Increased mannoprotein content in wines produced by *Saccharomyces kudriavzevii x Saccharomyces cerevisiae* hybrids

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Abstract

Several wine quality aspects are influenced by yeast mannoproteins on account of aroma compounds retention, lactic-acid bacterial growth stimulation, protection against protein haze and astringency reduction. Thus selecting a yeast strain that produces high levels of mannoproteins is important for the winemaking industry. In this work, we observed increased levels of mannoproteins in *S. cerevisiae* x *S. kudriavzevii* hybrids, compared to the *S. cerevisiae* strain, in wine fermentations. Furthermore, the expression of a key gene related to mannoproteins biosynthesis, *PMT1*, increased in the *S. cerevisiae* x *S. kudriavzevii* hybrid. We showed that artificially constructed *S. cerevisiae* x *S. kudriavzevii* hybrids also increased the levels of mannoproteins. This work demonstrates that either natural or artificial *S. cerevisiae* x *S. kudriavzevii* hybrids present mannoprotein overproducing capacity under winemaking conditions, a desirable physiological feature for this industry. These results suggest that genome interaction in hybrids generates a physiological environment that enhances the release of mannoproteins.

Keywords: winemaking, *Saccharomyces*, hybrids, polysaccharides, *PMT1*

1. Introduction

The use of selected yeast starters is an essential modern practice in the winemaking industry as it allows the quality of wines to be maintained, and reduces problems like stuck fermentations and undesired contaminations. Wine yeast selection, including non-*Saccharomyces* species, has included the capacity of yeast strains to release mannoproteins (Caridi, 2006; Dupin et al., 2000; Domicio et al, 2014). These highly glycosylated proteins are present mostly in the yeast cell wall and have been associated with good quality and technological traits of wines, which include: retention of aroma compounds, increased body and mouthfeel, protection against protein and tartaric instability, stimulation of lactic acid bacteria growth, reduced astringency and foam quality improvement in mannoproteins (Caridi, 2006; Dupin et al., 2000). The mannoprotein content of wines can be technologically increased by adding enzymatic preparations, which enhance the release of mannoproteins, as SIHAZYM Fine™ enzyme (a pectolytic enzyme compound with secondary ß-glucanase activity, Eaton) and also by wine ageing with yeast lees (Juega et al., 2015) and other new techniques (Bychkov et al., 2010). However, these practices require careful management and are subjected to normative limitations to avoid off-flavours and wine spoilage due to excessive hydrolysis of the enzymes and possible contaminations after adding additives. An interesting alternative in this context is the use of selected yeasts that overproduce mannoproteins. However, it is a difficult complex character to be used as a selection criterion, especially for screening large numbers of strains. The strategies based on mutations of specific genes or improved recombinant strains for mannoprotein release have been developed (González-Ramos and González, 2006; González-Ramos et al., 2008; González-Ramos et al., 2009; Quirós et al., 2010). Nevertheless, the strictly regulated use of GMOs (Genetic Modified Organisms) in food applications, particularly

in wine, in most countries, and often consumer rejection, limit the practical usefulness of these approaches. In order to overcome this limitation, other non-GMO-producing methodologies can be used to generate wine strains that offer good fermentative features and release of mannoproteins.

In this work we explored the ability of natural hybrids of *S. cerevisiae* x *S. Kudriavzevii* to release mannoproteins. The promising results observed in released mannoproteins in microvinifications in natural hybrids *S. cerevisiae* x *S. Kudriavzevii,* prompted us to investigate the expression of *PMT1*, a recombinant gene involved in mannoprotein formation and presumably responsible for this increase. Finally we evaluated the mannoprotein release ability in *S. cerevisiae x S. kudriavzevii* artificial hybrids, which can be generated to obtain non-GMO desired strains.

