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Influence of *Torulaspora delbrueckii* in varietal thiol (3-SH and 4-MSP) release in wine sequential fermentations

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ABSTRACT

In last years, non-*Saccharomyces* yeasts have emerged as innovative tools to improve wine quality, being able to modify the concentration of sensory-impact compounds. Among them, varietal thiols released by yeasts, play a key role in the distinctive aroma of certain white wines. In this context, *Torulaspora delbrueckii* is in the spotlight because of its positive contribution to several wine quality parameters. This work studies the physiological properties of an industrial *T. delbrueckii* strain, for the production of wines with increased thiol concentrations. *IRC7* gene, previously described in *S. cerevisiae,* has been identified in *T. delbrueckii,* establishing the genetics basis of its thiol-releasing capability. Fermentations involving *T. delbrueckii* showed improvements on several parameters (such as glycerol content, ethanol index, and major volatile compounds composition), but especially on thiols release. These results confirm the potential of *T. delbrueckii* on wine improvement, describing new metabolic features regarding the release of cysteinylated aroma precursors.

1. Introduction

Chemical composition of wines, and therefore, their sensorial characteristics, are strongly conditioned by components released by yeasts during alcoholic fermentation. Thus, the genetic and physiological diversity of oenological yeasts will be determinant in the aromatic properties of wine (Swiegers and Pretorius, 2007).

Saccharomyces cerevisiae, due to its importance in alcoholic fermentation, has been extensively studied to understand its role in wine properties, and how intraspecific genetic and physiological variations can modify the quality of wine (Pretorius, 2000). In this context, non-*Saccharomyces* yeasts, originally considered responsible for microbial-related problems in wine production, have been studied due to their physiological and metabolic abilities (Jolly et al., 2014) with a high enzymatic potential (Belda et al., 2016a), which may be useful in winemaking. They play a critical role during the early stages of fermentation by producing metabolites involved in the final chemical properties of wine.

Among the aromatic metabolites released by yeasts, sulfur-containing compounds are of great importance, with a strong influence in wine organoleptic properties because of their low detection thresholds. "Tropical volatiles thiols" stand out as the most important sulfur compounds in the varietal aroma of white wines. This group includes 4-methyl-4-sulfanylpentan-2-one (4-MSP), 3-sulfanylhexan-1-ol (3-SH) and its acetylated derivative 3-sulfanylhexyl acetate (3-SHA) (formerly, in order of mention: 4-mercapto-4-methylpentane-2-one (4-MMP), 3-mercaptohexan-1-ol (3-MH) and 3-mercaptohexyl acetate (3-MHA)) with detection thresholds of 3 ng/L, 60 ng/L and 4 ng/L, respectively (Swiegers et al., 2005).

It is known that 4-MSP and 3-SH exist in grapes in their non-volatile precursor forms, conjugated with cysteine (Tominaga et al., 1996) or glutathione (Fedrizzi et al., 2009). Carbon-sulfur lyase enzymes from yeasts are necessary to cleave the cysteine-glutathione conjugated precursor, releasing the correspondent volatile thiols (Dubourdieu et al., 2006). Therefore, the physiological properties of the yeast strain used to conduct the fermentation is a key factor affecting thiols release.

The genetic basis of thiols production from their cysteinylated and glutathionylated precursors are not clearly established (Cordente et al., 2015; Santiago and Gardner, 2015). However, it was demonstrated that a cystathionine-β-lyase enzyme, encoded by *IRC7* gene in *S. cerevisiae*,

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is determinant for cysteinylated (mainly cys-4MSP) precursor cleavage (Roncoroni et al., 2011; Thibon et al., 2008). Two alleles have been reported for *IRC7* gene: a full-length version and a 38-bp deletion form, the latter encoding for a less functional enzyme (Roncoroni et al., 2011). Most of *S. cerevisiae* strains have the deleted allele of the *IRC7* gene (Belda, et al., 2016b). In addition, under fermentation conditions, most *S. cerevisiae* strains are only able to release around 10% of the thiol precursors available in grape (Coetzee and du Toit, 2012), probably because of a low enzyme affinity for complex cysteinylated substrates and to Nitrogen Catabolism Repression (NCR), affecting both the genes responsible for the precursor assimilation (Subileau et al., 2008) and their later cleavage (Thibon et al., 2008).

