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

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**Abstract**

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 *S. cerevisiae* x *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).

**Keywords**: yeast hybridization, rare-mating, spore-to-spore mating, wine yeast.

## 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; González-Ramos et al., 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 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 involves about 1,200 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 (Sc1) that was been selected as a good mannoproteins producer. We develop an intraspecific hybrid between the two commercial strains Sc1 and Sc2 by rare mating that give rise to non-GMO strains. After the genomic stabilisation we obtain a strain that overproduce mannoprotein and show 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 *Saccharomyces 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).

121 Strains were maintained in GPY-agar medium (% w/v: yeast extract 0.5, peptone 0.5,
122 glucose 2, agar-agar 2).
123
124 *2.2Fermentation experiments*
125 *2.2.1 Synthetic must fermentation*
126 All the strains were used in synthetic must fermentations. Fermentations were carried
127 out in 100-ml bottles containing 80ml of synthetic must (Rossignol et al., 2003). The
128 sugar concentration in the must (50% glucose + 50% fructose) was adjusted to 200 g/l.
129 Must was inoculated independently with the different yeast strains to reach an initial
130 population of $2*10^6$ CFU/ml and was maintained without aeration at 20°C. The
131 fermentation process was monitored by the quantification of the total sugar
132 concentration. For this purpose, 1-mL aliquots of must were taken every 2 days and the
133 sugar concentration was determined enzymatically (the glucose-fructose determination
134 kit, Symta, Madrid, Spain). Fermentations were considered as stopped when the sugar
135 amount was the same during 3 measures. Each fermentation experiment was done twice.
136 The sugar consumption data obtained from each fermentation were fitted by the
137 following exponential decay function: $Y = D + S * e^{(-K * t)}$ as previously used by
138 Arroyo-López et al. (2009). In this function, "Y" is the total amount of sugar present in
139 must, "t" is the time in days, "D" is the asymptotic value when $t \rightarrow \infty$, "S" is the
140 estimated value of change, and "K" is the kinetic constant ($days^{-1}$) which defines the
141 maximum fermentation rate. Equations were fitted by the linear and non-linear
142 regression procedures with the Statistica 7.0 software package (StatSoft, Tulsa, OK,
143 USA), and by minimizing the sum of the squares of the difference between the
144 experimental data and the fitted model. Fit adequacy was checked by the proportion of
145 variance explained by the model ($R^2$) in relation to the experimental data. The obtained

146 equations were used to calculate the time required to consume 50% of the initial sugar
147 content present in must ($t_{50}$) and the time needed to consume almost all the amount of
148 sugars leaving a residual amount of 2g/L ($t_2$). $t_2$ wasn't obtained in the stuck
149 fermentations.

150 *2.2.2 Natural must fermentations*

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

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

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

Were 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$^{TM}$ XP Carbohydrate H+ 8μm (Thermo Fisher Scientific) and it was protected by a HyperREZ$^{TM}$ 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.

195

196 *2.4Analysis of polysaccharides and mannoproteins*

197 *2.4.1 Synthetic must*

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

202 The concentration of the total mannoproteins and polysaccharides in the eluted fraction
203 was determined against a standard curve of commercial mannan (Sigma, Tres Cantos,
204 Spain) according to the phenol-sulphuric acid method as described by Segarra et al.
205 (1995). Five replicates were performed for each determination. Standard curve of
206 commercial mannan was:

207 mannan (mg/L) = (A490nm – 0.0473) / 0.0106

208 For the specific detection of mannoproteins, supernatants were resolved by SDS-
209 PAGE(Laemmli, 1970). Proteins were transferred to a nitrocellulose membrane using
210 the Mini Protean transfer system (Bio-Rad) following the manufacturer's directions.
211 The mannoproteins present in the membrane were detected by the use of peroxidase-
212 conjugated concanavaline A (Sigma) as described by Klis et al. ( 1998): incubate the
213 membrane during 1h in blocking solution (BSA 3% prepared in PBS-Tween20); wash,
214 during 5 min, two times, with PBS-Tween20 ($NaH_2PO_4$ 100mM, NaCl 100mM,
215 Tween20 0.1% v/v, pH 6.8, adjusted with NaOH); incubate 1h with hybridization
216 solution (2.5mM $CaCl_2$, 2.5mM $MgCl_2$, 1µg/ml Concanavaline A solved in blocking
217 solution); wash, during 5 min, two times, with PBS-Tween20; wash, during 10 min, one
218 time, with PBS-Tween20; remove all the PBS-Tween20 solution and incubate during
219 1min with 1ml/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.

mannan (mg/L)=( mannose (mg/L)+0.9296)/0.7205

*2.5Protein 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 3,000g, and were filtered through a 0.45 μm PVDF filter. The stability of the bentonite-treated wines was assayed by incubating 5-mL aliquots (5aliquots of 5ml 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 polysaccharides 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 5mL of GPY medium at 25ºC. DNA was extracted following the methodology proposed by Querol et al.(1992), was resuspended in 50 μl of de-ionised 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 2μg were 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.

*2.7.2Microarray 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 artifacts 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

293 the microarray experiments). GO terms showing significant values (z-score >2 and *p*-
294 value <0.05) were sorted according to their corresponding GO category.
295
296 *2.8 qRT-PCR analysis*
297 PCR primers for interesting genes (*MNN*10, *YPS*7, *HXT*9, *HXT*11 and *HXK*1) were
298 designed according to the available genome sequences of *S. cerevisiae* (laboratory and
299 wine) strains, using PrimerBlast software from NCBI web site. Specificity, efficiency,
300 and accuracy of the primers were tested and optimized by standard PCRs. Primers
301 showing specific amplification (*MNN*10, *YPS*7 and *HXK*1) were used in the subsequent
302 quantitative real-time PCR (qRT-PCR) analysis. Primer sequences are listed in Suppl.
303 Mat. Table 1.
304 *2.8.1 Gene copy number estimation.*
305 Parental and R2IVo cells were grown overnight (o/n) in 5mL of GPY medium at 25ºC.
306 For every strain, DNA was extracted, in duplicate, from $10^6$ CFU according to Querol et
307 al. (1992). DNA was purified using phenol. qRT-PCR was performed with gene-
308 specific primers (200 nM) in a 10-µl reaction mixture, using the LightCycler 480 SYBR
309 Green I Master (Roche Applied Science, Germany) in a LightCycler 480 System
310 (Roche Applied Science, Germany) device. All samples were processed for melting
311 curve analysis, amplification efficiency, and DNA concentration determination using
312 the LightCycler 480 1.5.0 software. For every strain, DNA extracted from $10^6$ CFU and
313 serial dilutions ($10^{-1}$ to $10^{-5}$) were used for a standard curve. The copy number for each
314 gene was estimated by comparing the DNA concentration for S288c (haploid *S.*
315 *cerevisiae* strain).
316 *2.8.2 Expression analysis.*

317 Expression of selected genes was studied along a fermentation in synthetic must.
318 Fermentations were carried out as in 2.2.2 and samples were taken at 24h (end latency-
319 beginning of the exponential sugar consumption phase), 55h (middle of the exponential
320 sugar consumption phase) and 120h (end of the exponential sugar consumption phase-
321 beginning of the stationary consumption phase). When collected, samples were washed
322 with cold DEPC water and frozen immediately until their use.
323 Frozen cells were lysed with zymolyase (Seikagaku corporation) and total RNA was
324 extracted using the High Pure RNA Isolation Kit (Roche Applied Science, Germany).
325 RNA was reversed transcripted to cDNA with Reverse Transcriptase Core kit
326 (EuroGentec) following instructions from the manufacturer: 200 ng of RNA are used as
327 template and oligo $d(T)_{15}VN$ at 2,5 µM as final concentration in a reaction volume of
328 10µl. The reverse transcription reaction was setup in a TECHN
329 E PCR System: 10 min at 25ºC, 45 min at 48ºC and 5 min at 95ºC. mRNA level of the
330 three genes, in different strains and conditions, was quantified by qRT-PCR with gene-
331 specific primers (200 nM) in a 10 µl reaction, using the LightCycler 480 SYBR Green I
332 Master (Roche Applied Science, Germany) in a LightCycler® 480 System (Roche
333 Applied Science, Germany) device. All samples were processed for melting curve
334 analysis, amplification efficiency and DNA concentration determination using
335 LightCycler® 480 1.5.0 software. A mix of all samples and serial dilutions ($10^{-1}$ to $10^{-5}$)
336 were used as standard curve. The mean of gene expression from constitutive genes
337 *ACT*1 and *RDN*18 was used to normalize the amount of mRNA and absolute values are
338 represented.
339
340
341 **3. Results**

342 *3.1 Fermentation performance in synthetic must*

343 As a first selection step, all the stable hybrids along with the two parental strains were
344 evaluated for fermentative features (see Table 1 and Suppl. Mat. F1). Fermentations
345 were carried out at 20°C and were monitored by measuring the sugar content until
346 constant values were reached for 3 consecutive days. Table 1 shows the fermentation
347 parameters calculated for all the evaluated strains, including the maximum fermentation
348 rate (K), the time required to consume 50% w/v of the total sugars ($t_{50}$)and the time
349 needed to reach 2% w/v of the residual sugars($t_2$),as described in Materials and Methods
350 section.

351 Although no differences between both parental strains were detected in both the K and
352 $t_{50}$ parameters, Sc1 parental was unable to complete fermentation and showed an
353 estimated $t_2$ that was more than twice as high as Sc2 (Table 1).

354 As a general trend, no differences in the fermentation parameters were observed
355 between the hybrids obtained by rare-mating and those obtained by spore-to-spore
356 mating (Table 1). Strain R2 Io obtained the highest K value among the hybrids, higher
357 than the values obtained for both parental strains. Hybrids R2 IIIa and R2 IVo gave a
358 higher *K* value than parental Sc2,but no differences with parental Sc1were seen (Table
359 1).The same three hybrid strains (R2 Io, R2 IIIa and R2 IVo) achieved the lowest values
360 for $t_{50}$, although only hybrid R2 Io exhibited significant differences for this value as
361 compared to both parental strains (Table 1). Finally, strains R2 Io and R2 IVo also
362 showed the lowest $t_2$ values.