2. Materials and methods

2.1. Yeast strains and growth media

Saccharomyces cerevisiae strain T73 (Querol et al. 1992), VRB and K1M (commercial strains from Lallemand S.A.S.) were used as wine yeast model strains. Type strain IFO1802 was used as the *S. kudriavzevii* representative strain and W27 as a natural *S. cerevisiae* x *S. kudriavzevii* hybrid. Artificial stable hybrids R1, R3, S5 and S8 were previously generated and stabilised (Pérez-Través et al., 2012). Artificial hybrids R11, R13, S15 and S44 were chosen from Pérez-Través et al., (2012) and were stabilised herein following the same methodology: each selected hybrid strain was individually inoculated in 15 mL of synthetic must (Salmon and Barre, 1998) and incubated at 20°C without shaking. After fermentation (approximately 15-20 days), an aliquot was used to inoculate a new tube that contained the same sterile medium and was incubated under the same conditions. After five successive fermentations, an aliquot of the fifth

89 fermentation was seeded on GPY-agar plates and incubated at 20°C. Ten yeast colonies
90 were randomly picked and characterised by mtDNA-RFLP, inter-δ sequences and
91 RAPD (using primer R3) analyses, as well as DNA content. Simultaneously, the same
92 colonies were inoculated in synthetic must and, after these individual fermentations, ten
93 colonies from each one were analysed by the same methods. We considered a
94 genetically stable hybrid when the colonies recovered after individual fermentation
95 maintained the same molecular pattern for the three markers and the same DNA content
96 as the previously inoculated (original) culture (Pérez-Través et al., 2012).
97 Yeast cells were maintained in GPY medium (2% glucose, 0.5% Bacto peptone and 0.5%
98 yeast extract) at 30ºC. Microvinifications were carried out in MS300 synthetic media to
99 simulate standard grape juice (Bely et al. 2003). Overnight precultures were inoculated
100 at 0.5×10^6 cells/ml density, measured by determining OD_{600}, in 100-ml bottles with gas
101 interchange with 100 ml MS300. Batch fermentations were performed at 20ºC with
102 gentle agitation (100 rpm) in triplicate. Sugar consumption was determined by DNS
103 method (Robyt and Whelan, 1972).
104
105 2.2. Quantification of polysaccharides in wine fermentations
106 For the indirect quantification of mannoproteins, we performed the method described by
107 Segarra et al. (1995), based on the determination of polysaccharides released from
108 mannoproteins. Monosaccharides, determined by DNS method (Robyt and Whelan,
109 1972), were removed from the cultures' supernatants by two gel filtration in Econo-Pac
110 columns (Bio-Rad, Alcobendas, Spain) following the manufacturer's recommendations.
111 The concentration of the total polysaccharides in the eluted fraction was determined
112 against a standard curve of commercial mannan (Sigma, Tres Cantos, Spain) according
113 to the phenol-sulphuric acid method using a UV Mini 1240 spectrophotometer

(Shimadzu). Five replicates were performed per determination. A standard curve of commercial mannan ranging from 20 to 250 mg/L was as follows:

mannan (mg/L) = (A490nm – 0.01) / 0.091

Total polysaccharides were normalized with cell dry weight calculated from the OD_{600} measured values.

2.3. Gene expression determination

Frozen cells were lysed and homogenised by vortexing 6 times in LETS buffer (10 mm Tris pH 7.4, 10 mM lithium-EDTA, 100 mM lithium chloride, 1% lithium lauryl sulphate) with acid-washed glass beads (0.4-0.6 mm; Sigma-Aldrich) for 30 alternating with ice incubation. Total RNA was extracted by the phenol:chloroform method. Purified RNA was converted into cDNA and the expression of the *PMT1* gene was quantified by qRT-PCR (quantitative real-time PCR). Primers were designed to amplify the genes from both species. Next, 1 mg of RNA was mixed with 0.5 mM dNTPs and 50 pmol Oligo(dT) in 10 ml. The mixture was heated to 65ºC for 5 min and quenched on ice. Ten mM dithiothreitol (DTT), 50 U of RNase inhibitor (Invitrogen) and 1x First Strand Buffer (Invitrogen) and water to 20 ml were added to the mixture, which was incubated at room temperature for 2 min. After adding 200 U Superscript III (Invitrogen), samples were incubated at 42ºC for 50 min and the reaction was inactivated after 15 min at 70ºC. Agarose gel electrophoresis was used to check for genomic DNA contamination. qRT-PCR was performed with gene-specific primers (200 nM) in a 20-µl reaction using Light Cycler FastStart DNA MasterPLUS SYBR green (Roche Applied Science, Germany) in a LightCycler 2.0 System (Roche Applied Science, Germany). All the samples were processed for the melting curve analysis, amplification efficiency and DNA concentration determination. A mixture of all the