β-lyase activity, as the main activity related to thiol release, appeared to be scarce in most *non-Saccharomyces* species, and also as a strain-dependent characteristic (Belda et al., 2016a,b; Zott et al., 2011). Some *Torulaspora delbrueckii* strains stand out because of their thiol production activity in wine fermentations, increasing the levels of 3-SH, but without any detectable effect in 4-MSP production (Renault et al., 2016; Zott et al., 2011).

Several studies showed the advantages of *T. delbrueckii* in winemaking (Azzolini et al., 2015), achieving low concentrations of acetic acid (Bely et al., 2008), reduced ethanol concentration and increased amounts of mannoproteins in wines, causing a remarkable impact on their mouthfeel properties (Belda et al., 2015), but also by affecting the overall aroma profile of wines enhancing the fresh fruit odor descriptors and decreasing the perception of vegetal flavours (Ramirez et al., 2016; Renault et al., 2015).

Because of the lack of genomic information available from non-conventional yeasts, studies about the genetic determinants of the role of non-*Saccharomyces* species to wine quality are scarce. Thus, this work aims to study the physiological properties of an industrial *T. delbrueckii* strain in white wine fermentations, unveiling the genetic basis of its contribution to volatile thiol production with incidence not only in 3-SH, but also in 4-MSP release.

2. Materials and methods

2.1. Yeast strains and molecular identification

S. cerevisiae Viniferm Diana, *S. cerevisiae* Viniferm Revelacion and *T. delbrueckii* Viniferm NS-TD were obtained from Agrovin S.A. (Alcázar de San Juan, Spain). To avoid differences between starter cultures, all the strains were grown in a predefined must-based medium (12.5% concentrated must (final concentration 50 g/L glucose + fructose), 1% yeast extract, 0.5% proteose peptone no.3, pH 3.5) at 25 °C for 48 h, for their later use in microvinifications, as described in Belda et al. 2015.

As a control of the culture purity, yeast strains were identified according to Kurtzman and Robnett (1997) by sequence analysis of the 26S large subunit rRNA gene (primers NL-1 5′-GCATATCAATAAGCGGAGGAAAAG-3′ and NL-4 5′-GGTCCG TGTTTCAAGACGG′). In the case of *S. cerevisiae* strains, their implantation during fermentation was certified by the PCR-amplification of the interdelta region (Legras and Karst, 2003) using delta12 (5′-TCAACAATGGAATCCCAAC-3′) and delta21 (5′- CATCTTAACACCGTATATGA-3′) primers.

For the study of the *IRC7*-orthologous gene in *T. delbrueckii*, the following yeasts from the Complutense Yeast Collection were used: *T. delbrueckii* NS-G-9 (GenBank accession number: KT922978.1), *T. delbrueckii* NS-G-27 (GenBank accession number: KT922996.1), *T. delbrueckii* NS-G-46 (GenBank accession number: KT923014.1), *T. delbrueckii* NS-G-62 (GenBank accession number: KT923030.1), *T. delbrueckii* NS-G-66 (GenBank accession number: KT923034.1), *T. delbrueckii* NS-G-71 (GenBank accession number: KT923039.1), *T. delbrueckii* NS-G-72 (GenBank accession number: KT923040.1), *T. delbrueckii* NS-PDC-169 (GenBank accession number: KT922633.1). Additionally, *S. cerevisiae* Viniferm PDM from Agrovin S.A., was used as a control for heterozygous *IRC7* alleles.

