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Spontaneous must fermentation: Identification and biotechnological properties of wine yeasts

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ABSTRACT

During the spontaneous alcoholic fermentation there is a succession of different strains over the course of winemaking. The aim of the present study was to select a yeast strain with appropriate enological qualities and adapted to the ecological environment of the cellar to be used as starter culture. For that purpose yeast strains were isolated at different stages of fermentation. A total of 240 *Saccharomyces* spp isolates were characterized by DNA mitochondrial restriction analysis, which delivered 21 different molecular profiles which were then evaluated.

Enological characteristics, nitrogen requirements, protein haze stabilization, and enzymatic activities were evaluated. Microvinifications were carried out with the finalist strains and chemical and sensory analyses were performed for selecting the most adequate *Saccharomyces* strain for that winery. Finally, two selected strains were used by the wine cellar in two consecutives vintages.

1. Introduction

Today, most wine is produced using selected commercial strains of *Saccharomyces* sp and even small wineries select yeasts from their own environment for use as starter cultures.

Certain criteria need to be met in order to guarantee the desirable features of the yeast strains selected. The most important of these are: tolerance to ethanol; exhaustion of sugar potential and high fermentation activity; growth at high sugar concentrations; resistance to, and low production of, sulfur dioxide; low production of hydrogen sulfide and low volatile acidity; resistance to killer toxin; good enzymatic profile (Nikolaou, Soufleros, Bouloumpasi, & Tzanetakis, 2006). All these characteristics should go together with adequate flavor wines (Lambrechts & Pretorius, 2000).

Even when these criteria are met, inoculated active dry yeasts occasionally fail to predominate during the fermentation process. Sometimes starter cultures – although correctly used – may be displaced by indigenous winery yeasts (Barrajón, Arévalo-Villena, Rodriguez-Aragón, & Briones, 2009; Capece et al., 2010). Therefore, it is advisable to use a starter obtained from the winery itself, provided that it has good winemaking properties and is able to guarantee alcoholic fermentation throughout the process (Suárez-Lepe & Morata, 2012).

Extensive ecological surveys using molecular methods of identification have been carried out with the aim of selecting new yeasts better adapted to local fermentation conditions.

The winery involved in this study is a family cellar, located at the heart of the Spain's most important viticulture and enological region. It elaborates high quality wines using grapes from its own 150-ha vineyard. It produces both white (Airén, Sauvignon Blanc, Chardonnay and Muscat) and red grape varieties (Tempranillo, Cabernet Sauvignon, Merlot, Syrah, Petit Verdot, Malbec, Cabernet Franc and Garnacha).

This winery has traditionally used spontaneous fermentation. The aim of this study is to know the yeast population diversity (*Saccharomyces* and non-*Saccharomyces*) in spontaneous fermentation and the enological properties of the *Saccharomyces* strains, in order to select those strains with adequate characteristics and well adapted to the cellar environment to be used as starters in winemaking. On the other hand, the behavior and implantation of the selected strain/s in the cellar was evaluated in subsequent vintages.

2. Material and methods

2.1. Sampling and isolation

Samples were taken from seven different spontaneous fermentation process; both red (Cabernet Sauvignon, Tempranillo,

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Petit Verdot and Syrah) and white varieties (Chardonnay, Airén and Sauvignon Blanc) were sampled. The cellar only used one commercial yeast as starter culture to address the fermentation process in red grape musts and two in white musts.

The sampling was carried out at different stages of fermentation (1070 g/L of density—Start-, 1030 g/L —Halfway- and 1000 g/L — End-), obtaining a total of twelve samples in red wines and nine in the white ones.

Samples and/or serial dilutions were streaked onto YPD (glucose 20 g/L, yeast extract 10 g/L, peptone 20 g/L, agar 20 g/L) agar plates with added tetracycline (0.25%) and sodium propionate (0.025%) to inhibit bacteria and mold growth, respectively. Plates were incubated at 28 °C for 48 h; those displaying around 100 colonies were replicated on lysine agar medium (Oxoid), to distinguish between *Saccharomyces* sp and non-*Saccharomyces* sp yeasts.

Twenty *Saccharomyces* colonies per sample were chosen at random from the YPD plates for each grape variety, obtaining a total of 240 and 180 isolates in the red and white wines respectively. On the other hand, all colonies grown in Lysine medium were purified for their identification at species level.

2.2. Genetic diversity of yeasts

2.2.1. Identification of non-Saccharomyces yeasts

Identification of isolates at species level was performed by PCR (ITS-5.8S rDNA)-RFLP. Amplification and restriction of ribosomal DNA was carried out as previously described by Fernández-González, Úbeda, and Briones (2000). Amplified DNAs were digested with 3 restriction endonucleases: *Hin*fI, *Cfo*I and *Hae*III (Boehringer Mannheim).

PCR and RFLP fragment lengths were used for identification of yeasts using Quidy (a program developed in our laboratory) and www.yeast-id.com (Valencia University and CSIC, Spain).

