Population of indigenous yeast strains from Prieto Picudo grapes in different growing areas of Denomination of Origin «Tierra de León»

José Manuel ÁLVAREZ-PÉREZ*, Enrique GARZÓN-JIMENO, Juan José R. COQUE

Research Institute of Vineyard and Wine. University of León. Avda. Portugal, 41. 24071, León, Spain.

*Corresponding author, e-mail: jmalvp@unileon.es

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ABSTRACT

Many different studies about autochthonous yeast populations and their potential use as starters in the fermentation process have been conducted. However it is not clear enough which is the most suitable environment to sampling with the purpose of isolating the best indigenous yeast populations. Most researches had carried out the isolation in fermentative environments (cellars), although other studies sampled in natural habitats (vineyards). Yeast isolation was made from Prieto Picudo grape samples taken from different vineyards which are spread in the cultivation area of this variety. Different yeast species have been identified unequivocally by molecular techniques. Major were yeast species associated to this natural habitats (named "indigenous yeast"), although a *Saccharomyces cerevisiae* strain was isolated too. Furthermore this *Saccharomyces* 3d strain was isolated in two different vineyards (with a separation area of approximately 50 km). Accordingly, this strain could be considered as a "terroir strain". Small-scale fermentations were made with this strain. Their oenological properties were compared with the average properties of a collection of yeast strains isolated in our laboratory in fermentative habitats (cellars). Results show that the *Saccharomyces* 3d strain couldn't produce a complete fermentation, therefore is not suitable as a starter in an industrial process. Therefore, the isolation of autochthonous yeast strains must be done in fermentative habitats (cellars) in order to achieve success.

Keywords: *autochthonous yeasts, indigenous yeasts, Prieto Picudo, Saccharomyces cerevisiae, wine.*

Introduction

Prieto Picudo is a red grape variety quite grown almost exclusively in southern Leon (Castilla y León, north west Spain). The production area is located between two rivers (Cea and Esla), which influence climatic conditions of this region. Wines produced with this grape variety are included in the Denomination of Origin (DO) "Tierra de León" which currently comprises 40 cellars. Due to its youth (DO distinction was conferred in 2007) the grape production and number of bottles sold are in a constant increased, so an increment of 52 and 90 per cent, respectively, was achieved when compared 2000 and 2013 vintages (data provided by DO "Tierra de León"). Traditionally this variety is used in the elaboration of a singular

semi-sparkling rosé wine, commonly named as "Rosado de aguja" because of the addition of grape brunches in the final stages of the fermentation process (this technique is locally known as "madreo"). Currently, the cellars also elaborate a great variety of red wines which are suitable for ageing. In fact, the first "Gran Reserva" wine (60 months ageing, 18 in oak) elaborated had a wide acceptance by consumers. The characterization of the aroma profile of Prieto Picudo rosé wines has recently been reported (Álvarez-Pérez *et al.*, 2012). These wines are characterized by a complex aroma, pointing out the high levels of some thiols (i.e. 4-mercapto-4-methyl-2-pentanone) which are similar to those found in white wines from Sauvignon Blanc, where these compounds are

considered varietal markers. Population analysis of yeasts in different cellars together with the influence in the final aroma and organoleptic properties of a wine by different autochthonous yeast strains was achieved in a recent study (Álvarez-Pérez *et al.*, 2014). This study confirms the existence of a local and unique population in each cellar. Thereby, by an adequate selection process, each winery could produce wines with exclusive aromatic properties.

Several studies have linked the association of yeast population to a fermentation process and their influence in the final organoleptic properties of wines (for a complete review see Swiegers *et al.*, 2005). There are approximately 100 genera of yeasts, however 15 of these are related to winemaking processes (Kurtman and Fell, 1998). According to their habitats, it is possible distinguish between yeasts associated to the grape surface (vineyards) and yeasts associated to the equipment surfaces of a winery (cellars) (Bisson and Lucy Joseph, 2009). Yeast population in grape surface is highly dependent on several factors like climatic conditions, cultivation methods, sanitary conditions, etc. Values from 10^4 - 10^6 cells/cm² are estimated in grape surfaces (Fleet *et al.*, 2002). *Saccharomyces* yeasts are in very low concentrations compared to the levels of other yeast genera. Thus, population is dependent on the maturation state of grape berries. Basidiomycete yeasts are predominant in early stages of maturation, giving way to ascomycete yeasts during the maturation process (Bisson and Lucy Joseph, 2009). In wineries, *Saccharomyces cerevisiae* is the predominant yeast in the installation surfaces, although it is possible to detect other yeasts like *Candida*, *Cryptococcus* or *Brettanomyces*, depending on the surface and its cleanliness (Pretorius, 2000). In a fermentation process, there are several selective pressures (nutrient depletion, anaerobic conditions, increasing level of ethanol) which favor yeast with the most efficient fermentative catabolism, like *S. cerevisiae* (Martini, 1993) and other closely related species such as *S. bayanus* (Pretorius *et al.*, 1999).

