Sc2 maintained it during all the experiment and Sc1 decreased its expression at 120 h.

For *HXK*1 gene results (Fig. 6C and Suppl. Mat. Table 5A and B) indicated that at 24 h the strains showed the lower relative expression values of all the experiment, nowadays, relative expression values of Sc1 were higher than those showed by Sc2 and R2 IVo. At 55 h the three strains increased their relative expression values, but all presented similar values. At 120 h Sc1 maintained its expression value and Sc2 and R2 IVo increased their relative expression values; this increase was 7–10 folds the expression values showed at 24 h.

This results indicated that the higher mannoprotein production of R2 IVo could be due to the maintenance of the increased expression of *MNN*10 during long time than Sc1 and to the higher expression values of *YPS*7 in the middle of the fermentation (55 h point) as is shown in Fig. 6A and B. The improvement in the fermentation kinetics could be due to the higher increment in the expression of *HXK*1, showed in Sc2 too, at the end of the fermentation (120 h), see Fig. 6C.

4. Discussion

During the winemaking process, other than products and by-products of sugar metabolism, yeast cells release cell constituents, like proteins and polysaccharides, which also contribute to wine quality. A number of studies have been published in recent decades that have

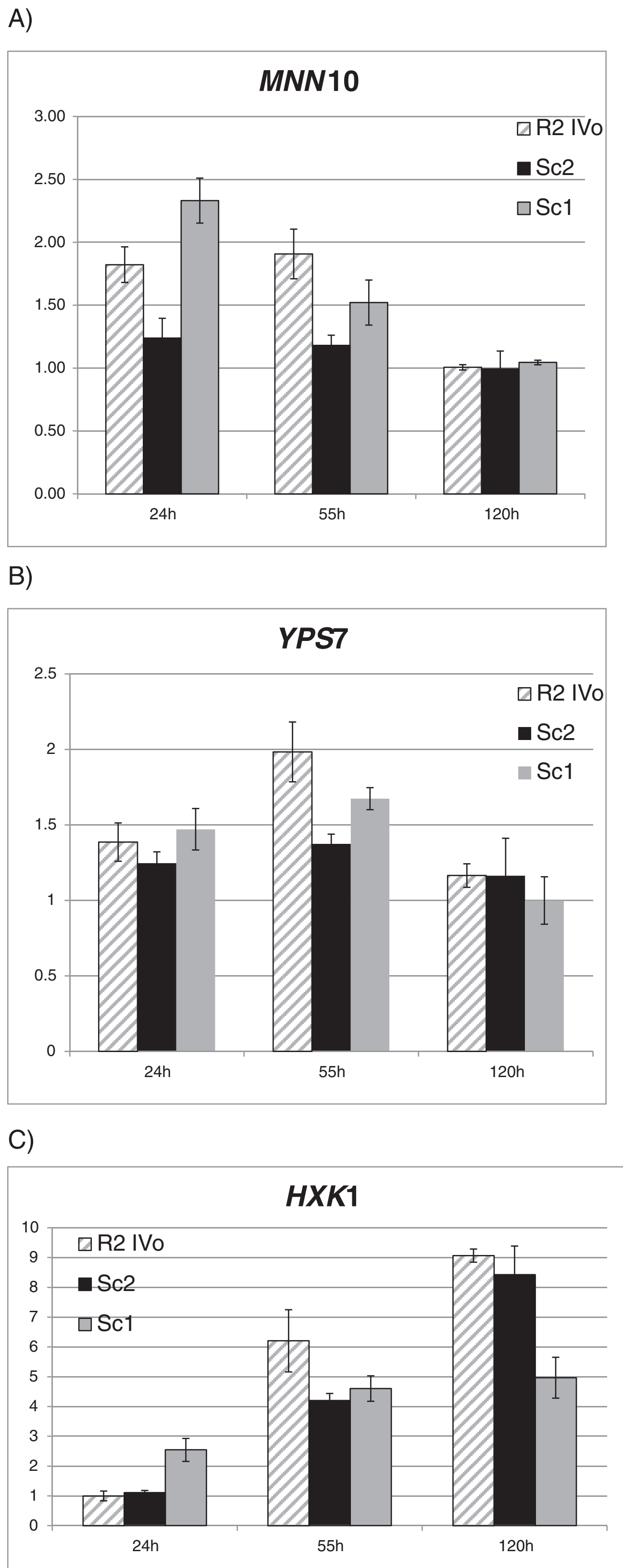

Fig. 6. Relative expression of the genes A) *MNN*10, B) *YPS*7 and C) *HXK*1 during fermentation. Expression of each one of the genes was related to the lower expression value obtained for this gene in all the experiment.

