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Research Paper

Saccharomyces cerevisiae and non-*Saccharomyces* yeasts in grape varieties of the São Francisco Valley

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Abstract

The aims of this work was to characterise indigenous *Saccharomyces cerevisiae* strains in the naturally fermented juice of grape varieties Cabernet Sauvignon, Grenache, Tempranillo, Sauvignon Blanc and Verdejo used in the São Francisco River Valley, northeastern Brazil. In this study, 155 *S. cerevisiae* and 60 non-*Saccharomyces* yeasts were isolated and identified using physiological tests and sequencing of the D1/D2 domains of the large subunit of the rRNA gene. Among the non-*Saccharomyces* species, *Rhodotorula mucilaginosa* was the most common species, followed by *Pichia kudriavzevii*, *Candida parapsilosis*, *Meyerozyma guilliermondii*, *Wickerhamomyces anomalus*, *Kloeckera apis*, *P. manshurica*, *C. orthopsilosis* and *C. zemplinina*. The population counts of these yeasts ranged among 1.0 to 19 x 10^5 cfu/mL. A total of 155 isolates of *S. cerevisiae* were compared by mitochondrial DNA restriction analysis, and five molecular mitochondrial DNA restriction profiles were detected. Indigenous strains of *S. cerevisiae* isolated from grapes of the São Francisco Valley can be further tested as potential starters for wine production.

Key words: Brazilian wines, *Saccharomyces cerevisiae*, indigenous strains, non-*Saccharomyces*, mitochondrial DNA restriction analysis.

Introduction

Winemaking in the semi-arid region of the São Francisco River Valley, northeastern Brazil has grown rapidly in recent years, leading to the production of approximately 7 million litres of wine per year. The ability to produce two to three crops of grapes (*Vitis vinifera* L.) per year due to favourable weather conditions distinguishes this region from traditional winemaking areas in Brazil and the world. This region has an annual average temperature of 26 °C and water available for irrigation. These vineyards are cultivated on the banks of the São Francisco River between latitudes 8 and 9º South and between the Brazilian states of Bahia and Pernambuco. The cultivars used in this region are Cabernet Sauvignon, Grenache, Tempranillo, Sauvignon Blanc, Syrah and Verdejo (Santos, 2008).

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Several authors have investigated the origin of *Saccharomyces cerevisiae* strains that are responsible for spontaneous grape must fermentation (Esteve-Zarzoso *et al.*, 2000; Clemente-Jimenez *et al.*, 2004; Schuller *et al.*, 2005, Valero *et al.*, 2007; Wang and Liu, 2013). Some authors contend that *S. cerevisiae* comes from the microbial community resident in wineries. In the vineyard, yeasts may be transported from the soil to the grapes by insects or by the wind (Valero *et al.*, 2007). Autochthonous winery-resident strains of *S. cerevisiae* take over grape-resident yeasts and predominate in natural fermentations (Ciani *et al.*, 2004; Settanni *et al.*, 2012; Lederer *et al.*, 2013). Damaged grape berries are rich depositories of *S. cerevisiae*, demonstrating that the vineyard can be a natural reservoir of this yeast (Valero *et al.*, 2007). Moreover, the diversity of *S. cerevisiae* strains differs according to each plant and grape cluster. For this reason, it is not always possible to obtain the beverage with the same sensorial quality from spontaneous wine fermentation (Clemente-Jimenez *et al.*, 2004).

Indigenous strains of *S. cerevisiae* have been shown to be better adapted to their local environmental conditions and substrates than non-indigenous strains (Esteve-Zarzoso *et al.*, 2000; Fleet, 2008; Urso *et al.*, 2008; Capece *et al.*, 2012). These indigenous strains may contribute to the overall sensorial quality of wine because they are more competitive in their local environmental conditions and they assure the maintenance of the typical sensorial properties of the wines produced in a particular region (Schuller *et al.*, 2005; Valero *et al.*, 2007*;* Settanni *et al.*, 2012). During alcoholic fermentation, these *S. cerevisiae* strains can release various aroma compounds, which influence the organoleptic quality of wines (Capece *et al.*, 2012). Thus, the aims of this study were as follows: (i) to isolate and identify the yeasts from fermented musts of five grape varieties used in the production of wine in the São Francisco Valley; and (ii) to characterise strains of *S. cerevisiae* by mitochondrial DNA restriction analysis (mtDNA-RFLP).

Materials and Methods

Sampling and yeast isolation

Samples were collected between July and September 2008 at Fazenda Ouro Verde / Miolo in the semi-arid Sao Francisco Valley region, municipality of Casa Nova, Bahia, Brazil. The grape varieties used in this work were the red grapes Tempranillo, Cabernet Sauvignon and Grenache and the white grapes Sauvignon Blanc and Verdejo. In each vineyard, six sampling points were defined, and the distance between the points varied between 80 and 100 m.

Approximately 2 kg of grapes were collected aseptically in sterile plastic bags, transported to the laboratory in an ice bath and processed in no more than 48 h. From each sampling point, grapes were crushed, and the grape juice was fermented at 20 °C in small volumes (500 mL) (Schuller *et al.*, 2005). Fermentation evolution was monitored daily until the sugar content was reduced to 70 g/L, corresponding to the consumption of approximately 2/3 of the sugar content and/or before 15 days had passed.