363 Strains R2 IIIo and R8 IIIo displayed the same behaviour as Sc1, were unable to
364 complete fermentation, and their estimated $t_2$ values were higher than 42 days (Table 1),
365 according these data these strains suffered a stuck fermentation as was indicated in the
366 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 Sc1showed 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 2g/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 Figure 1. The aim of this selection step was to compare the production of mannoproteins (because the only polysaccharides presents 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 (67.1mg/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

392 strains released significantly bigger amounts of polysaccharides than both the parental
393 strains (Figure 1). The remaining two hybrid strains, R8 VIo and R8 VIIo, released a
394 similar amount of polysaccharides to parental Sc2. The maximum polysaccharides
395 content was detected in the medium inoculated with hybrid R2 IVo (100 mg/L). This
396 value represents an increase of around 1.5 times as compared to the values obtained
397 with parental Sc2, and of around 2 times if compared to parental Sc1.
398 To confirm that the total amount of polysaccharides was in accordance with the
399 presence of the mannoproteins in the medium, we carried out the specific detection of
400 mannoproteins in fermented synthetic musts using peroxidase-conjugated concavalin A.
401 As a general rule, the results obtained with this methodology confirmed those obtained
402 by the quantification of total polysaccharides. Even though this is a qualitative detection
403 method, our results clearly demonstrate that most hybrids released a larger amount of
404 mannoproteins than the parental strains.
405 Comparing in each gel the intensity of the bands of the hybrids versus the parental
406 strains, the fermentations carried out by hybrids R8IIa and S7 gave the largest amount
407 of mannoproteins, followed by those obtained with hybrids R2 Io, R2 IIo and R2 IVo
408 (Figure 2). Hybrids R2 Io and R2 IIo produced slightly different mannoprotein bands
409 patterns from those produced by the parental strains and the remaining hybrids (Figure
410 2). Finally, the amount of mannoproteins released by hybrid strains R8 VIo and R8 VIIo
411 was similar to that released by the other hybrids, which evidences similar mannoprotein
412 profiles (Figure 2). Nonetheless, these two hybrids produced a smaller amount of total
413 polysaccharides than the other hybrid strains (Figure 1).
414 Based on the results obtained from the total polysaccharides and mannoprotein release,
415 we selected hybrid strains R2 IVo, R8 IIa and S7 to evaluate their capacity to increase
416 the stabilisation of a white wine against protein haze.

417

418 *3.3 Protein haze stability of the wines fermented by the higher mannoprotein producer*
419 *hybrids.*

420 Fermentations of Sauvignon Blanc grape must were carried out with the three higher
421 mannoprotein producer hybrids and the two parental strains to evaluate the effect on
422 wine stability of the mannoproteins produced by each different strain. Chemical
423 analyses of the wines evidenced that parental Sc1 and hybrid S7 were unable to
424 consume all the fructose that was originally present in the must, and they left as much as
425 6.4 and 5.6 g/L of residual fructose, respectively (data not shown). After fermentation,
426 wines were subjected to the heat test for protein stability before and after bentonite
427 fining, as described in the Materials and Methods. Turbidity values close to 75 nefelos
428 (nephelometric turbidity units) were obtained for the wines fermented with Sc1, Sc2 and
429 R2 IVo without the addition of bentonite, while higher values were observed for hybrids
430 R8 IIa and S7 (Figure 3). R2 IVo and the R8 IIa hybrid strains showed the best
431 stabilisation profiles, with R2 IVo seemed to require slightly less bentonite for complete
432 stabilisation. The results for R8 IIa and Sc1 reveal lack of correlation between protein
433 instability before bentonite stabilisation and the response of the corresponding wine to
434 bentonite fining. Although no clear differences were obtained with this approach, we
435 can conclude that R2 IVo was the best in this test and was also the strain that produces
436 maximum levels of polysaccharides, for this reason this hybrid was selected for further
437 analysis.

438

439 *3.4 Measuring of the mannoprotein production in Verdejo fermentations.*

440 To ensure that R2 IVo hybrid produce higher amounts of mannoproteins than its
441 parental strains, we performed fermentation in Verdejo must. Fermentations were

442 carried out at 20°C and were monitored by measuring the sugar content until constant
443 values were reached for 3 consecutive days. Table 2 shows the fermentation parameters
444 calculated for all the evaluated strains, including the maximum fermentation rate (K),
445 the latency (l) and the time required to consume 95% w/v of the total sugars ($t_{95}$), as
446 well as the main chemical parameters (glucose, fructose, glycerol and ethanol). The
447 three strains finished the fermentation. Although Sc2 was the strain that showed the
448 higher Vmax and Sc1 and R2 IVo showed similar value of this parameter, the hybrid R2
449 IVo finished the process earlier than parental Sc1, indicating an improvement of the
450 fermentative capability.
451 As the natural must contains other polysaccharides different to mannoproteins and the
452 phenol sulphuric method detects polysaccharides in general, we used the methodology
453 described by Quirós et al (2012) in order to analyse the amount of mannoproteins
454 released by the selected strains. The results are shown in Figure 4. Sc2 was the strain
455 that lower amount of mannoproteins produced (~123mg/L), followed by Sc1. The
456 hybrid R2 IVo produced, statistically, more mannoproteins than both of its parental
457 strains (~157mg/L).
458 As a resume, hybrid strain R2 IVo exhibited good fermentative behaviour in both
459 synthetic and natural grape musts (Table 1 and 2; Suppl. Mat. F1), and released large
460 amounts of mannoproteins and polysaccharides that seem related with protection of
461 wine against protein haze (Figure 1, 2, 3 and 4). This strain seems to have inherited the
462 positive physiological features from each parental strain. In order to characterize the
463 potential genomic changes that may have occurred during hybrid generation and
464 stabilisation, and which could be related with the improved physiological features of
465 this strain, we performed array-comparative genomic hybridisation (CGH).
466

467 *3.5 Comparative genomic hybridisation analysis of hybrid R2 IVo and the parental*
468 *strains*

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

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

489 Parental Sc1 was characterised by an overrepresentation of the genes typically found in
490 wine yeast strains(Carreto et al., 2008), such as *MAL11*, *MAL13*, *CUP1*-1 and *CUP1*-2
491 (Figure 5 and Suppl. Mat. Table 2). This parental strain, characterised by its ability to

492 produce and release mannoproteins also displayed an overrepresentation of some of the
493 genes involved in oligosaccharides metabolism and processing (e.g., *SPR1),* which
494 codify for a glucan 1,3-beta glycosidase), *SWP1* (dolichyl- diphosphooligosaccharide
495 protein glycotransferase) and *IMA1* (a α-1,6-glucosidase).
496 The gene onthology (GO) analysis was carried out with the overrepresented genes
497 detected in each particular parental strain and the significant GO terms obtained were
498 sorted according to their corresponding GO categories (Suppl. Mat. Table 3). According
499 to that analysis, the terms related to disaccharides and oligosaccharides metabolism
500 were significantly overrepresented in parental strain Sc1,while terms related to
501 transposition were associated with parental Sc2 (Suppl. Mat. Table 3).
502 The comparative analysis, which derived from the competitive hybridisation of hybrid
503 R2 IVo versus each parental strain, evidenced that the hybrid maintained the copy
504 number of one parental strain or the other for several genes. The hybrid did not show
505 genes significantly overrepresented in relation to the two parental strains. However we
506 observed significantly overrepresented in the hybrid with regards to Sc1 (genes in red in
507 R2-IVo in Figure 5) that do not show differences in copy numbers with Sc2, indicating
508 that probably Sc2 has an intermediate copy number between Sc1 and R2-IVo for these
509 genes. Then, the hybrid possesses more copies of these genes than the two parentals.
510 The same explanation could be associated with genes in blue in R2-IVo in Figure 5,
511 with genes significantly overrepresented in the hybrid with regards to Sc2. According to
512 the data shown in Figure 5, the hybrid R2-IVo presented 25 overrepresented ORFs
513 against Sc1 and 65 different genes overrepresented against Sc2 (Figure 5 and Suppl.
514 Mat. Table 4). Both strains Sc1 and the hybrid shared nine overrepresented ORFs,
515 which included five annotated genes (*CUP1*-1 and 2, *RMD6*, *HXT15* and *SEO1*).
516 However, 18 ORFs, including six annotated subtelomeric genes (*HXT9*, *HXT11*, two

517 ORFs of *HXT12*, *FSP2*, *REE1* and *BSC3*) and eight genes corresponding to transposons,
518 were commonly overrepresented in both the hybrid and parental strain Sc2 (Figure 5
519 and Suppl. Mat. Tables 2 and 4).
520 Apart from the overrepresented ORFs shared between the hybrid and parental strains,
521 the hybrid exhibited 7 and 56 genes in significantly higher copy numbers than Sc1 and
522 Sc2, respectively (Figure 5 and Suppl. Mat. Table 4). In particular, those genes involved
523 in cell wall organisation and maintenance, like the endopeptidase coding gene YPS7
524 and the gene coding for α-1,6-mannosyltransferase *MNN10,* had significantly higher
525 copy numbers in the hybrid than in parental Sc2. No differences between hybrid and
526 Sc1 were observed for these ORFs, indicating a similar copy number between these two
527 mannoprotein higher producer strains.
528 In this case, the GO analysis was separately performed with the ratio data obtained from
529 the hybridisation of the hybrid versus parental Sc1 and parental Sc2 (Suppl. Mat. Table
530 3). According to this analysis, the terms related to transposition were also significantly
531 overrepresented in the hybrid as compared to parental Sc1, as were some other terms
532 related to carbohydrate metabolism and glycosidase activity (Suppl. Mat. Table 3). The
533 GO analysis done with the over/underrepresented genes between the hybrid and Sc2
534 evidenced an overrepresentation of the terms related to detoxification in the hybrid
535 genome.
536 If we consider its better fermentation performance, its greater mannoprotein release, and
537 its effects on protein haze protection, the R2 IVo hybrid strain proved to be the most
538 suitable strain for industrial purposes. These physiological properties may be related
539 with the genes of the HXT family (HXT9, HXT11, HXT12), which showed
540 significantly higher copy numbers in the hybrid and the strain Sc2. In addition, the
541 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 studied the expression of these three genes during fermentation. Results are shown in Figure 6 and Table S5.