In this study it is analyzed the population associated to different vineyards from different cellars, spread out of the production area of DO "Tierra de León". Yeasts were directly isolated from grapes of different locations and unequivocally identified by molecular techniques.

The results obtained were compared with the population of yeast isolated from fermentative habitats, specifically from the same cellars where the vineyards are located (Álvarez-Pérez *et al.*, 2014). The comparison was made by different fermentation assays in order to assess the behavior in a semi-industrial process.

MATERIALS AND METHODS

Grape samples

Grape samples were kindly provided from vineyards by different cellars of DO "Tierra de León", from vineyards belonging to each one. We selected for this study the same cellars that in previous studies (Álvarez-Pérez *et al.*, 2014): Gordonzello S.A. (Gordoncillo, León), Cooperativa de los Oteros (Pajares de los Oteros, León), Bodegas Pedro Casis (Gordaliza del Pino, León), Cooperativa Vinícola Ribera del Cea (Valderas, León), and Vinícola Valmadrigal (Castrotierra de Valmadrigal, León). The last one informed that they used grapes from Gordoncillo vineyards (the same grapes that Gordonzello S.A. winery), so there were not samples included from this cellar. Thus, four different samples were available for the analyses.

Yeast isolation from grape samples

Two different pre-weighted sterile bottles were filled out with the different grape samples and crushed in sterile conditions. One of these was supplemented with 5 % (w/w) of ethanol in order to enhance the isolation of *Saccharomyces* yeast. All samples were incubated at 10 °C. Every 15 days aliquots of 100 µL from serially diluted samples in sterile water were plated on WL nutrient agar (Sharlau, Barcelona, Spain) plates supplemented with 150 µg/mL chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) to avoid bacterial growth. Plates were incubated at 10 °C until the occurrence of microbial colonies. Several colonies, with different morphology, were randomly selected and replicated in YPD-Agar plates (Lodder, 1970), that were incubated at 28 °C for 2-3 days. Then they were stored at 4 °C until the analyses took place. Clones were named with a number indicating the sampling period (i.e. "0" stands for initial sampling, "1" first biweekly sampling and so on), followed by a consecutive letter for each sampling period (i.e. "2e" indicating a clone "e" isolated at the second biweekly period, that is to say, 1 month from the start of incubation).

Characterization of yeast species

Yeast identification was carried out by the D1-D2 regions of 26S rDNA sequencing (O'Donnell 1993; Villa-Carvajal *et al.*, 2004); and confirmed, when it was required, by RFLP (restriction fragment length polymorphism) analysis of the 5.8S-ITS-rDNA region (González *et al.*, 2006). Yeast total DNA was isolated from 5 mL cultures grown in YPD (Lodder, 1970) at 28 °C and 200 rpm for 24 h. Cells were harvested and suspended in 400 mL of solution A (sorbitol, 1M; EDTA, 0.1 M; pH 7.5). Next they were treated by adding 50 mL of Zymolyase-20T (ICN Biomedicals Inc., Morgan Irvine, CA, USA) and incubated at 37 °C for 1 h. Protoplasts were recovered by centrifugation, resuspended in 400 mL of solution B (EDTA, 20 mM; Tris-HCl, 50 mM; pH 7.4), and lysed by adding 10 mL of SDS 20% and incubation at 65 °C for 10 min. Next 160 mL of 5M potassium acetate were added and the preparation was strongly agitated and incubated on ice for 10 min. Cellular debris was pelleted by centrifugation at 13,000 rpm for 15 min. Supernatant was recovered and treated by adding 1 mL of RNase (10 mg/ml) (Fermentas, Glen Burnie, MD, USA) at 37 °C for 20 min. The sample was cleaned by phenol-chloroform extraction and the DNA present in the aqueous phase was precipitated by adding 1 volume of isopropanol. DNA was finally dissolved in 20 mL of TE buffer and preserved at -20°C until use.