2.2.2. Characterization of Saccharomyces strains

All *Saccharomyces* isolates (420 plus the active dry yeasts (ADY) used in the wine cellar) were subjected to mitochondrial DNA restriction analysis using the restriction endonuclease *Hin*fI (Boehringer Mannheim). The genetic profiles of all wild strain isolates were compared among them and with the ADY restriction pattern used in the cellar.

2.3. Enological characteristics of Saccharomyces strains

One representative strain was selected for each genetic profile, all of which were subjected to a battery of specific tests to determine their enological characteristics. The tests were carried out according to the winery interests.

In order to study fermentation kinetics (by measuring released CO_2), H_2S production (by lead acetate reduction) and foam formation (qualitative visual evaluation), microfermentations were performed at 25 °C or 16 °C — depending if the strains came from red or white wines — until constant weight was reached. The fermentations were carried out in 250 mL Erlenmeyer glass flasks equipped with valves containing SO_2 saturated water and filled with 220 mL of a commercial must adjusted to 250 g/L of sugar and 50 ppm of SO_2 (pH = 3.5). All the musts were inoculated with 10^6 cells/mL.

To evaluate the ability to start fermentation under adverse conditions (presence of ethanol, SO_2 and high sugar concentrations), tests were carried out using a synthetic must containing 339 g/L of sugar (30 Brix), 100 mg/L SO_2 or various concentrations of ethanol (8, 10 and 12% of ethanol (Úbeda, Briones, & Izquierdo, 1998)). All media were inoculated with 10^6 cells/mL and incubated at 28 °C for 72 h.

Flocculation properties were evaluated using the method proposed by Caillet (1991).

Finally, synthesis of, and resistance to, killer toxin (K_2) (Somers & Bevan, 1969) as well as biogenic amines formation were examined in the studied strains (Caruso et al., 2002).

2.3.1. White wines

Apart from the tests described above, the winery requested the assessment of some specific traits useful in white winemaking.

Cells vitality was measured using a μ Trac 4200 impedance analyzer (SY-LAB Instruments) and by following the method described by Barrajón, Arévalo-Villena, Úbeda, and Briones (2011).

Nitrogen requirements were evaluated in some strains from white wines (WA, WD, WE, WF, WI, WJ, WN, WO and WR), previously selected. Synthetic musts containing 250 g/L of sugars (66% of glucose and 34% of fructose), diammonium phosphate (DAP) and eight of the most consumed amino acids according to Barrajón, Giese, Arévalo-Villena, Úbeda, and Briones (2011) were inoculated with 10^7 cells/mL of each strain and incubated at 25 °C for 8 days. Two different nitrogen concentrations were tested: 173 mg N/L, by using the average concentration of the amino acids used in grape musts: Arg (350 mg/L), Phe (15 mg/L), Ser (36 mg/L), Leu (18 mg/L), Lys (28 mg/L), His (12 mg/L), Met (15 mg/L), Trp (310 mg/L) and 500 mg/L of $(NH_4)_2HPO_4$; and half concentration.

All the assays were sampled after 2 and 8 days of fermentation. The nitrogen content was analyzed by high performance liquid chromatography (HPLC) following Gómez-Alonso, Hermosín-Gutiérrez, and García-Romero (2007).

Residual sugars and glycerol were quantified by HPLC-IR (Jasco), using a Kromasil amino column, particle size 5 μm (250 × 4.6) and thermostatized at 25 °C. The ethanol content was analyzed by an enzymatic method (Boehringer Mannheim).

To study the capability for protein haze stabilization, 5 mL of supernatant of each culture were in contact (72 h/25 °C) with both a wine undergoing natural protein haze and an alcoholic solution of bovine serum albumin (10%). After that, the samples were heated at 90 °C/60 min, held at 4 °C/16 h, and then allowed to warm to room temperature. The increased turbidity was measured at 490 nm in a turbidimeter (Hanna Instruments Mod:HI 93703). Commercial mannoproteins (dose of 20 mg/mL) and bentonite (dose of 70 g/hL) were used as controls. All the assays and the appropriate controls were performed in triplicate.

2.4. Enzyme activity of enological interest

A volume of an over night incubated culture, containing 10^6 cells/mL was used to inoculate different substrates for testing the enzyme activities. These were: polygalacturonase (Fernández-González, Úbeda, Vasudevan, Cordero Otero, & Briones, 2004); protease (Fernández-González et al., 2000) and β-glucosidase (Arévalo-Villena, Úbeda Iranzo, Cordero Otero, & Briones Pérez, 2005), urease, laccase, cinnamate decarboxylase (Briones, Ubeda, Cabezudo, & Martin-Alvarez, 1995) and β-glucanase (Santos, Francisco Rey, Conde, Villanueva, & Nombela, 1979). Strains with reported activity or enzyme crudes were used as positive control, while the non-inoculated substrate was used as negative control.

2.5. Laboratory-scale microvinification

Strains displaying the most suitable winemaking properties together with enzyme activities of interest were selected for microvinification assays. Three liters of a Petit Verdot mash and