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Selection and use of pectinolytic yeasts for improving clarification and phenolic extraction in winemaking

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ABSTRACT

Pectinase enzymes have shown a considerable influence in both, sensitive and technological properties of wines. They can help to improve clarification process, releasing more color and flavor compounds entrapped in grape skin, facilitating the liberation of phenolic compounds. This work aims to find yeasts that, because of their native pectinases, can be applied on combined fermentations with *Saccharomyces cerevisiae* obtaining significant benefits over single-inoculated traditional fermentations. 462 yeast strains isolated from wineries were identified and tested for several enzymatic activities of recognized interest for enology industry. Considering the 7 identified species, only *Aureobasidium pullulans*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola* showed polygalacturonase activity. Because of its interest in winemaking, due to its reported incidence in wine flavor, the impact of *M. pulcherrima* as a source of pectinolytic enzymes was analyzed by measuring its influence in filterability, turbidity and the increase on color, anthocyanin and polyphenol content of wines fermented in combination with *S. cerevisiae*. Among the strains screened, *M. pulcherrima* NS-EM-34 was selected, due to its polygalacturonase activity, for further characterization in both, laboratory and semi-industrial scale assays. The kinetics concerning several metabolites of enological concern were followed during the entire fermentation process at microvinification scale. Improved results were obtained in the expected parameters when *M. pulcherrima* NS-EM-34 was used, in comparison to wines fermented with *S. cerevisiae* alone and combined with other pectinolytic and non-pectinolytic yeasts (*A. pullulans* and *Lachancea thermotolerans*, respectively), even working better than commercial enzymes preparations in most parameters. Additionally, *M. pulcherrima* NS-EM-34 was used at a semi-industrial scale combined with three different *S. cerevisiae* strains, confirming its potential application for red wine improvement on the mentioned sensorial and technological properties.

1. Introduction

Current research in the wine industry pursues different objectives in agronomic, biochemical and microbiological aspects. The hallmarks of enological microbiology are informed by three different targets: the sensory, technological and fermentative properties of microbial strains. The enzymatic properties of the different microorganisms involved in the winemaking process have been studied for a long time (Van Rensburg and Pretorius, 2000; Belda et al., 2016).

Pectinase enzymes have a considerable influence on both the sensory and technological properties of wines (Merín and Morata de Ambrosini, 2015). They can help to improve the clarification and filtration process, releasing more of the color and flavor compounds contained in the grape skin, and facilitating the liberation of phenolic compounds (Van Rensburg and Pretorius, 2000). The addition of commercial enzyme preparations, with filamentous fungi as the main source, can be costly for industry. Within this context, researchers have focused their attention on the native pectinases of yeasts (Alimardani-Theuil et al., 2011; Merín et al., 2011, 2015; Pretorius, 2000). It has been reported that at least 50% of the *Saccharomyces cerevisiae* enological strains tested had limited pectinolytic activity (Fernández-González et al., 2004) There has recently been increasing interest in the application of non-*Saccharomyces* wine yeasts, but the ability these yeasts have to secrete efficient pectinases needs to be studied in depth.

Traditionally, the commercial pectinases used in winemaking comprise the mixtures of polygalacturonase, pectate lyase and pectin methylesterase enzymes (Lang and Dornenburg, 2000). Of these, two types of polygalacturonases, endo- and exo-polygalacturonase, are mainly responsible for pectinolytic activity, and hence are enzymes of particular importance to industry. Furthermore, cold-active pectinolytic enzymes have a number of potential advantages such as their functionality during the prefermentative cold soak process that contributes to the color and flavor stability of wines (Merín and Morata de Ambrosini, 2015).

Combined fermentations using non-*Saccharomyces* and *S. cerevisiae* strains, as sequential inocula in wine fermentations, have a significant impact on the sensorial properties of wines (Ciani et al., 2010; Fleet,

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2008; Lambrechts and Pretorius, 2000). Most studies have been developed at laboratory scale but scarcely validated on an industrial or semi-industrial scale, questioning their applicability at cellar (Jolly et al., 2014). Some enzymatic activities related to aroma enhancement (glycosidases and β-lyase for terpene and thiol release, respectively) and the release of some interesting products such as glycerol and mannoproteins, among others, are the highlights that justify the increasing interest in these mixed fermentations (Ciani et al., 2010; Rojas et al., 2001). In this context, combined fermentations are a very useful tool to improve wine fermentations in which aromatic complexity of spontaneous fermentations and the safety of industrial targeted fermentations are joined (Ciani et al., 2010; Romano et al., 2003). The wine industry is currently demanding new yeast strains in order to innovate and improve wine quality. Within this context, positive results in industrial assays with selected yeast strains have an added value, and may contribute to the deployment of non-*Saccharomyces* strains in the enology industry.

Since the incidence of *M. pulcherrima* on overall wine quality in combined fermentations has been described (Parapouli et al., 2010), modifying wine aroma by releasing high amounts of esters (Sadoudi et al., 2012) or decreasing ethanol content of wines (Contreras et al., 2015; Quirós et al., 2014) and also the potential use of its antimicrobial activity (Oro et al., 2014), the study of its pectinolytic activity to improve clarification and phenolic extraction has not been carried out yet.

This work aims to validate the industrial use of a selected *M. pulcherrima* strain that improves different aspects of wine quality, such as polyphenol and anthocyanin content, color intensity, turbidity or filterability.