Genetic typing of *Saccharomyces cerevisiae* strains

S. cerevisiae strain typing was carried out by RFLP of mitochondrial DNA analysis (RFLP-mtDNA) by using different endonucleases (Querol *et al.*, 1992). Bands profiles were bioinformatics analyzed by using InfoQuest FP software package (Bio-Rad, Hercules, CA, USA). It was possible the comparison between the different polymorphisms obtained resulting in dendrograms (UPGMA) according to the coefficient of similarity (DICE algorithm) as reported in (Álvarez-Pérez *et al.*, 2014).

Pure micro fermentations

Micro fermentations were made by duplicate by using 500 mL bottles filled with 350 mL of sterile grape must. Prieto Picudo grape must was sterilized by filtration through 0.22 µm filters (Millipore, Billerica, EEUU). Each microvinification was inoculated with one milliliter of a yeast preinoculum (OD₆₀₀ = 1) from overnight cultures

(28 °C / 200 rpm). Wine fermentations were carried out at 20 °C and monitored by measuring the loss of weight every 24 hours until the end of the process (constant weight). After this, yeast cells were removed by centrifugation (5 min / 4000 rpm). Samples were stored at -20 °C until quantitative analyses were performed.

Chemical quantitative analyses

Wines obtained by pure micro fermentations were quantitatively analyzed. Levels of glucose, fructose, glycerol, ethanol and acetic acid in wines were measured by HPLC using an Agilent 1200 series (Agilent Technologies, Santa Clara CA, USA) chromatograph endowed with a refraction index detector (RID), and a variable wavelength detector (VWD). The instrument was equipped with a HyperREZ XP Carbohydrate H⁺ column (8 µm particle size, 300 x 7.7 mm) and a HyperREZ XP carbohydrate H⁺ Guard pre-column (Thermo Scientific, Waltham, USA), kept at 50 °C. Analysis, from a 1:5 dilution of the wines and a 1:25 dilution of grape must were carried out after filtering the samples through 0.45 µm cellulose acetate filters (Costar, Washington DC, USA). The detection of sugars (glucose, fructose), glycerol and ethanol, was achieved by using a RID detector (positive polarity) at a flow rate of 0.8 mL/min with 4 mM H₂SO₄ as mobile phase (injection volume 25 µL). Quantifications of products were performed according to area peaks referring to calibration curves obtained with standard products.

Mathematical data processing

The fermentation process was controlled by measuring of the weight loss each 24 hours (as described above). Graphical representation of cumulative weight loss with regard to the time elapsed in each point leads a curve that reports the behavior of a particular yeast strain (see Fig. 1). However, normalization was required for comparative purposes. Sugar content (mainly glucose and fructose) of the initial must and residual sugars in different wines obtained was achieved by HPLC analyses. A new variable, Z (weight loss cumulative percentage) was defined. The curve obtained by representing this new variable (Z, percentage) versus time elapsed (days) was fitted to a non-linear model (re-parameterized Gompertz equation) (Gompertz, 1825) by using Statistica 7 software (StatSoft, Inc., IL, EEUU). This model is defined by three empirical parameters: Latency time (l), maximum fermentation rate

Fig.1. (a) Normalization process to the fermentation curves (Cumulative weight loss vs Fermentation time); (b) Each fermentation curve was adjusted to a non-linear model. The behavior in a fermentation process is described by the empirical parameters obtained (A , m , l).

(m) and percentage of sugars fermented (A). The behavior of each strain was related to the values of these parameters (see Fig. 1b).