demonstrated the positive contribution of yeast mannoproteins to wine attributes (Caridi, 2006). Based on those reports, different experimental approaches have been proposed for the isolation and/or development of yeast strains that are able to secrete larger amounts of mannoproteins (González-Ramos et al., 2009, 2010; Quirós et al., 2010). However, some of these methods are based on genetic engineering and could face regulatory constraints and consumer distrust. Others involve random mutagenesis and can face a risk of an unintended genetic modification of the desirable oenological features of the original wine yeast strain. In this work, we were able to combine by hybridisation techniques the desirable oenological features of two commercial *S. cerevisiae* strains in a single strain: Sc1, with a high capacity to release polysaccharides, including mannoproteins; and Sc2, with excellent fermentative performance at industrial level. The strains obtained by making full use of these natural hybridisation processes do not face the regulatory and marketing restrictions that GMO microorganisms do.

The literature frequently mentions that hybrids can inherit particular physiological features in new combinations, which can be even higher than those of the parents. *S. cerevisiae* × *Saccharomyces kudriavzevii* interspecific hybrids can retain the fermentation vigour of *S. cerevisiae* and the ability to produce particular aromatic compounds from *S. kudriavzevii*; whilst *S. cerevisiae* × *Saccharomyces uvarum* hybrids can display the capacity to ferment at both low and high temperatures and to produce intermediate amounts of minor fermentative compounds (Sipiczki, 2008). Most of the stable hybrids analysed in this work give intermediate values between both parental strains for fermentation kinetics parameters K, t_{50} and t_2. In some cases, hybrids (particularly R2 Io, R2 IIIa and R2 IVo) gave even higher K values and lower t_{50} and t_2 values than parental Sc2, which was selected for its excellent fermentative behaviour (Table 1).

Strain Sc1, selected for its high mannoprotein release capacity, gave the lowest values of total polysaccharides produced (evaluated by the phenol/sulphuric method) when compared with parental Sc2 and all the tested hybrids, in a synthetic must fermentation. However, mannoprotein specific staining indicated similar or bigger mannoprotein content for Sc1. These differences indicate that Sc2 could be releasing other polysaccharides different to mannoproteins being the total mannoprotein release or the mannoprotein/total polysaccharide ratio higher in Sc1, and that mannoprotein releasing – instead of the total polysaccharide release – is better related to the technological properties. It has been reported that not only the total amount of mannoproteins, but also their specific kind, has been associated with beneficial activity in wine (Moine-Ledoux and Dubourdieu, 1999; Waters et al., 1994). In this work, most hybrids exhibited similar mannoprotein patterns to the parental strains. As we wanted to improve parental traits, we selected for posterior analysis strains with similar bands but with higher intensity than the ones showed by the parental strains.

In this work, we chose protein haze stabilisation as a model application to detect interesting hybrid strains given its amenability to laboratory-scale experimentation. These methods are based on the haze susceptibility of Sauvignon Blanc (González-Ramos et al., 2009). Using this method we could see that the wine obtained with strain R2 IVo responded considerably better to bentonite-fining treatments, although the resolution of this method is not the best according to our data. These results have been confirmed with a quantitative method (Quirós et al., 2012) in Verdejo must. When mannoproteins were quantified at the end of this fermentation, it was revealed that Sc1 produced more mannoproteins than Sc2 (as was said by the producers). The selected hybrid R2 IVo released more mannoproteins than both of its parental strains, indicating that this trait was improved not only for the parental Sc2, but it was also improved with respect to the parental Sc1.