Comparing the relative expression of *MNN*10 gene, of the same strain at different time point (Suppl. Mat. Table 5B) the hybrid R2 IVo maintained a high relative expression value at 24h and 55h, diminishing at 120h; Sc1 diminished its expression at 55h 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 point (Suppl. Mat. Table 5B), showed that the R2 IVo increased its expression values at 55h, Sc2 maintained it during all the experiment and Sc1decreassed its expression at 120h.

For *HXK*1 gene results (Figure 6C and Suppl. Mat. Table 5A and B) indicated that at 24h 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 55h the three strains increased their relative expression values, but all presented similar values. At 120h 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 24h.

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 (55h point) as is shown in Figure 6A and B. Whilst 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 (120h), see Figure 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 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; González-Ramos et al., 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; Sc2, with excellent fermentative performance at industrial level. The strains obtained by making full use of

591 these natural hybridisation processes do not face the regulatory and marketing
592 restrictions that GMO microorganisms do.
593 The literature frequently mentions that hybrids can inherit particular physiological
594 features in new combinations, which can be even higher than those of the parents. *S.*
595 *cerevisiae* x *S. kudriavzevii* interspecific hybrids can retain the fermentation vigour of *S.*
596 *cerevisiae* and the ability to produce particular aromatic compounds from *S.*
597 *kudriavzevii*; while *S. cerevisiae* x *S. uvarum* hybrids can display the capacity to
598 ferment at both low and high temperatures and to produce intermediate amounts of
599 minor fermentative compounds (Sipiczki, 2008). Most of the stable hybrids analysed in
600 this work give intermediate values between both parental strains for fermentation
601 kinetics parameters K, $t_{50}$ and $t_2$.In some cases, hybrids (particularly R2 Io, R2 IIIa and
602 R2 IVo) gave even higher K values and lower $t_{50}$ and $t_2$ values than parental Sc2, which
603 was selected for its excellent fermentative behaviour (Table 1).
604 Strain Sc1, selected for its high mannoprotein release capacity, gave the lowest values
605 of total polysaccharides produced (evaluated by the phenol/sulphuric method) when
606 compared with parental Sc2 and all the tested hybrids, in a synthetic must fermentation.
607 However, mannoprotein specific staining indicated similar or bigger mannoprotein
608 content for Sc1. These differences indicate that Sc2 could be releasing other
609 polysaccharides different to mannoproteins being the total mannoprotein release or the
610 mannoprotein/total polysaccharides ratio higher in Sc1, and that mannoproteins
611 releasing –instead of the total polysaccharides release- are better related to the
612 technological properties. It has been reported that not only the total amount of
613 mannoproteins, but also their specific kind, has been associated with beneficial activity
614 in wine(Moine-Ledoux and Dubourdieu, 1999; Waters et al., 1994). In this work, most
615 hybrids exhibited similar mannoprotein patterns to the parental strains. As we wanted to

616 improve parental traits, we selected for posterior analysis strains with similar bands but
617 with higher intensity than the ones showed by the parental strains.
618 In this work, we chose protein haze stabilisation as a model application to detect
619 interesting hybrid strains given its amenability to laboratory-scale experimentation.
620 These methods are based on the haze susceptibility of Sauvignon Blanc(González-
621 Ramos et al., 2009). Using this method we could see that the wine obtained with strain
622 R2 IVo responded considerably better to bentonite-fining treatments, although the
623 resolution of this method is not the best according our data. These results have been
624 confirmed with a quantitative method (Quirós et al., 2012) in Verdejo must. When
625 mannoproteins were quantified at the end of this fermentation, was revealed that Sc1
626 produced more mannoproteins than Sc2 (as was said by the producers). The selected
627 hybrid R2 IVo released more mannoproteins than both of its parental strains, indicating
628 that this trait was improved not only for the parental Sc2, but it was also improved with
629 respect to the parental Sc1.
630 Many studies have shown that extensive genome rearrangements and gene duplication
631 occur in organisms, particularly yeasts, during adaptation to changing environments.
632 These changes can partially explain the hybrid improvement achieved in this work. It is
633 well-known that microarrays data can be used to reflect such genome changes (Dunham
634 et al., 2002; Dunn et al., 2005; Peris et al., 2012). The experiments carried out to detect
635 specific alterations in the gene copy number in the selected hybrid, which might explain
636 some of the inherited physiological properties and hybrid improvement, evidenced a
637 number of overrepresented genes in the three strains compared (Sc1, Sc2 and R2 IVo).
638 The genes associated with cell wall organisation could be held responsible for the
639 increased ability of strains to produce and release polysaccharides. In our study, gene
640 *MNN10,* which codifies for a subunit of a Golgi mannosyltransferase complex, was

overrepresented in the hybrid genome if compared to parental Sc2, while 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 glucans 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 glycosilation 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 mannoproteins 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 (Figure 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.

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693 **5. Acknowledgements**

694 This work has been supported by grants AGL2012-39937-CO2 (01 and 02), and
695 AGL2009-07327 from the Spanish Government and FEDER to A. Querol, E. Barrio
696 and R. Gonzalez, respectively and to PROMETEO grant (Project
697 PROMETEOII/2014/042) from the Generalitat Valenciana to A.Q. L. P-T. and C. L.
698 wish to acknowledge the CSIC and the Spanish Ministry of Education and Science
699 (MEC) for an I3P fellowship and a postdoctoral contract, respectively. We are grateful
700 to Pilar Morales and Manuel Quirós for their help with mannoprotein characterisation
701 and to Ana Cristina Adam for her help with qRT-PCR assays.

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## Figure legends

**Figure 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 polysaccharides value.

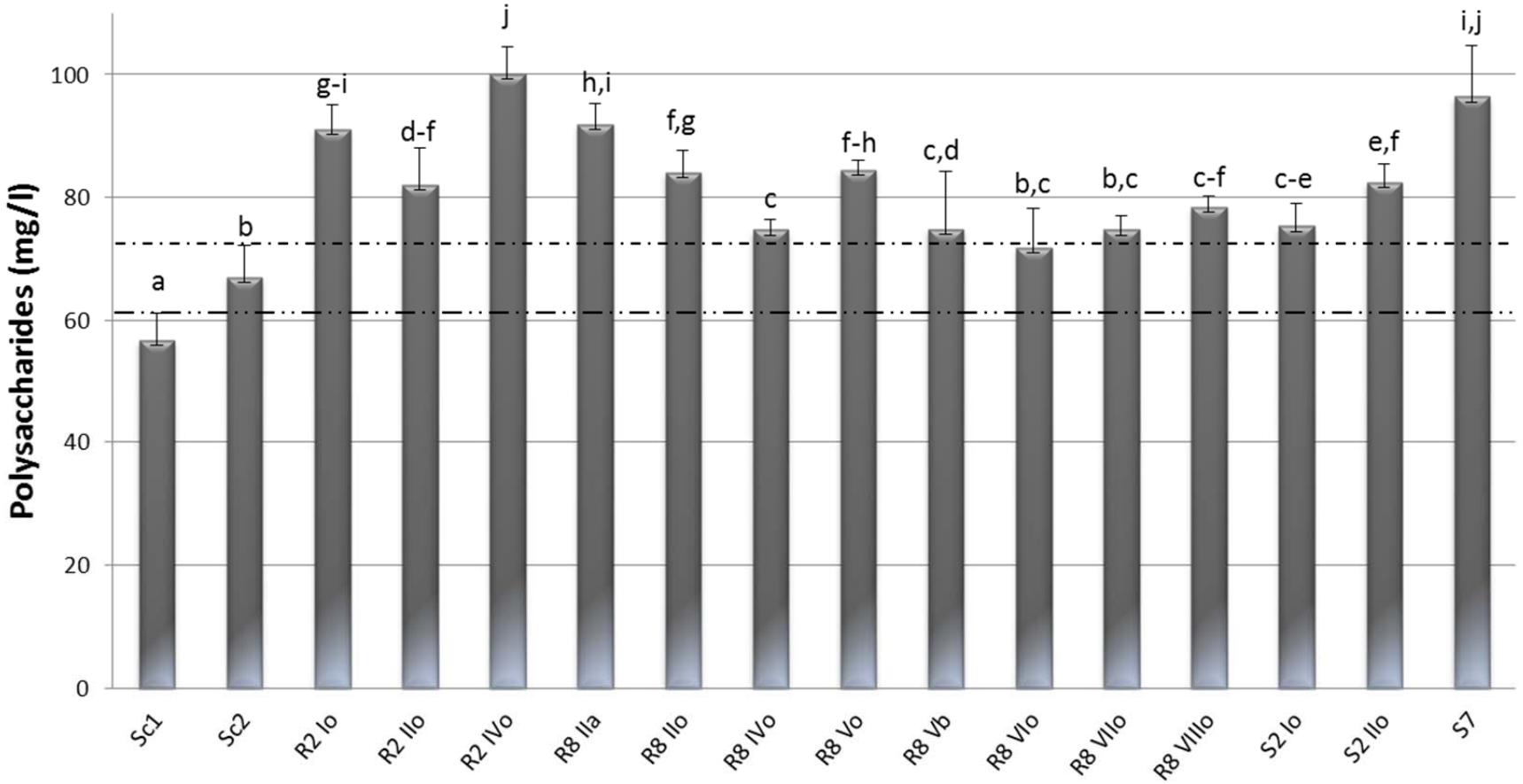
**Figure 2:** Mannoproteins released during fermentation of a Synthetic must by the hybrid strains compared to their parental. The identities of the strains are indicated in each panel. A and B: hybrids obtained by rare-mating methodology; C: hybrids obtained by spore to spore mating. Arrows in A indicate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

**Figure 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.

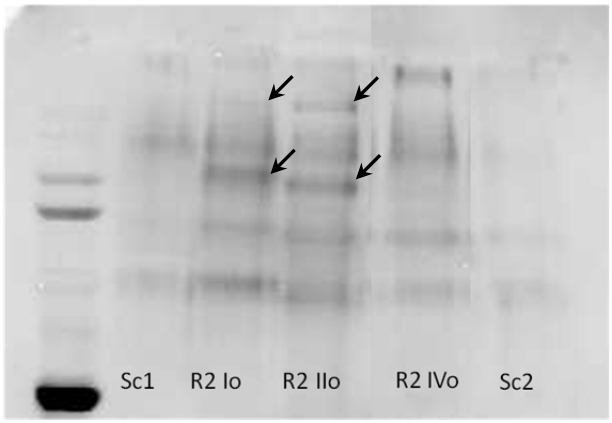
**Figure 4:** Final concentrations of released mannoproteins by hybrid, parental and control strains in verdejo must. Bars not sharing the same letter were significantly different according to one way ANOVA and Tukey test (α=0.05).