RESULTS AND DISCUSSION

Yeast strains genetic characterization

In previous studies (Álvarez-Pérez *et al.*, 2014) we analyzed the population of yeast associated to a fermentative environment. Thus, 900 yeasts were isolated and characterized from Prieto Picudo grape must samples provided by different wineries (see section 2.1). A collection of 119 *Saccharomyces* yeasts was obtained: 118 *Saccharomyces cerevisiae* and a *S. cerevisiae* x *S. kudriavzevii* hybrid yeast. In the present study we analyzed the population of yeasts associated to the natural environment, consisting of different vineyards of Prieto Picudo grapes spread out of the cultivation area of the DO "Tierra de León". A total of 58 yeast strains, in 7 different samplings (initial sample and six different biweekly samplings), were selected and analyzed. Yeast identification was made by the D1-D2 regions of 26S rDNA sequencing. Table 1 summarizes the identification results.

In the initial samplings wild yeasts were identified and those which predominate at the surface of grapes. According to different authors the main genera of yeasts associated

to grape surface were isolated and identified: *Hanseniaspora uvarum* and *Metschnikowia pulcherrima* (Beltran *et al.*, 2002; Combina *et al.*, 2005; Hierro *et al.*, 2006; Bisson and Lucy Joseph, 2009). Also, different species of basidiomycete *Cryptococcus* were identified together with *Rhodotorula* and dimorphic fungi *Aureobasidium* as described by some authors (Prakitchaiwattana *et al.*, 2004). Two different *Cystofilobasidium*, *C. capitatum* and *C. lari-marini*, were identified according NCBI blast. However, according to a taxonomic study (Sampaio *et al.*, 2001) based on DNA-DNA reassociation experiments it has been reported that *C. lari-marini* must be regarded as a synonym of *C. capitatum*.

In a third sampling (6 weeks after starting the incubation) one of the yeast isolated, named 3d, was identified as *Saccharomyces cerevisiae*. In the fourth sampling three of the yeasts analyzed (named 4a, 4d and 4i) were again identified as *S. cerevisiae*. In the fifth sampling only the 5b clone was identified as *S. cerevisiae*. In the final sixth sampling (12 weeks after starting the incubation) most of the yeasts analyzed were unequivocally identified as *S. cerevisiae* (see Tab. 1). Isolation of this yeast in natural environmental is possible, even at low levels (Martini *et al.*, 1996; Prakitchaiwattana *et al.*, 2004). Our data support this statement because they show that population of *Saccharomyces* yeast increase with incubation

Tab. 1. Yeast isolated at low temperature (10 °C) from Prieto Picudo grape samples from different locations. The sampling period, vineyard where it was isolated and if the original sample was supplemented with ethanol are indicated. Furthermore, the fragment size amplified by using NL1 and NL4 primers it is also reported. Yeasts were identified according to NCBI nucleotide BLAST.