Many studies have shown that extensive genome rearrangements and gene duplication occur in organisms, particularly yeasts, during adaptation to changing environments. These changes can partially explain the hybrid improvement achieved in this work. It is well-known that microarray data can be used to reflect such genome changes (Dunham

et al., 2002; Dunn et al., 2005; Peris et al., 2012). The experiments carried out to detect specific alterations in the gene copy number in the selected hybrid, which might explain some of the inherited physiological properties and hybrid improvement, evidenced a number of overrepresented genes in the three strains compared (Sc1, Sc2 and R2 IVo).

The genes associated with cell wall organisation could be held responsible for the increased ability of strains to produce and release polysaccharides. In our study, gene *MNN10*, which codifies for a subunit of a Golgi mannosyltransferase complex, was overrepresented in the hybrid genome if compared to parental Sc2, whilst no differences in copy numbers were observed between R2 IVo and Sc1. The overrepresentation of *MNN10* might be associated with the better mannoprotein release in these strains. Indeed, deletion of either Mnn10p or its homologue Mnn11p results in defects in the mannan synthesis in vivo. An analysis of the enzymatic activity of the complexes isolated from mutant strains suggests that Mnn10p and Mnn11p are responsible for the majority of the complex's α-1,6-polymerizing activity (Jungmann et al., 1999). Additionally, the same behaviour was observed for gene *YPS7*, which codifies for a protease related to cell wall glucan incorporation and retention. *YPS7* also forms part of the transcriptional response to cell wall stress and is required during severe cell wall stress in *S. cerevisiae* (Krysan et al., 2005). Finally, *SWP1*, which codifies for an oligosaccharyl transferase subunit required for N-linked glycosylation of proteins in the endoplasmic reticulum, was overrepresented in mannoprotein producer parental Sc1 if compared to Sc2, and Sc1 and hybrid R2 IVo present a similar copy number for this gene, which may also be related with the increased mannoprotein synthesis for hybrid R2 IVo. A combination of the genes associated with cell wall organisation obtained from parental Sc1 and the similar duplications in some genes like *SWP1* to parental Sc2 can justify that the hybrid is even better than both the parental ones for these properties.

An initial set of genes with an altered copy number has been associated with telomeric or subtelomeric regions in different chromosomes (Fig. 5). Brown et al. (2010) suggested that these regions are "hotbeds for genomic evolution and innovation". Both telomeric and subtelomeric genes evolve faster than their internal counterparts, and they are frequently the sites of gene duplications (Ames et al., 2010). According to different authors, differences in the copy number of several telomeric genes are very important for adaptation and to overcome different environmental stresses (Carreto et al., 2008; Dunham et al., 2002). In our work, the subtelomeric genes belonging to the *HXT* family (*HXT9*, *HXT11*, *HXT12*) had significantly higher copy numbers in the hybrid. This set of subtelomeric genes was also overrepresented in strain Sc2. Although sugar utilisation *HXT* genes are virtually identical to each other, which allows the possibility of cross-hybridisation and makes it impossible to know which particular gene(s) is(are) overrepresented in the pair Sc2 vs. R2 IVo, this difference can be related to the best fermentation performance of both Sc2 and R2 IVo (Table 1). In this sense, Lin and Li (2011) found a strong correlation between the copy number of *HXT* genes and fermentative strain behaviour.

Furthermore, alterations in the copy number of glycolytic genes or the genes responsible for sugar transportation can be associated with the strains' improved fermentation performance. In this sense, parental strain Sc2, characterised for its good fermentative performance, had a significantly higher copy number of genes *GPM1* and *HXK1* than Sc1, but no differences with the hybrid R2 IVo (also showing good fermentation performance) were detected. In particular, the *HXK1* gene has been reported to be expressed when yeast cells are grown on a fermentable medium using glucose, fructose or mannose as a carbon source (Bisson and Fraenkel, 1983).

Our work demonstrates that hybridisation combined with stabilisation under winemaking conditions is an effective approach to obtain yeast strains with both improved mannoprotein producing capacity and fermentation performance, which are physiological features that genetically depend on the coordinated expression of numerous different genes (polygenic features). A hybrid with both features improved was selected and a number of genes potentially responsible for the improvement of the hybrid generated in this work have been postulated.

Acknowledgements

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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.2015.04.004.

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