139 samples and serial dilutions (10^{-1} to 10^{-5}) was used as a standard curve. The constitutive
140 *ACT1* gene expression was used to normalise the amount of mRNA. The average and
141 standard deviation of independent biological triplicates are represented.
142
143 2.6. Statistical analysis
144 All the experiments were performed at least in triplicate and the data are represented as
145 averages ± standard deviations. To statistically determine the significant data, Student *t*-
146 tests were performed with the Excel software using a *p*-value of 0.05.
147
148 3. Results
149 3.1. Increased levels of mannoproteins during winemaking in the *S. kudriavzevii x S.*
150 *cerevisiae* natural hybrid.
151 In this study we aimed to evaluate the capacity of *S. kudriavzevii x S. cerevisiae* hybrids,
152 used mainly in fermentations in north European regions due to his cryophilic behaviour,
153 to release mannoproteins. For this purpose, we performed microvinifications in
154 synthetic must with hybrid strain W27 and, representing strains of their parental species,
155 the *S. cerevisiae* T73 strain and *S. kudriavzevii* IFO1802. In the samples taken after the
156 50% sugar consumption (sample A; IFO1802 82,8 g/l, day 11; W27 75,9 g/l, day 9; T73
157 92, 8 g/L, day 11) and at the end of fermentation (sample B, residual sugars below 2 g/l
158 for all strains), we used the indirect method described in Quirós et al. (2011) to evaluate
159 the capacity of the different strains to release mannoproteins in wine media (Figure 1A).
160 The results showed that the *S. cerevisiae* strain produced intermediate levels of
161 mannoproteins after the 50% sugar consumption and that these levels were maintained
162 until the end of fermentation. The *S. kudriavzevii* strain presented a different pattern
163 since it released low levels of mannoproteins at the first time point (0.38 mg/mg dw),

which significantly increased at the last time point (0.65 mg/mg dw). Hybrid strain W27 presented significantly higher levels than those reached by the pure strains at both time points.

3.2 Differential expression of a protein O-mannosyltransferase gene *PMT1*

We have shown that there is a genomic recombination in strain W27, located in the promoter of the *PMT1* gene (Belloch et al., 2009), which encodes an enzyme that plays a key role in the mannosylation of proteins. Thus strain W27 contains a normal *PMT1 S. cerevisiae*-like allele and another recombinant allele with the *S. cerevisiae* coding region and the *S. kudriavzevii* promoter (Belloch et al., 2009). In order to check whether this genomic composition correlates with the mannoprotein release, we studied the regulation of this gene in the strains studied in this work during microvinifications (Figure 1B). Unexpectedly, the results show that *PMT1* gene expression was not activated after 3 or 14 days in the *S. kudriavzevii* IFO1802 strain, but showed activation in the *S. cerevisiae* T73 strain at both time points. In strain W27, *PMT1* was activated after 14 days of microvinification. These results argue against our hypothesis since the presence of a *PMT1* recombinant allele in W27 did not seem to drastically affect its gene expression regulation compared to the *S. cerevisiae* allele.

3.3. Increased levels of mannoproteins during winemaking in artificial *S. kudriavzevii x S. cerevisiae* hybrids.

We constructed artificial *S. kudriavzevii x S. cerevisiae* hybrids, which frequently contain alleles of each parental for every gene with no chromosomal recombinations (D. Peris, personal communication). Then we checked the genomic composition of *PMT1* alleles by PCR, as described in Belloch et al. (2009), and observed no recombinant

alleles, but at least one copy of each parental (results not shown). Next we performed microvinifications with all the strains (Figure 1C), including the natural and artificial hybrids, under the same above-described conditions and we measured mannoprotein release at the end of fermentation (Figure 1D). The results show that *S. kudriavzevii x S. cerevisiae natural* hybrid produced increased mannoprotein levels (1.52 mg/mg dw) compared to the *S. cerevisiae* strains (0.30-0.39 mg/mg dw). Compared to the *S. kudriavzevii* strain, artificial hybrids showed lower levels for some of them (S5, R13, S15 and S44) and higher levels for other strains (R1, R3, S8 and R12). Finally, we observed that the artificial hybrids showed significantly lower levels than natural hybrid W27. These results suggest that *S. kudriavzevii x S. cerevisiae* genomic hybridisation favors the release of mannoproteins under microvinification conditions and highlights the importance of the W27 *PMT1* recombination in this phenotype.