Sample	Sampling	Vineyard/Winery	EtOH	Size (bp)	Identification by sequencing (BLAST)
Oa	0	Gordonzello	N	680	<i>Cryptococcus laurentii</i>
Ob	0	Gordonzello	N	680	<i>Cryptococcus terrestris</i>
Oc	0	Gordonzello	N	680	<i>Cystofilobasidium capitatum</i>
Od	0	Gordonzello	N	680	<i>Cryptococcus amylorentus</i>
Oe	0	Gordonzello	N	680	<i>Rhodotorula nothofagi</i>
Of	0	Gordonzello	N	660	<i>Cryptococcus flavecens</i>
Og	0	Gordonzello	N	660	<i>Cryptococcus flavecens</i>
Oh	0	Gordonzello	N	660	<i>Aureobasidium pullulans</i>
Oi	0	Gordonzello	N	650	<i>Cryptococcus flavecens</i>
Oj	0	Pedro Casis	N	650	<i>Cryptococcus victoriae</i>
Ok	0	Coop. Los Oteros	N	680	<i>Cryptococcus sp.</i>
Ol	0	Coop. Vin. Ribera Cea	N	680	<i>Cryptococcus victoriae</i>
Om	0	Coop. Vin. Ribera Cea	N	650	<i>Aureobasidium pullulans</i>
On	0	Pedro Casis	N	600	<i>Metschnikowia pulcherrima</i>
Oo	0	Pedro Casis	N	600	<i>Metschnikowia pulcherrima</i>
1a	1	Pedro Casis	N	650	<i>Cryptococcus victoriae</i>
1b	1	Pedro Casis	N	600	<i>Metschnikowia pulcherrima</i>
1c	1	Coop. Vin. Ribera Cea	N	630	<i>Aureobasidium pullulans</i>
1e	1	Coop. Vin. Ribera Cea	N	650	<i>Filobasidium capsuligenum</i>
1h	1	Pedro Casis	N	630	<i>Cryptococcus victoriae</i>
2a	2	Pedro Casis	N	600	<i>Hanseniaspora uvarum</i>
2b	2	Pedro Casis	N	550	<i>Cryptococcus amylorentus</i>
2c	2	Gordonzello	N	650	<i>Cryptococcus amylorentus</i>
2d	2	Gordonzello	N	650	<i>Cystofilobasidium lari-marini</i>
3a	3	Pedro Casis	N	550	<i>Metschnikowia fructicola</i>
3c	3	Pedro Casis	N	630	<i>Hanseniaspora uvarum</i>
3d	3	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
3e	3	Pedro Casis	Y	550	<i>Metschnikowia fructicola</i>
3f	3	Pedro Casis	Y	550	<i>Metschnikowia sp.</i>
3g	3	Pedro Casis	Y	550	<i>Metschnikowia fructicola</i>
3h	3	Coop. Vin. Ribera Cea	N	650	<i>Leucosporidium scottii</i>
3i	3	Gordonzello	N	650	<i>Filobasidium capsuligenum</i>
3j	3	Gordonzello	N	650	<i>Cystofilobasidium capitatum</i>
4a	4	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
4b	4	Pedro Casis	N	650	<i>Hanseniaspora uvarum</i>
4c	4	Pedro Casis	N	600	<i>Metschnikowia pulcherrima</i>
4d	4	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
4e	4	Gordonzello	N	650	<i>Cystofilobasidium capitatum</i>
4f	4	Pedro Casis	Y	600	<i>Metschnikowia pulcherrima</i>
4g	4	Pedro Casis	Y	600	<i>Metschnikowia pulcherrima</i>
4h	4	Coop. Vin. Ribera Cea	N	650	<i>Hanseniaspora uvarum</i>
4i	4	Coop. Vin. Ribera Cea	N	650	<i>Saccharomyces cerevisiae</i>
4j	4	Coop. Vin. Ribera Cea	N	600	<i>Metschnikowia pulcherrima</i>
5a	5	Coop. Vin. Ribera Cea	N	650	<i>Hanseniaspora uvarum</i>
5b	5	Coop. Vin. Ribera Cea	N	650	<i>Saccharomyces cerevisiae</i>
5c	5	Pedro Casis	Y	550	<i>Metschnikowia fructicola</i>
5d	5	Pedro Casis	Y	650	<i>Metschnikowia aff. fructicola</i>
6a	6	Coop. Vin. Ribera Cea	N	650	<i>Saccharomyces cerevisiae</i>
6b	6	Coop. Vin. Ribera Cea	N	650	<i>Saccharomyces cerevisiae</i>
6c	6	Coop. Vin. Ribera Cea	N	650	<i>Saccharomyces cerevisiae</i>
6d	6	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
6e	6	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
6f	6	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
6g	6	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
6h	6	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
6i	6	Pedro Casis	N	650	<i>Saccharomyces cerevisiae</i>
6j	6	Pedro Casis	Y	550	<i>Metschnikowia aff. fructicola</i>
6k	6	Pedro Casis	Y	650	<i>Saccharomyces cerevisiae</i>

N: No ethanol in grape sample; Y: 5 % (w/w) ethanol in grape sample

time, displacing autochthonous yeasts of grape surface.

In order to perform the strain characterization of *Saccharomyces* strains, RFLP-mtDNA analyses were carried out. These analyses indicated that all *S. cerevisiae* strains were the same since their fingerprints were identical (Fig. 2). This data was very striking, since the different *S. cerevisiae* clones had been isolated from different vineyards

in a wide geographical region. Thus this strain, named 3d (as the first clon isolated), was isolated from samples of two different locations separated more than 50 kilometres from Pedro Casis winery vineyards (Gordaliza del Pino, León) and vineyards of Cooperativa Vinícola Ribera del Cea winery vineyards (Valderas, León). A comparison between this strain and the collection of 119 *Saccharomyces* strains isolated from fermentative environments

Fig. 2. RFLP-mtADN analyses of *Saccharomyces cerevisiae* yeasts isolated. M is the molecular weight ladder of 1 kb (Fermentas, Maryland, USA).