2. Materials and methods

2.1. Isolation and molecular identification of yeast strains

Grape samples were collected from different districts in the Spanish Designation of Origin (DO) Ribera del Duero. Samples were taken from *Vitis vinifera* L.cv. Tempranillo grapes during the 2013 and 2014 harvests, at appropriate ripeness and in good sanitary conditions. After pressing, a suitably diluted aliquot of grape must was spread onto lysine agar medium (Oxoid) plates at 28 °C for 48 h. Four hundred and sixty-two yeast colonies were taken and restreaked on the same medium to obtain pure cultures. All the isolates were conserved at −80 °C and deposited in the Complutense Yeast Collection. These isolates were identified by partial sequencing of the 26S large subunit rRNA gene. Total genomic DNA was extracted using the isopropanol method (Querol et al., 1992), and the DNA for sequencing was amplified by using an Eppendorf Mastercycler apparatus, with forward NL-1 primer (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse NL-4 primer (5′-GGT CCG TGT TTC AAG ACG G-3′) (Kurtzman and Robnett, 1997). The sequences obtained were analyzed and compared by BLAST-search for yeast identification (BLAST; www.ncbi.nlm.nih.gov).

Six yeast strains, three *S. cerevisiae* and three non-*Saccharomyces*, were selected for the conducted trials of this study at a microvinification scale and an industrial scale. The *S. cerevisiae* strains were: *S. cerevisiae* CVA (Genbank accession number KT222660) and VRI (Genbank accession number KT222662) from CYC (Complutense Yeast Collection, Madrid, Spain) and Viniferm RVA (Genbank accession number KT222661) from Agrovin S.A., (Alcázar de San Juan, Spain). The non-*Saccharomyces* strains were: *Metschnikowia pulcherrima* NS-EM-34 (Genbank accession number KT222665), *Aureobasidium pullulans* NS-O-82 (Genbank accession number KT222663) and *Lachancea thermotolerans* NS-G-32 (Genbank accession number KT222664) from CYC.

2.2. Enzymatic characterization of yeast strains

The 462 yeast strains were screened for polygalacturonase, protease, cellulase and β-glucosidase activities. Polygalacturonase activity was determined in polygalacturonate agar medium containing 1.25% polygalacturonic acid (Sigma), 0.67% yeast nitrogen base (YNB, Difco), 1% glucose and 2% agar, adjusted to a final pH 3.5, as previously described (Strauss et al., 2001).

Protease activity was evaluated on YPD plates containing 2% skim milk powder (Sigma-Aldrich). The plates were incubated for five days at 30 °C. A clear zone around the colony identified protease activity (Strauss et al., 2001).

Cellulase production was determined on YPGE plates (containing 1% yeast extract, 2% peptone, 3% glycerol and 2% ethanol) with 0.4% carboxymethylcellulose, as previously described (Teather and Wood, 1982).

β-glucosidase activity was evaluated as reported by Villena et al. (2005), on a medium containing 0.5% cellobiose (4-O-β-D-glucopyranosyl-D-glucose), 0.67% yeast nitrogen base (Difco) and 2% agar.

2.3. Pectinolytic activity on microvinifications

A microvinification assay was conducted to confirm the pectinolytic activity of *M. pulcherrima*, in sequential fermentations combined with the commercial *S. cerevisiae* Viniferm RVA strain. *M. pulcherrima* NS-EM-34 and *A. pullulans* NS-O-82 strains were used as polygalacturonase active strains, and *L. thermotolerans* NS-G-32 as a negative control. These non-*Saccharomyces* strains were selected among the complete yeast collection analyzed due to their pectinolytic properties and reported enological usage (Jolly et al., 2014). Initial cellular concentrations in must were of about 10^6 cells/ml for every strain in sequential fermentations with an inocula ratio of 1:1. Forty-eight hours after the inoculation of non-*Saccharomyces* strains, *S. cerevisiae* Viniferm RVA was used to develop sequential fermentations.

Additionally, in order to compare with usual industrial practices, two commercial enzyme preparations, Enozym Clar and Enozym Lux (Agrovin S.A.) with high and medium polygalacturonase activity, respectively, were used as positive controls. The time of action of both enzymes was four hours prior to inoculation, according to the manufacturer instructions. After this time, *S. cerevisiae* RVA was inoculated. All assays were compared with a control assay inoculated solely with *S. cerevisiae* RVA.

Furthermore, two temperature conditions were evaluated in the assays; first, applying a controlled prefermentative cold soak (12 °C during the first 48 h, and 25 °C during the remainder of the fermentation) and, second, an assay at a constant temperature of 25 °C from the start without prefermentative cold soak.

The assays were conducted, in triplicate, by using 50 ml Falcon® tubes containing 40 g of Tempranillo crushed and destemmed grapes in their own juice. The cap was immersed daily during vinification to simulate winemaking procedures.

The Color Intensity (CI), Total Polyphenol Index (TPI) and Anthocyanin Content (AC) of the wines were determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) with a 1 cm path-length quartz cuvette. The CI value was calculated as the sum of the absorbances at 420, 520, and 620 nm (Glories, 1984). TPI was measured spectrophotometrically at 280 nm using wine 1/100 (*v*/*v*) diluted with distilled water (Ribéreau-Gayon et al., 2006). AC was analyzed by determining the absorbance at 520 nm of wine 1/100 (*v*/*v*) diluted with 1% (*v*/*v*) of HCl (Ruiz-Hernández, 2004).

Wine filterability was measured by filtration through a 0.22 μm filter (25 mm diameter) applying a vacuum force of 0.1 bars, as described by Haight and Gump (1994) with slight modifications, and expressed as the seconds needed to filtrate 1 ml of wine. Additionally, the turbidity of wines produced in microvinifications was evaluated by measuring the nephelometric turbidity units on a nephelometer (2100N Turbidimeter, Hach, Loveland, USA). All the experiments were conducted in triplicate.