**Figure 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 regards to Sc2.

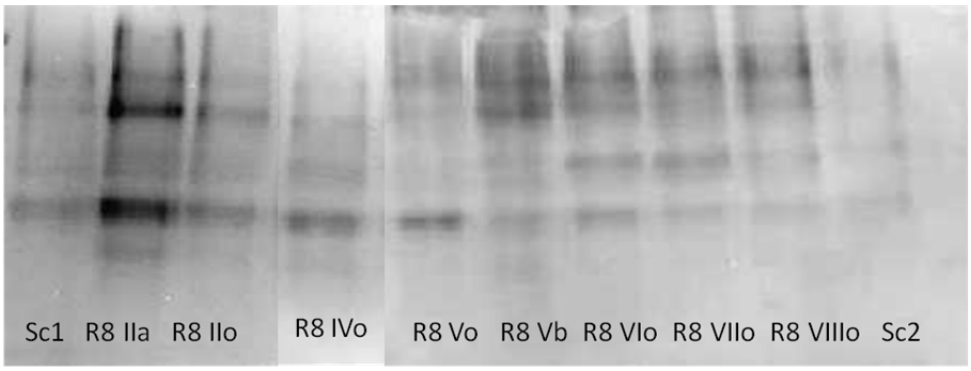
**Figure 6:** Relative expression of the genes A) *MNN*10, B) *YPS*7 and C) *HXK*1 during a fermentation. Expression of each one of the genes was related to the lower expression value obtained for this gene in all the experiment.


Polysaccharides (mg/l)
0
20
40
60
80
100
a
b
g-i
d-f
j
h,i
f,g
c
f-h
c,d
b,c
b,c
c-f
c-e
e,f
i,j
Sc1
Sc2
R2 Io
R2 IIo
R2 IVo
R8 IIa
R8 IIo
R8 IVo
R8 Vo
R8 Vb
R8 VIo
R8 VIIo
R8 VIIIo
S2 Io
S2 IIo
S7

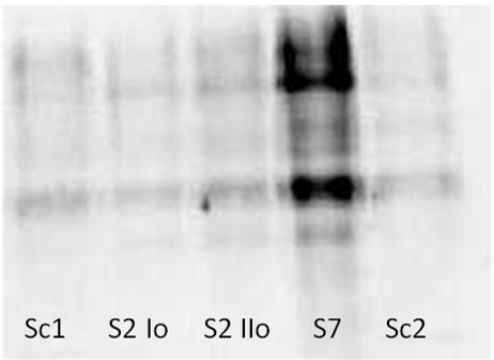
A

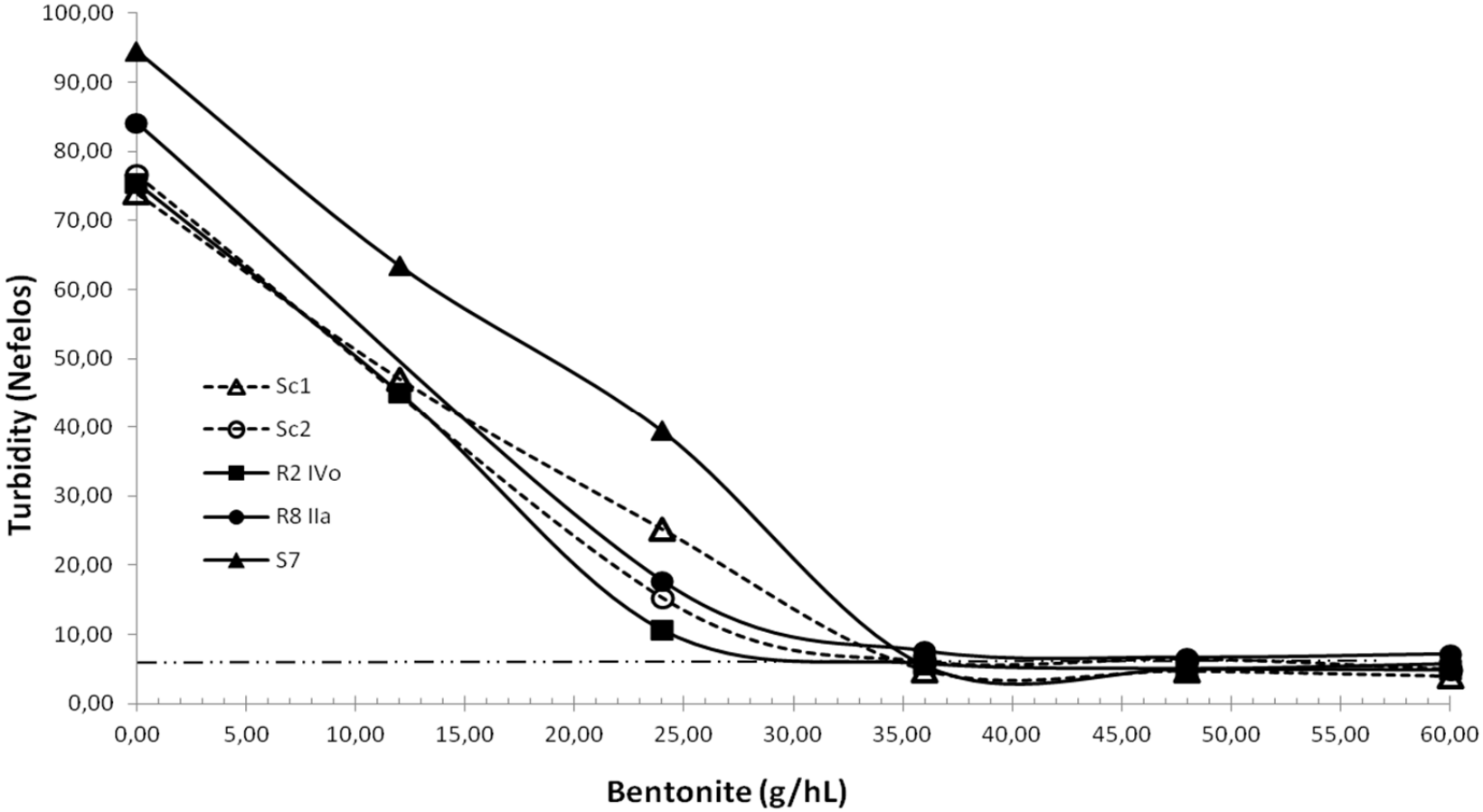

Sc1
R2 Io
R2 IIo
R2 IVo
Sc2

B


Sc1
R8 IIa
R8 IIo
R8 IVo
R8 Vo
R8 Vb
R8 VIo
R8 VIIo
R8 VIIIo
Sc2

C


Sc1
S2 Io
S2 IIo
S7
Sc2


100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
Turbidity (Nefelos)
Sc1
Sc2
R2 IVo
R8 IIa
S7
0,00
5,00
10,00
15,00
20,00
25,00
30,00
35,00
40,00
45,00
50,00
55,00
60,00
Bentonite (g/hL)