Fig.3. Comparison among the collection of yeast isolated from Prieto Picudo cellars (industrial environment) and the yeast isolated from grapes (3d strain). Dendrogram (left) based on RFLP-mtDNA analyses with the restriction endonuclease *AluI* (centre) of all strains (right) is shown. The grape yeast isolated is highlighted in bold.

(Álvarez-Pérez *et al.*, 2014) was made. A bioinformatics analysis (as described above) leads us to ensure that 3d strain was different from any other of the industrial collection (Fig. 3). Thereby we discarded a possible contamination of samples in our laboratory.

The fact that an unique strain of *Saccharomyces cerevisiae* had been isolated from different grape samples belonging to different cultivation areas (vineyards associated to different wineries) suggests the existence of a strain that can be considered as representative of an enological region or “terroir”. Similar results were obtained by other authors in other regions (Vezinhet *et al.*, 1992; Versavaud *et al.*, 1995). The population of fermentative *Saccharomyces* strains in vineyards is very low (Martini *et al.*, 1996; Prakitchaiwattana *et al.*, 2004), in fact a *Saccharomyces* strain is rarely isolated from intact berries (Martini, 1993). In wine cellar acts differently since *Saccharomyces cerevisiae* is prevalent on these surfaces (Martini *et al.*, 1996). This demonstrates the selective effect of grape juice and wine as growth substrates (Martini, 1993). In fact, some authors severed that cells of *S. cerevisiae* are rarely isolated from natural surfaces (including grapes) while natural fermentations of musts is carried out by winery resident flora (Martini *et al.*, 1996). Yeast strains are non-motile, so they rely on aerosols, animal vectors, mainly insects, and human activity for their natural dispersal (Mortimer and Polsinelli, 1999). Therefore is unclear the origin of 3d strain in different vineyards. Insects may have an important role in the spread of this strain in the cultivation region.

Saccharomyces cerevisiae has been one of the most important model microorganism in research, but above all this specie is one of the microorganisms most appreciated microorganisms by humans because of its utility in the production of several foods and drinks (Stefanini *et al.*, 2012). Today, we have broad knowledge about its genetic and phenotypic traits, however its origin and evolution process are still unclear. Some authors predict a natural origin (Martini, 1993) of wine yeast strains while others attribute its origin to fermentative environments, man-made niches, like wineries (Fay and Benavides, 2005). The first genetic diversity characterization of *S. cerevisiae* isolated from several sources showed clear differences between wild (natural origin) and domesticated

(fermentative origin) strains (Fay and Benavides, 2005). A most recent study (Liti *et al.*, 2009) over the complete genome of 36 *S. cerevisiae* unveils five “clean” lineages, where one of them comprises a number of wine strains as well as European non-wine strains (called wine/European population). The other lineages corresponded to lineages from other sources and different origins. Maybe, a natural origin of *Saccharomyces* strains will be assumed, although a domestication process, due to fermentation processes, may explain the differential properties among strains according to their habitat.

Oenological characterization of the *Saccharomyces* strain

As indicated in the previous section we analyzed the yeast population in grapes from different cultivation areas under DO “Tierra de León”. Only one strain of *Saccharomyces cerevisiae* was isolated between populations of wild yeasts normally associated to this natural environment. This strain was used in the elaboration of experimental wines (pure microvinifications, see above) that were analyzed by HPLC. In a previous study (Álvarez-Pérez *et al.*, 2014) the population of yeast associated to fermentative habitat was also analyzed in this way. A comparison of the performance of the isolated grape yeast (3d strain) against fermentative yeasts provided experimental information about different biochemical features of this microorganism in relation to its habitat. The values of the 3d strain, for each parameter, are indicated by a cross in a box-plot graph (Fig. 4) that informs about collection of yeasts previously analyzed (Álvarez-Pérez *et al.*, 2014).