2.2. IRC7 genotype identification

S. cerevisiae Viniferm Diana and *S. cerevisiae* Viniferm Revelacion were used as homozygous strains for the short and long *IRC7* alleles, respectively. This was confirmed using the PF6 (5′-AGCTGGTCTGGAGAAAATGG-3′) and PR7 (5′-TCTTCTGCGAGACG-TTCAAA-3′) primers (Roncoroni et al., 2011) with the following PCR conditions: initial denaturing step of 2 min at 94 °C followed by 35 cycles of 94 °C for 15 s, 56 °C for 30 s and 72 °C for 1 min, and then a final extension step at 72 °C for 5 min. The PCR products were run on 2% agarose gels with an appropriate molecular weight marker and the size of the amplified products was checked.

In this work, the identification of the orthologous *IRC7* gene in *T. delbrueckii* was performed. Using the information available at GenBank-NCBI (www.ncbi.nlm.nih.gov/genbank/), we identified the putative *IRC7*-orthologous gene in *T. delbrueckii* (GenBank accession number: XM_003681410.1) and, based on that sequence, we designed the following primer pair for the study of the correspondent polymorphic region in this species: ITdF 5′-AGCTGGGCTCAAGGATATGC-3′ and ITdR 5′-GTGACTCTTGAGACGCTCAA-3′. The PCR reaction conditions were an initial denaturing step of 2 min at 94 °C followed by 35 cycles of 94 °C for 15 s, 57 °C for 30 s and 72 °C for 1 min, and then a final extension step at 72 °C for 5 min. The PCR products were run on 2% agarose gels with an appropriate molecular weight marker and the size of the amplified products was checked.

Clustal Omega software was used to carry out alignments of the protein sequences, and MUSCLE software was used to estimate phylogenetic distances between protein sequences, keeping default settings (Fig. S1).

2.3. Microvinifications and growth kinetics

All fermentations were prepared using the must from *Vitis vinifera* L.cv. Verdejo grapes and processed accordingly to the methods described in previous works (Benito et al., 2012). The must was frozen at − 30 °C for 6 months and then defreezed for its use in fermentations. The viability of indigenous yeasts was measured in the defreezed must, showing values under 10^3 viable cells/mL. The thiol precursors content in grape must was evaluated according to the method described by Roland et al. (2010) and the values were: 0,1 μg/L of Cys-4MSP; not detectable quantity of Glu-4MSP (limit of detection: 0,006 μg/L); 44 μg/L of Cys-3SH and 649 μg/L of Glu-3SH.

2.4. 3.5 L of débourbage fresh must were placed into 5 L glass fermentation vessels, leaving enough space for carbon dioxide emission. The composition of the medium was: 223 g/L sugar, 214 mg/L yeast assimilable nitrogen, 1.89 g/L malic acid and pH was 3.31

Four fermentation assays were performed, in static conditions, by triplicate: (1) inoculation with *S. cerevisiae* Viniferm Diana (ScD); (2) inoculation with *S. cerevisiae* Viniferm Revelacion (ScR); (3) sequential inoculation with *T. delbrueckii* followed by *S. cerevisiae* Viniferm Diana (TD…ScD) after four days, and; (4) sequential inoculation with *T. delbrueckii* followed by *S. cerevisiae* Viniferm Revelacion (TD…ScR) after four days.

Cultures of *T. delbrueckii* NS-TD were adjusted in order to reach an initial cellular concentration in must of 10^6 cells/mL, developing sequential cultures with an inoculum of 10^6 cells/mL of the *S. cerevisiae* strains at day 4. Growth kinetics were followed by plating 50 μL of the appropriate dilution on Sabouraud glucose agar with chloramphenicol (for total yeast counts) and lysine media (for non-*Saccharomyces* yeasts counts). Colonies were counted after growth at 30 °C for 48 h.

All fermentation processes were carried out at 20 °C. Once fermentation of sugars was completed (glucose + fructose concentration lower than 4 g/L), 50 mg/L of sulfur dioxide was added to the wine in potassium metabisulphite (Panreac, Barcelona, Spain) form. Then, they

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