4. Discussion

Mannoprotein accumulation has been studied in recent years given several of these molecules' beneficial aspects in wine environments. Thus wine researchers have focused on finding yeast strains that have the ability to release large amounts of mannoproteins during winemaking. In this work, we found that *S. kudriavzevii x S. cerevisiae* hybrids produced increased amounts of mannoproteins in microvinifications compared to other *S. cerevisiae* standard strains. We also noted that this phenotype was observed in natural hybrids, which could indicate long-term adaptation and involves genomic recombinations in specific genes as *PMT1*, which increased expression is important to explain increased mannoprotein levels. However, we also found this phenotype in newly generated artificial hybrids with no genomic recombinations, which

suggests that the combination of both genomes is important for the ability to increase released mannoproteins.

Mannosylation of proteins is an essential evolutionary conserved posttranslational modification for cells. Many secretory proteins are mannosylated in the endoplasmic reticulum and glycosylated later in the Golgi. In yeast, there are six mannosyl transferases (dolichyl phosphate mannose-dependent protein O-mannosyltransferases, Pmt1p-6p) divided into three subfamilies. Pmt1p subfamily members (Pmt1p and Pmt5p) interact with the Pmt2p subfamily (Pmt2p and Pmt3p), Pmt1p-Pmt2p, and also with Pmt5p-Pmt3p heterodimers as predominant complexes, although distinct combinations and interactions with other complexes have been found (Hutzler et al., 2007; Nakatsukasa et al., 2004). A comparison of sequence similarity between the *S. cerevisiae* and *S. kudriavzevii* alleles gave values that ranged from 94.4% (*PMT1*) to 84.2% (*PMT6*). In contrast, the residues that have been described as being important for complex formation (Lommel et al., 2010) are all conserved. This suggests that interspecies mannosyl transferase complexes formation can be established and that they can possibly have new enzyme kinetics. This could well explain the mannoprotein accumulation results observed herein since these interspecies complexes could provide increased levels of mannoproteins, which could be finally released into winemaking media. Finally, it is interesting to highlight that the artificially generated interspecific hybrids generated by non-GMO techniques can be easily and directly transferred to the winemaking industry given the lack of consumers and legal drawbacks associated with genetically modified foods.

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Figure Legends

Figure 1. *S. cerevisiae, S. kudriavzevii* and hybrids polysaccharides level during winemaking and *PMT1* expression. A) Comparison of the released mannoproteins at the middle (50% sugar consumption (sample A)) or at the end of the fermentation (sample B) of a synthetic must for the *S. cerevisiae* T73 strain (white bars), the *S. kudriavzevii* IFO1802 strain (black bars) and the *S. cerevisiae* x *S. kudriavzevii* natural hybrid W27 strain (grey bars). In all cases, polysaccharide determination method (A and D) generated low variability among the biological replicates (below 10 %). B) mRNA

levels of the *PMT1* gene, measured during synthetic must fermentation for the three strains, after 3 or 14 days of fermentation. C) Sugar consumption profile during must fermentation for the strains: *S. cerevisiae* T73, VRB and K1M; *S. kudriavzevii* IFO1802; *S. cerevisiae* x *S. kudriavzevii* natural (W27) and artificial (R1, R3, R11, R13, S5, S8, S15 and S44) hybrid strains. The % of the total sugar content was significantly adjusted ($p<0.05$) to exponential (sigmoidal for ISO1802 and W27) curves. D) Comparison of released mannoproteins at the end of the fermentation for strains: *S. cerevisiae* T73, VRB and K1M (white bars); *S. kudriavzevii* IFO1802 (black bars); *S. cerevisiae* x *S. kudriavzevii* natural (W27) and artificial (R1, R3, R11, R13, S5, S8, S15 and S44) hybrid strains (grey bars).