Wines elaborated with the 3d strain (grape isolated) had high levels of residual sugars (10.20 g/L) when compared with those elaborated with yeast isolated from fermentative environment. Most of yeasts produced wines with low sugar levels (Fig. 4a). This is in accordance with the ethanol content in the final wine, since 13.06 % vol. is slightly lower to ethanol content in relation to other Prieto Picudo laboratory wines in the same way elaborated. This value is lower than minimum in the box-plot graph (Fig. 4b). Glycerol content (6.11 g/L) can be considered as normal according to several authors (Ough *et al.*, 1972; Scanes *et al.*, 1998). In fact, the 3d value almost coincides with the median in the corresponding box-plot graph (Fig. 4c). In relation to experimental fermentation

Fig.4. Box-plot graphs of different oenological parameters of fermentative collection yeast (Álvarez-Pérez *et al.*, 2014). Shown with a cross the values obtained with the "3d" yeast strain. (a) Residual sugar content (g/L). (b) Ethanol content (% vol.). (c) Glycerol content (g/L).

Fig.5. Box-plot graphs of empirical parameters of fermentation behavior of fermentative yeast collection (Álvarez-Pérez *et al.*, 2014). Shown with a cross the values obtained with the "3d" yeast strain. (a) Percentage of sugars fermented (Z, %). (b) Maximum fermentation rate (m, g/L day).

parameters evidenced again great difference between the isolated grape strain and the collection of fermentative yeasts. The percentage fermented (A) by 3d strain was slightly higher to Q1 in the corresponding box-plot graph (Figure 5a), that it is to say that only the 25 % of fermentative strains showed a weaker performance in these conditions. The same conclusion can be applied to the fermentation rate parameter (m), whose value is close to Q1 in the graph (Figure 5b).

During biomass production and the alcoholic fermentation the yeast cells are subjected to several stresses, highlighting the osmotic stress mainly due to high sugar concentration and the increasing level of ethanol (Querol *et al.*, 2003). A particular characteristic of industrial yeast is the presence of polymorphic chromosomes (Codón *et al.*, 1998) due to several spontaneous mutations like the recombination between transposable Ty elements (Rachidi *et al.*, 1999), mitotic crossing

over and gene conversions and others (Querol *et al.*, 2003). These mechanisms are a response to very selective pressures (mentioned above) in order to achieve faster adaptation to environmental changes (Puig and Pérez-Ortín, 2000). This adaptation is commonly called as "domestication". By these reasons the physiological behavior of a yeast strain isolated from natural environmental diverge from those whose principal niche is an industrial environment (Liti *et al.*, 2009). Our experimental results are in accordance with previous observations. The strain isolated from vineyard showed a weaker performance in relation to fermentation yeasts from different wineries of DO "Tierra de León" (Álvarez-Pérez *et al.*, 2014). For this reason, the isolation of yeast strains with the aim of being used in industrial fermentations is not suitable. Selection of autochthonous yeast strains of a particular winery is the right methodology to ensure success as has been demonstrated in a previous study (Álvarez-Pérez *et al.*, 2014). In fact, a commercial wine

was elaborated using autochthonous yeast. This wine has exclusive aromatic properties clearly distinguishable from other commercial wines, both of this or other wineries.

CONCLUSIONS

The results of this research are relevant in two ways. One of them it is the population studies of *Saccharomyces* strains over different habitats. This study revealed that *Saccharomyces* strains are in low proportion over wild yeast in natural habitat. Furthermore, the oenological studies showed that wild *Saccharomyces* do not have efficient catabolic system associated to fermentation processes in relation to autochthonous strains of cellars. It was noticeable that the same *S. cerevisiae* strain (named 3d) was isolated in different vineyard dispersed over cultivation area. This strain may be considered like “terroir” of this cultivation area.

The final aim of yeast isolation and/or selection is aimed at its possible industrial application. One strategy used is the isolation of yeast in natural habitats like vineyards. In fact, some companies offer this service to the wineries. The results shown that the “wild” *S. cerevisiae* strain was unable to produce a complete fermentation (high levels of residual sugar in the final wine) therefore could not be used as a good starter in a wine elaboration process. Only *S. cerevisiae* strains isolated from fermentative habitats (domestication process) may be used for this purpose if their oenological properties are suitable. So, companies or institutions that offer this service to wineries incur in an economic and scientific fraud. This research studies may be useful highlight the importance of a correct isolation and selection of yeast strains with potential industrial properties.

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