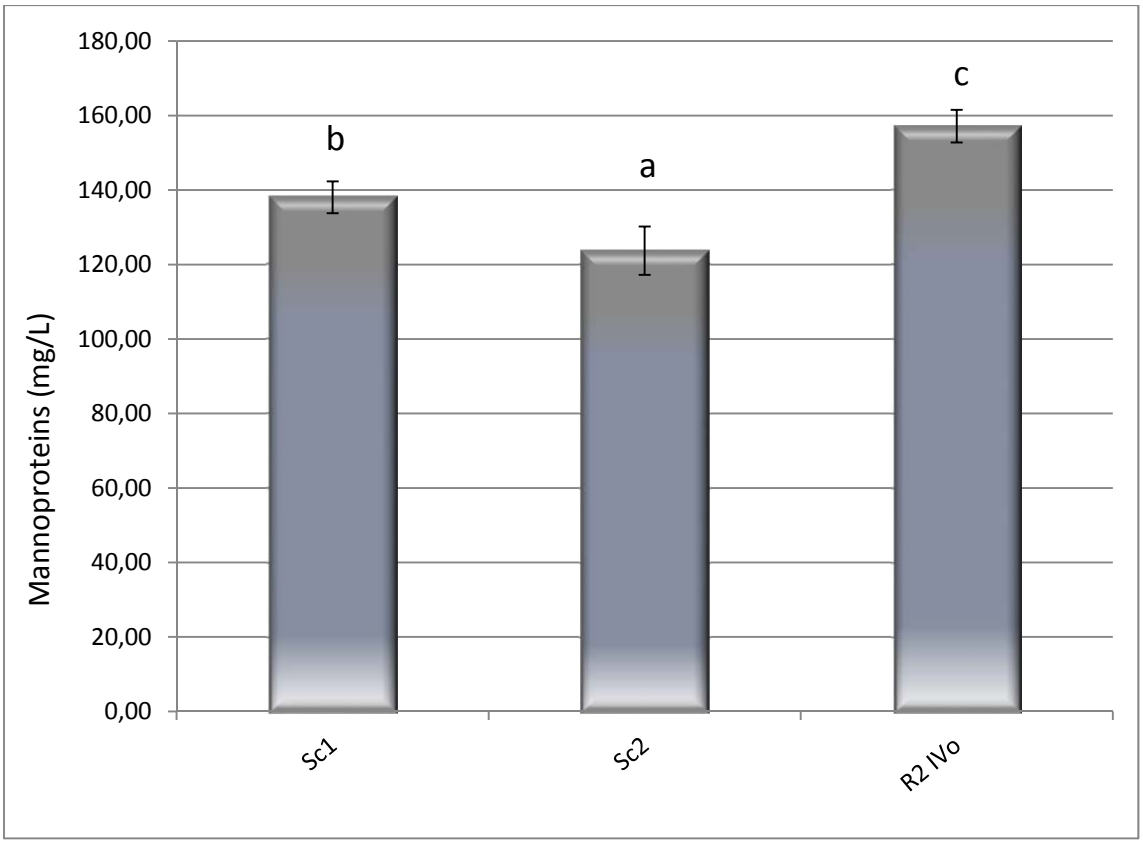
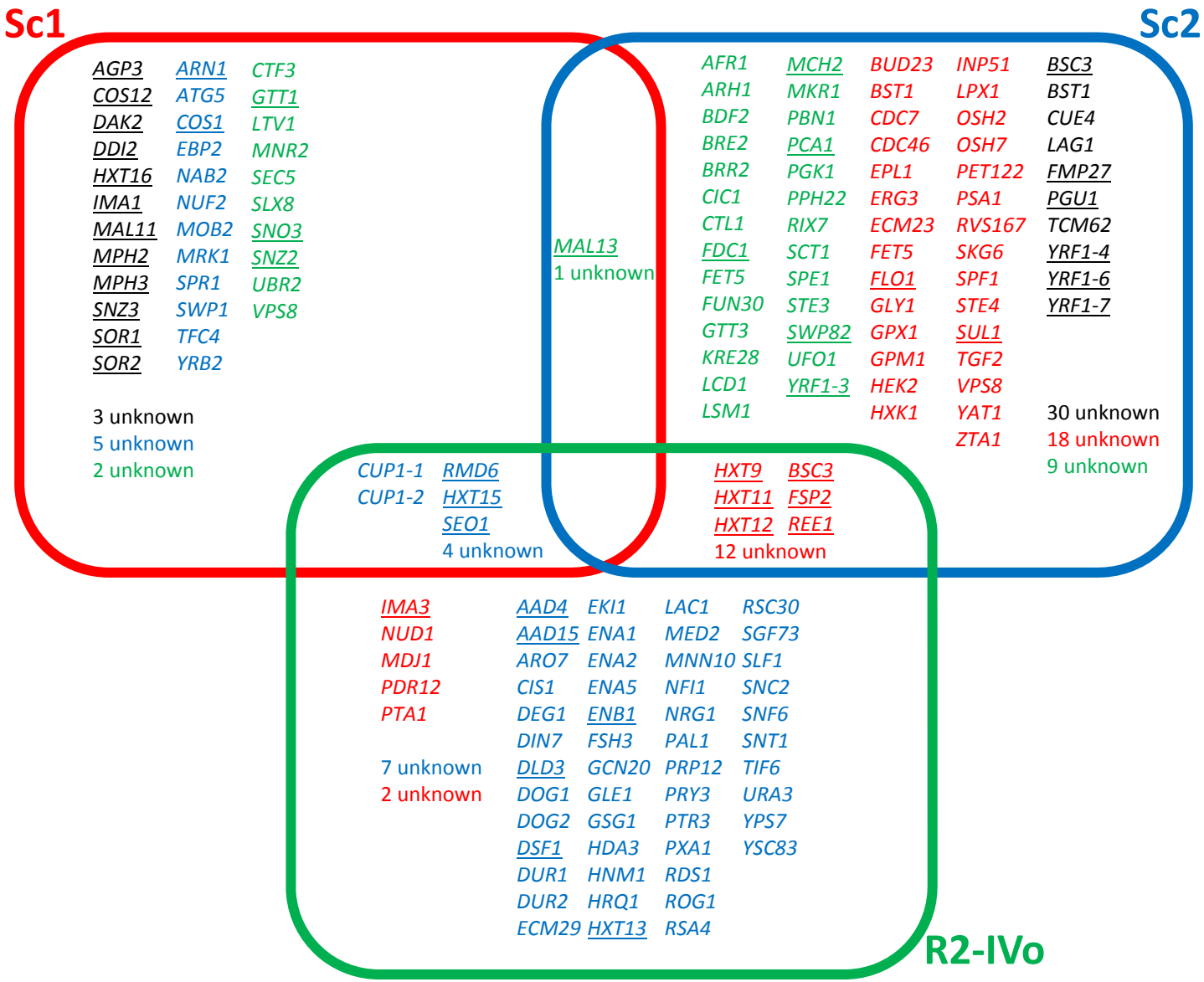
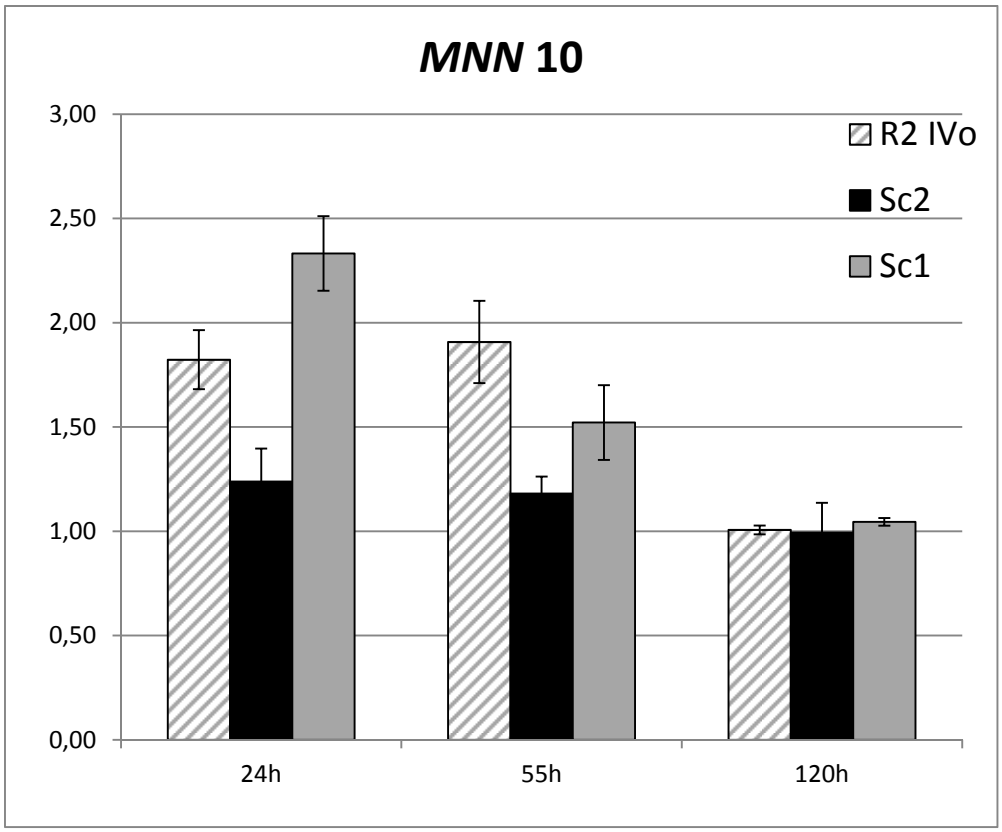

180,00
160,00
140,00
120,00
100,00
80,00
60,00
40,00
20,00
0,00
Mannoproteins (mg/L)
b
a
c
Sc1
Sc2
R2 IVo

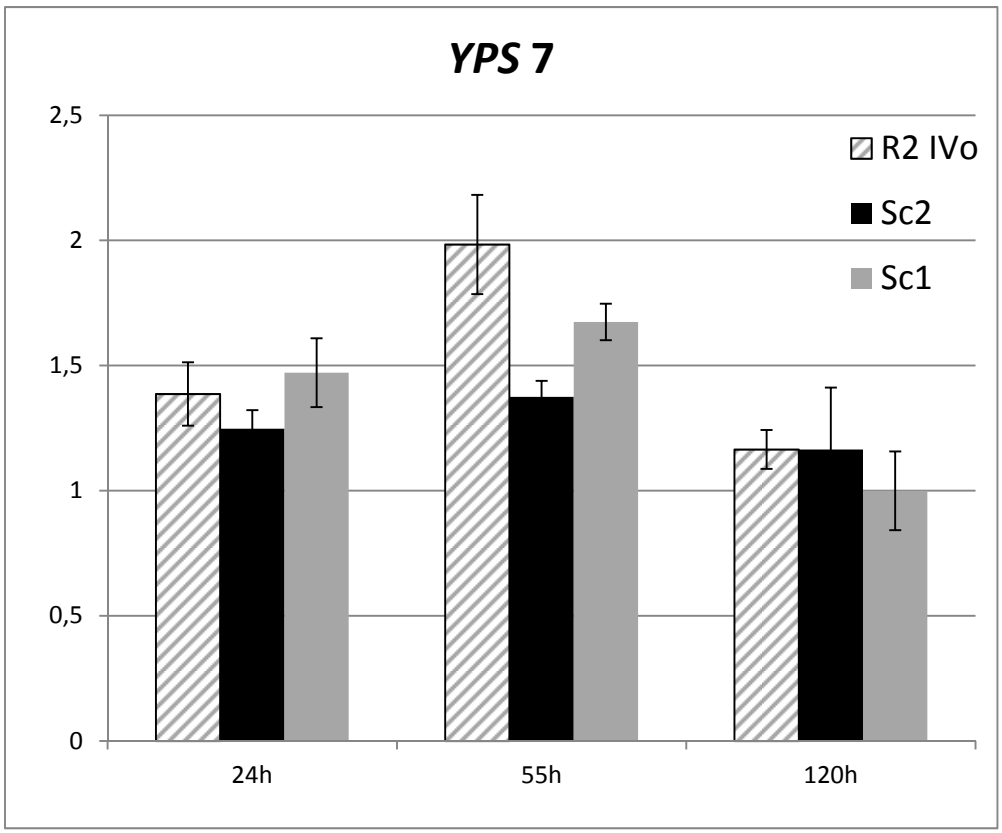
**Figure 4.**


Sc1
AGP3 ARN1 CTF3
COS12 ATG5 GTT1
DAK2 COS1 LTV1
DDI2 EBP2 MNR2
HXT16 NAB2 SEC5
IMA1 NUF2 SLX8
MAL11 MOB2 SNO3
MPH2 MRK1 SNZ2
MPH3 SPR1 UBR2
SNZ3 SWP1 VPS8
SOR1 TFC4
SOR2 YRB2
3 unknown
5 unknown
2 unknown
MAL13
1 unknown
Sc2
AFR1 MCH2 BUD23 INP51 BSC3
ARH1 MKR1 BST1 LPX1 BST1
BDF2 PBN1 CDC7 OSH2 CUE4
BRE2 PCA1 CDC46 OSH7 LAG1
BRR2 PGK1 EPL1 PET122 FMP27
CIC1 PPH22 ERG3 PSA1 PGU1
CTL1 RIX7 ECM23 RVS167 TCM62
FDC1 SCT1 FET5 SKG6 YRF1-4
FET5 SPE1 FLO1 SPF1 YRF1-6
FUN30 STE3 GLY1 STE4 YRF1-7
GTT3 SWP82 GPX1 SUL1
KRE28 UFO1 GPM1 TGF2
LCD1 YRF1-3 HEK2 VPS8
LSM1 HXK1 YAT1 30 unknown
ZTA1 18 unknown
9 unknown
CUP1-1 RMD6
CUP1-2 HXT15
SEO1
4 unknown
HXT9 BSC3
HXT11 FSP2
HXT12 REE1
12 unknown
IMA3 AAD4 EKI1 LAC1 RSC30
NUD1 AAD15 ENA1 MED2 SGF73
MDJ1 ARO7 ENA2 MNN10 SLF1
PDR12 CIS1 ENA5 NFI1 SNC2
PTA1 DEG1 ENB1 NRG1 SNF6
DIN7 FSH3 PAL1 SNT1
7 unknown DLD3 GCN20 PRP12 TIF6
2 unknown DOG1 GLE1 PRY3 URA3
DOG2 GSG1 PTR3 YPS7
DSF1 HDA3 PXA1 YSC83
DUR1 HNM1 RDS1
DUR2 HRQ1 ROG1
ECM29 HXT13 RSA4
R2-IVo

A)


MNN 10
3,00
2,50
2,00
1,50
1,00
0,50
0,00
R2 IVo
Sc2
Sc1
24h
55h
120h

B)


YPS 7
2,5
2
1,5
1
0,5
0
R2 IVo
Sc2
Sc1
24h
55h
120h

C)

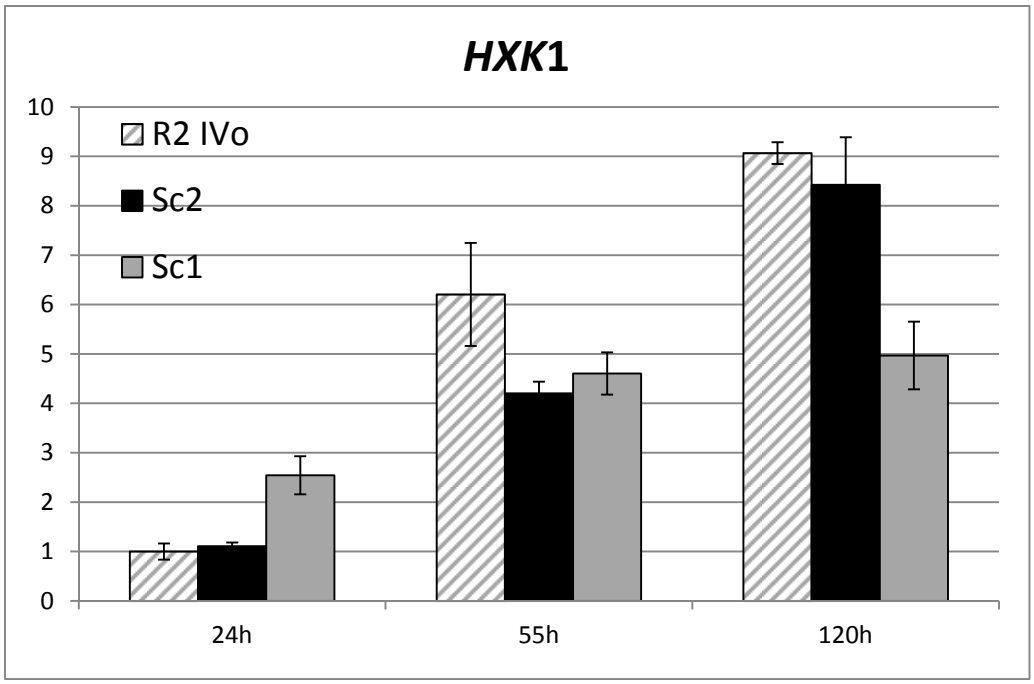


**Table 1:** Main kinetic parameters of the fermentations carried out with both parental and hybrid strains on synthetic must at 20°C and chemical analysis of the final fermented products.

| 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±0.004$^{d-f}$** | **6.76±0.05$^{b-f}$** | **stuck** | **0.38±0.11$^{a}$** | **9.2±0.78$^{f}$** | **5.18±0.11$^{a-d}$** | **12.13±0.05$^{d-f}$** |
| **Sc2** | **Parental** | **0.082±0.004$^{a-d}$** | **7.15±0.08$^{b-g}$** | **22.58±0.69$^{a.b}$** | **bdl** | **2.18±0.25$^{a}$** | **5.83±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±0.15$^{a-c}$ | bdl | 2.03±0.32$^{a}$ | 5.55±0.07$^{c-e}$ | 11.78±0.11$^{b-f}$ |
| R2 IIo | Rare-mating | 0.100±0.005$^{b-f}$ | 6.5±0.38$^{a-e}$ | 27.11±1.03$^{b-e}$ | bdl | 2.40±0.50$^{a}$ | 5.45±0.07$^{c-e}$ | 12.29±0.08$^{e.f}$ |
| R2 IIIa | Rare-mating | 0.117±0.005$^{e-g}$ | 5.89±0.29$^{a-c}$ | stuck | bdl | 5.18±0.39$^{c.d}$ | 5.55±0.07$^{c-e}$ | 12.69±0.02$^{f}$ |
| R2 IIIo | Rare-mating | 0.102±0.020$^{b-f}$ | 6.25±0.51$^{a-d}$ | stuck | bdl | 7.00±0.42$^{e}$ | 5.20±0.00$^{a-d}$ | 12.03±0.06$^{c-f}$ |
| **R2 IVo** | **Rare-mating** | **0.120±0.013$^{f.g}$** | **5.40±0.56$^{a.b}$** | **16.85±2.42$^{a}$** | **bdl** | **1.75±0.21$^{a}$** | **5.60±0.00$^{c-e}$** | **12.13±0.10$^{d-f}$** |
| R2 Vlo | Rare-mating | 0.104±0.006$^{c-f}$ | 6.81±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±0.05$^{f-h}$** | **27.21±3.49$^{b-e}$** | **0.31±0.44$^{a}$** | **2.38±0.30$^{a}$** | **5.63±0.13$^{c-e}$** | **11.77±0.41$^{b-f}$** |
| R8 IIo | Rare-mating | 0.080±0.008$^{a-d}$ | 8.06±0.59$^{e-h}$ | 32.89±2.42$^{d.e}$ | bdl | 2.37±0.68$^{a}$ | 5.67±0.07$^{c-e}$ | 12.36±0.18$^{f}$ |
| R8 IIIo | Rare-mating | 0.095±0.003$^{a-f}$ | 7.50±0.46$^{c-h}$ | stuck | bdl | 4.58±0.46$^{b.c}$ | 5.76±0.14$^{c-e}$ | 12.23±0.20$^{d-f}$ |
| R8 IVo | Rare-mating | 0.082±0.016$^{a-d}$ | 7.84±0.99$^{d-h}$ | 30.23±2.05$^{c-e}$ | bdl | 2.65±0.48$^{a}$ | 5.68±0.07$^{c-e}$ | 12.33±0.24$^{f}$ |
| R8 Vo | Rare-mating | 0.072±0.003$^{a-c}$ | 8.93±0.53$^{h}$ | 31.55±1.83$^{d.e}$ | bdl | 2.48±0.11$^{a}$ | 5.13±0.13$^{a-c}$ | 12.49±0.01$^{f}$ |
| R8 Vb | Rare-mating | 0.071±0.004$^{a.b}$ | 8.59±0.37$^{g.h}$ | 28.30±0.15$^{b-e}$ | bdl | 1.71±0.24$^{a}$ | 5.70±0.00$^{c-e}$ | 11.03±0.02$^{a-c}$ |
| R8 VIo | Rare-mating | 0.070±0.003$^{a.b}$ | 8.51±0.07$^{f-h}$ | 30.84±1.03$^{c-e}$ | bdl | 2.38±0.01$^{a}$ | 5.64±0.332$^{c-e}$ | 11.62±0.19$^{a-f}$ |
| R8 VIIo | Rare-mating | 0.071±0.006$^{a.b}$ | 8.33±0.39$^{f-h}$ | 30.22±1.04$^{c-e}$ | bdl | 2.00±0.11$^{a}$ | 6.09±0.19$^{e}$ | 12.23±0.16$^{d-f}$ |
| R8 VIIIo | Rare-mating | 0.086±0.001$^{a-e}$ | 7.55±0.16$^{c-h}$ | 33.18±3.89$^{e}$ | bdl | 2.58±0.62$^{a}$ | 5.36±0.24$^{b-d}$ | 11.19±0.29$^{a-d}$ |
| S2 Io | Spore to spore | 0.073±0.006$^{a-d}$ | 7.88±0.36$^{d-h}$ | 28.13±0.97$^{b-e}$ | bdl | 2.05±0.03$^{a}$ | 5.20±0.17$^{a-d}$ | 11.24±0.36$^{a-e}$ |
| S2 IIo | Spore to spore | 0.070±0.007$^{a.b}$ | 8.13±0.24$^{e-h}$ | 28.81±1.83$^{b-e}$ | bdl | 2.27±0.52$^{a}$ | 5.53±0.08$^{c-e}$ | 12.29±0.25$^{e.f}$ |
| **S7** | **Spore to spore** | **0.091±0.008$^{a-f}$** | **6.87±0.31$^{b-g}$** | **25.35±0.38$^{b-d}$** | **bdl** | **2.27±0.06$^{a}$** | **5.55±0.07$^{c-e}$** | **11.78±0.11$^{a.b}$** |

Ω- Extracted from Pérez-Través et al 2015

$- Values expressed as mean ± standard deviation. Values not shearing the same superscript letter within the column are significantly different (ANOVA and Tukey HSD test, α=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 (0,05g/L).

In **bold** are indicated those strains chosen to be used in the following selection steps.

Table S1. Primers used for qRT-PCR analysis.

| Gene | | Primer |
|---|---|---|
| **HXK1** | Forward | TCCAATGATTCCCGGTTGGG |
| | Reverse | ACCGCTCAACTTGACCAACA |
| **YPS7** | Forward | GACTTTCTGAGCCCAGCCTT |
| | Reverse | TCCACATAAGTGGCCGCAAT |
| **MNN10** | Forward | GCCTATGCGAAGAGACATGGA |
| | Reverse | GGAAACTCCCTGAACGTCTG |

Table S2. Genes upper represented in the parental strains Sc1 and Sc2 when a comparison between them is made.

| Strain | Gene | Function | Process |
|---|---|---|---|
| Sc1 | *AGP3* | amino acid transporter activity | amino acid transport |
| | *ARN1* | siderochrome-iron transporter activity | iron-siderochrome transport |
| | *ATG5* | unknown | protein-vacuolar targeting; autophagy |
| | *COS1* | | unknown |
| | *COS12* | | |
| | *CUP1-1* | copper ion binding | response to copper ion |
| | *CUP1-2* | | |
| | *DAK2* | glycerone kinase activity | glycerol catabolism; response to stress |
| | *DDI2* | unknown | unknown |
| | *EBP2* | unknown | rRNA processing |
| | *HXT15* | mannose transporter activity; fructose transporter activity; glucose transporter activity | hexose transport |
| | *HXT16* | | |
| | *IMA1* | hydrolase activity, hydrolyzing O-glycosyl compounds | unknown |
| | *MAL11* | alpha-glucoside:hydrogen symporter activity; maltose:hydrogen symporter activity; trehalose transporter activity | alpha-glucoside transport; trehalose transport |
| | *MOB2* | protein kinase activator activity | establishment and/or maintenance of cell polarity (sensu Saccharomyces); regulation of exit from mitosis; protein amino acid phosphorylation |
| | *MPH2* | carbohydrate transporter activity; maltose porter activity | carbohydrate transport |
| | *MPH3* | | |
| | *MRK1* | glycogen synthase kinase 3 activity | proteolysis and peptidolysis; protein amino acid phosphorylation; response to stress |
| | *NAB2* | poly(A) binding | poly(A)+ mRNA-nucleus export; mRNA polyadenylation |
| | *NUF2* | structural constituent of cytoskeleton | microtubule nucleation; chromosome segregation |
| | *RMD6* | unknown | unknown |
| | *SEO1* | transporter activity | transport |
| | *SNZ3* | protein binding | pyridoxine metabolism; thiamin biosynthesis |
| | *SOR1* | L-iditol 2-dehydrogenase activity | mannose metabolism; fructose metabolism |
| | *SOR2* | oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor | hexose metabolism |
| | *SPR1* | glucan 1,3-beta-glucosidase activity | sporulation (sensu Saccharomyces) |
| | *SWP1* | dolichyl-diphosphooligosaccharide-protein glycotransferase activity | N-linked glycosylation |
| | *TFC4* | RNA polymerase III transcription factor activity | transcription initiation from Pol III promoter |
| | *YRB2* | structural molecule activity | protein-nucleus export; nuclear pore organization and biogenesis; ribosomal protein-nucleus import; mRNA-binding (hnRNP) protein-nucleus import; snRNP protein-nucleus import; NLS-bearing substrate-nucleus import; tRNA-nucleus export; snRNA-nucleus export; |
| | *12 unknown* | | |
| Sc2 | BSC3 | unknown | unknown |
| | BST1 | unknown | vesicle organization and biogenesis; ER-associated protein catabolism |
| | BUD23 | S-adenosylmethionine-dependent methyltransferase activity | bud site selection |
| | CDC46 | chromatin binding; ATP dependent DNA helicase activity | pre-replicative complex formation and maintenance; DNA replication initiation; DNA unwinding; establishment of chromatin silencing |
| | CDC7 | protein serine/threonine kinase activity | protein amino acid phosphorylation; regulation of DNA replication; DNA replication initiation |
| | CUE4 | unknown | unknown |
| | ECM23 | unknown | cell wall organization and biogenesis; pseudohyphal growth |
| | EPL1 | histone acetyltransferase activity | regulation of transcription from Pol II promoter; histone acetylation |
| | ERG3 | C-5 sterol desaturase activity | ergosterol biosynthesis |
| | FET5 | ferroxidase activity | iron ion transport |
| | FLO1 | cell adhesion molecule activity | flocculation |
| | FMP27 | unknown | unknown |
| | FSP2 | alpha-glucosidase activity | |
| | GLY1 | threonine aldolase activity | glycine biosynthesis; threonine catabolism |
| | GPM1 | phosphoglycerate mutase activity | glycolysis; gluconeogenesis |
| | GPX1 | glutathione peroxidase activity | response to oxidative stress |

| Strain | Gene | Function | Process |
|---|---|---|---|
| Sc2 | HEK2 | mRNA binding | telomerase-dependent telomere maintenance; mRNA localization, intracellular |
| | HXK1 | hexokinase activity | fructose metabolism |
| | HXT11<br>HXT12<br>HXT9 | galactose transporter activity; mannose transporter activity; fructose transporter activity; glucose transporter activity | hexose transport |
| | INP51 | inositol-polyphosphate 5-phosphatase activity | dephosphorylation; endocytosis; cell wall organization and biogenesis; phosphatidylinositol biosynthesis |
| | LAG1 | protein transporter activity | replicative cell aging; ceramide biosynthesis |
| | LPX1 | lipase activity | peroxisome organization and biogenesis |
| | OSH2<br>OSH7 | oxysterol binding | steroid biosynthesis |
| | PET122 | translation regulator activity | protein biosynthesis |
| | PGU1 | polygalacturonase activity | pectin catabolism; pseudohyphal growth |
| | PSA1 | mannose-1-phosphate guanylyltransferase activity | GDP-mannose biosynthesis; protein amino acid glycosylation; cell wall mannoprotein biosynthesis |
| | REE1 | unknown | unknown |
| | RVS167 | cytoskeletal protein binding | polar budding; response to osmotic stress; endocytosis |
| | SKG6 | unknown | unknown |
| | SPF1 | ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism | calcium ion homeostasis; protein amino acid glycosylation |
| | STE4 | heterotrimeric G-protein GTPase activity | signal transduction during conjugation with cellular fusion |
| | SUL1 | sulfate transporter activity | sulfate transport |
| | TCM62 | chaperone activity | protein complex assembly |
| | TGF2 | general RNA polymerase II transcription factor activity | transcription initiation from Pol II promoter |
| | VPS8 | unknown | late endosome to vacuole transport |
| | YAT1 | carnitine O-acetyltransferase activity | alcohol metabolism; carnitine metabolism |
| | YRF1-4<br>YRF1-6<br>YRF1-7 | DNA helicase activity | telomerase-independent telomere maintenance |
| | ZTA1 | unknown | unknown |
| | 60 unknown | | |

Table S4. Genes overrepresented in the hybrid in CGH analysis.

| Comparison | Gene | Function | Process |
|---|---|---|---|
| R2 vs Sc1 | *BSC3* | unknown | unknown |
| | *FSP2* | alpha-glucosidase activity | |
| | *HXT9* | galactose transporter activity; mannose transporter activity; fructose transporter activity; glucose transporter activity | hexose transport |
| | *HXT11* | | |
| | *HXT12* | unknown | |
| | *IMA3* | oligo-1,6-glucosidase activity | disaccharide catabolic process |
| | *MDJ1* | co-chaperone activity | proteolysis and peptidolysis; protein folding |
| | *NUD1* | structural constituent of cytoskeleton | microtubule nucleation |
| | *PDR12* | organic acid transporter activity; xenobiotic-transporting ATPase activity | organic acid transport; propionate metabolism; transport |
| | *PTA1* | cleavage/polyadenylation specificity factor activity | tRNA processing; mRNA polyadenylation; mRNA cleavage; transcription termination from Pol II promoter, poly(A) independent; transcription termination from Pol II promoter, poly(A) coupled |
| | *REE1* | unknown | unknown |
| | *14 unknown* | | |
| R2 vs Sc2 | *AAD4* | aryl-alcohol dehydrogenase activity | aldehyde metabolism |
| | *AAD15* | | |
| | *ARO7* | chorismate mutase activity | aromatic amino acid family biosynthesis |
| | *CIS1* | unknown | regulation of CDK activity |
| | *CUP1-1* | copper ion binding | response to copper ion |
| | *CUP1-2* | | |
| | *DEG1* | pseudouridylate synthase activity | RNA processing |
| | *DIN7* | nuclease activity | DNA repair |
| | *DLD3* | D-lactate dehydrogenase (cytochrome) activity | lactate metabolism |
| | *DOG1* | 2-deoxyglucose-6-phosphatase activity | glucose metabolism |
| | *DOG2* | 2-deoxyglucose-6-phosphatase activity | response to stress; glucose metabolism |
| | *DSF1* | unknown | unknown |
| | *DUR1* | allophanate hydrolase activity; urea carboxylase activity | urea metabolism; allantoin catabolism |
| | *DUR2* | | |
| | *ECM29* | unknown | cell wall organization and biogenesis |
| | *EKI1* | choline kinase activity; ethanolamine kinase activity | phosphatidylethanolamine biosynthesis |
| | *ENA1* | ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism | sodium ion transport |
| | *ENA2* | | |
| | *ENA5* | | |
| | *ENB1* | ferric-enterobactin transporter activity | ferric-enterobactin transport |
| | *FSH3* | unknown | unknown |
| | *GCN20* | unknown | regulation of translational elongation |
| | *GLE1* | unknown | poly(A)+ mRNA-nucleus export |
| | *GSG1* | unknown | ER to Golgi transport; meiosis |
| | *HDA3* | histone deacetylase activity | regulation of transcription, DNA-dependent; histone deacetylation |
| | *HNM1* | choline transporter activity | choline transport |
| | *HRQ1* | ATP-dependent 3'-5' DNA helicase activity | DNA duplex unwinding; DNA strand renaturation |
| | *HXT13* | mannose transporter activity; fructose transporter activity; glucose transporter activity | hexose transport |
| | *HXT15* | | |
| | *LAC1* | protein transporter activity | aging; ceramide biosynthesis |
| | *MED2* | RNA polymerase II transcription mediator activity | transcription from Pol II promoter |
| | *MNN10* | alpha-1,6-mannosyltransferase activity | N-glycan processing; mannan metabolism; cell wall mannoprotein biosynthesis; actin filament organization |
| | *NFI1* | unknown | chromosome condensation |
| | *NRG1* | DNA binding; transcriptional repressor | regulation of transcription from Pol II promoter; |

| Comparison | Gene | Function | Process |
|---|---|---|---|
| | | activity | glucose metabolism; invasive growth (sensu Saccharomyces); response to pH |
| | *PAL1* | unknown | unknown |
| | *PRP12* | exonuclease activity | rRNA processing; mitochondrial genome maintenance |
| | *PRY3* | unknown | unknown |
| | *PTR3* | amino acid binding | chemosensory perception |
| | *PXA1* | ATP-binding cassette (ABC) transporter activity | fatty acid transport |
| | *RDS1* | transcription factor activity | response to xenobiotic stimulus |
| | *RMD6* | unknown | unknown |
| | *ROG1* | lipase activity | lipid metabolism |
| | *RSA4* | unknown | ribosomal large subunit assembly |
| | *RSC30* | DNA binding | regulation of transcription, DNA-dependent |
| R2 vs Sc2 | *SEO1* | transporter activity | transport |
| | *SGF73* | unknown | histone acetylation |
| | *SLF1* | RNA binding | regulation of translation; copper ion homeostasis |
| | *SNC2* | v-SNARE activity | vesicle fusion; endocytosis; Golgi to plasma membrane transport |
| | *SNF6* | general RNA polymerase II transcription factor activity | chromatin remodeling |
| | *SNT1* | NAD-dependent histone deacetylase activity; NAD-independent histone deacetylase activity | negative regulation of meiosis; histone deacetylation |
| | *TIF6* | unknown | processing of 27S pre-rRNA; ribosomal large subunit biogenesis |
| | *URA3* | orotidine-5'-phosphate decarboxylase activity | pyrimidine base biosynthesis |
| | *YPS7* | aspartic-type endopeptidase activity | unknown |
| | *YSC83* | unknown | |
| | *11unknown* | | |

Table S5. Homogeneous groups obtained in the expression analysis of *MNN*10, *YPS*7 and *HXK*1 genes.

A) Comparisons of the different strains at the same time point.

| Gene | *MNN*10 | | | *YPS*7 | | | *HXK*1 | | |
|---|---|---|---|---|---|---|---|---|---|
| | **R2IVo** | **Sc2** | **Sc1** | **R2IVo** | **Sc2** | **Sc1** | **R2IVo** | **Sc2** | **Sc1** |
| **24h** | b | a | c | a | a | a | a | a | b |
| **55h** | b | a | a,b | b | a | a,b | a | a | a |
| **120h** | a | a | a | a | a | a | b | b | a |

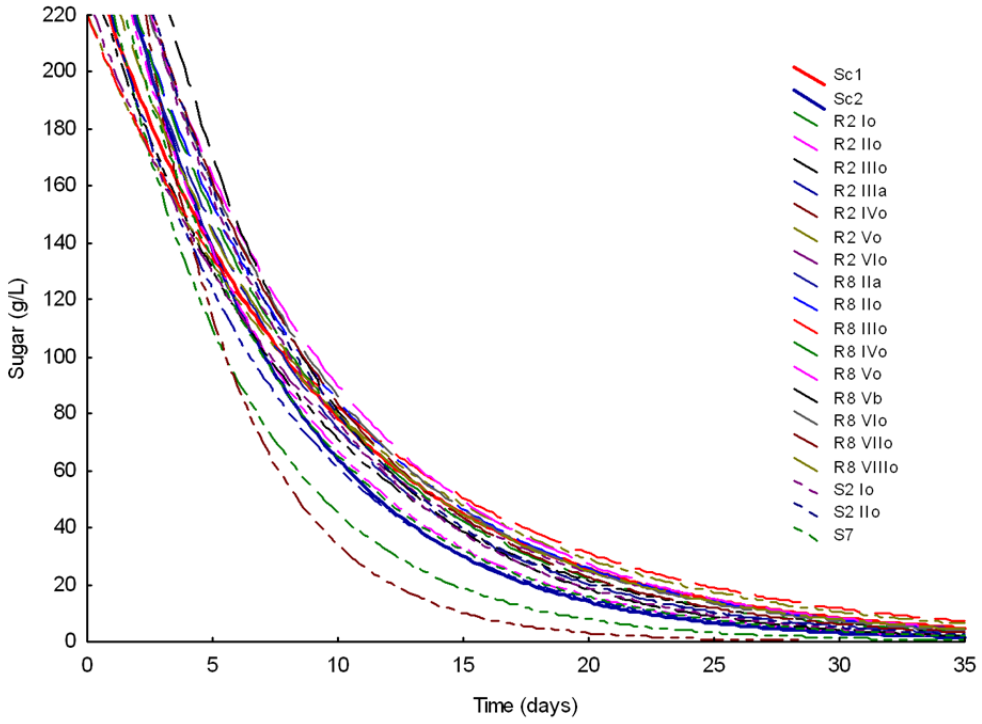
Homogeneous groups obtained by ANOVA and Tukey HSD test, α=0.05, n=3

B) Comparisons of the same strain at different time point.

| Gene | *MNN*10 | | | *YPS*7 | | | *HXK*1 | | |
|---|---|---|---|---|---|---|---|---|---|
| | **24h** | **55h** | **120h** | **24h** | **55h** | **120h** | **24h** | **55h** | **120h** |
| **R2IVo** | b | b | a | a | b | a | a | b | b |
| **Sc2** | a | a | a | a | a | a | a | b | c |
| **Sc1** | b | a | a | b | b | a | a | b | b |

Homogeneous groups obtained by ANOVA and Tukey HSD test, α=0.05, n=3

a)




b)

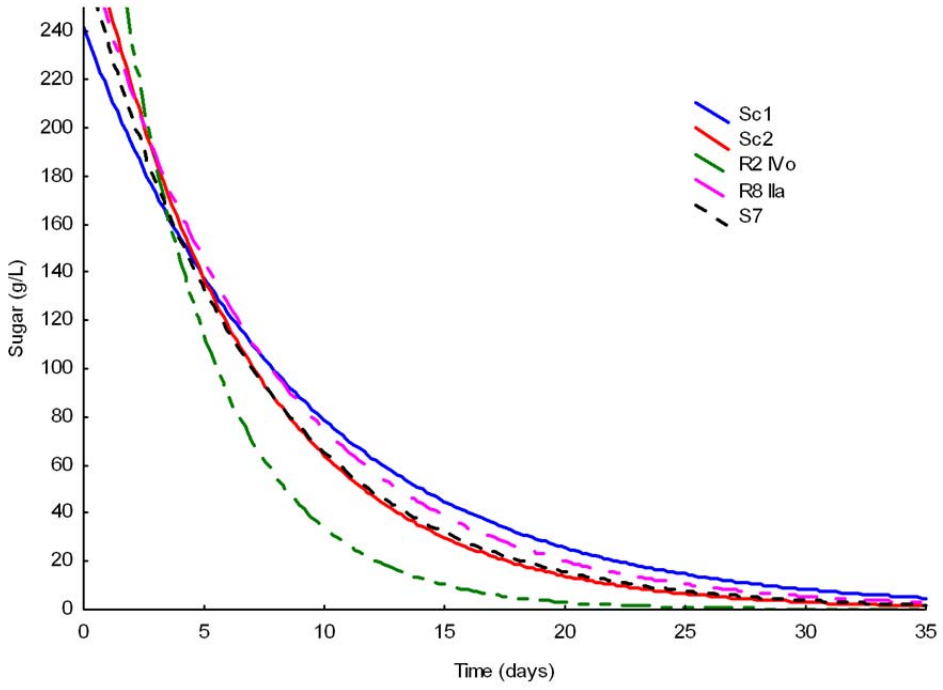



**Fig S1**.Sugar consumption in synthetic must. a) all the strains; b) selected and parental strains.