Serial 10-fold dilutions of the samples were inoculated (0.1 mL) in triplicate on YM agar (yeast extract-malt extract agar, glucose 1%, malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, agar 2% and chloramphenicol 0.01%) for the isolation of *S. cerevisiae* strains and other yeasts and on lysine agar (yeast carbon base 1.17%, lysine 0.056%, agar 2% and 0.01% chloramphenicol) for isolation of only non-*Saccharomyces* yeasts. Plates containing between 30 and 300 yeast colonies were examined. From each grape fermentation, ten colonies of the most prevalent yeast morphotype on the YM plates were purified, and each different yeast morphotype was also counted and purified for later identification. From the lysine agar plates, each different yeast morphotype was counted and purified for later identification.

Identification and molecular characterisation of yeasts isolated from fermented must

The yeasts were identified by the standard methods of Kurtzman *et al.* (2011). Yeast identities were confirmed by sequencing the D1/D2 variable domains of the large subunit of the rRNA gene; the D1/D2 divergent domains were PCR-amplified as described by Lachance *et al.* (1999) using the primers NL-1 (5'-GCATATCAATAAGCG GAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAA GACGG-3'). Identities of badisiomycetous species were also confirmed by sequencing the intergenic transcribed spacer (ITS) 1-5.8S-ITS2 region of the large-subunit rRNA gene (10). The amplified DNA was concentrated and purified with WizardSV columns (Promega, USA) and then sequenced in a MegaBACE 1000 automated sequencing system (Amersham Biosciences, USA). The sequences were edited with the program DNAMAN, version 4.1 (Lynnon Bio-Soft, Canada). Existing sequences for other yeasts were retrieved from GenBank.

All isolates previously identified as *S. cerevisiae* were compared using mitochondrial DNA (mtDNA) restriction analysis. To verify whether the strains found in this study were indigenous, comparisons were made with five commercial strains of *S. cerevisiae* and one commercial strain of *S. bayanus* used in the São Francisco Valley region. The names of the supplier companies have been substituted by capital letters from A to F. These strains were *S. cerevisiae* (A, B, C, D, E) and *S. bayanus* (F). The mtDNA of isolates was purified as described by Querol *et al.* (1992), and digested with *Hinf*I restriction endonuclease (Invitrogen, USA). The restriction fragments were separated by agarose gel electrophoresis, stained with ethidium bromide, visualised under UV-light and photographed.

Results

A total of 215 yeast isolates, 155 of *S. cerevisiae* and 60 of non-*Saccharomyces* yeasts, were obtained from five varieties of wine grapes in small scale fermentations (Figure 1).

The fermentation times of the cultivars from Verdejo, Sauvignon Blanc and Tempranillo grapes were between 7 and 8 days, whereas the fermentation times for cultivars from Cabernet Sauvignon and Grenache grapes were between 13 and 15 days. The grape cultivars Tempranillo, Verdejo and Sauvignon Blanc had a total of 98 yeast isolates comprised of 56 *S. cerevisiae* isolates and 42 non-*Saccharomyces* isolates. The grape varieties Cabernet Sauvignon and Grenache had a total of 117 yeasts comprised of 99 *S. cerevisiae* isolates and 18 non-*Saccharomyces* isolates. Among the non-*Saccharomyces* species isolated from the grape musts, *Rhodotorula mucilaginosa* was the most common species, followed by *Pichia kudriavzevii*, *Candida parapsilosis*, *Meyerozyma guilliermondii*, *Wickerhamomyces anomalus*, *Kloeckera apis*, *P. manshurica*, *C. orthopsilosis* and *C. zemplinina.* The population counts of these yeasts ranged among 1.0 to 19 x 10^5 cfu/mL.

A total of 155 isolates of *S. cerevisiae* were compared by mitochondrial DNA restriction analysis. The profiles P1 (represented by strains LMA-V68 and LMA-V132), P2 (represented by strain LMA-V80), P3 (represented by strain LMA-V148), P4 (represented by strain LMA-V152) and P5 (represented by strains *S. cerevisiae* E and strain LMA-V65) were found among the 155 isolates of *S. cerevisiae* obtained in this study (Figure 2). Figure 3 shows the distribution of these five molecular profiles among the grape varieties fermented in laboratory scale. Figure 4 shows the molecular profiles of commercial strains *S. cerevisiae* A (profile P6), *S. cerevisiae* B (P7), *S. cerevisiae* C (P8), *S. cerevisiae* D (P5), *S. bayanus* F (P8) and *S. cerevisiae* E (P5). These mtDNA profiles were compared with indigenous strains LMA-V68 (P1), LMA-V80 (P2) and LMA-V65 (profile P5, isolated from fermented grapes), as shown in the same figure. The commercial yeast *S. cerevisiae* D and *S. cerevisiae* E showed the same restriction profile (P5) as yeast LMA-V65 (Figure 4), which was isolated from grapes in our study. Commercial strains *S. cerevisiae* C and *S. bayanus* F have identical restriction mtDNA profiles (profile P8).

In this study, the molecular profile P1 was prevalent among the strains of *S. cerevisiae* isolated from São Francisco Valley, comprising 45.8% of the total 155 strains tested, followed by the profile P5 (41.3%), P4 (7.1%), P3 (3.2%) and P2 (2.6%) (Figure 2). The molecular profile P1 was found in all five grape cultivars studied. The molecular profile P5 was found in the grape varieties Grenache, Cabernet Sauvignon (in the highest percentage) and Sauvignon Blanc. This molecular profile was identical to the commercial strains *S. cerevisiae* D and E. The strains that have the molecular profile P3 were found in grape cultivars Tempranillo and Sauvignon Blanc, and the molecular profile P2 was found only in Cabernet Sauvignon.

Discussion

The differences in the number of yeasts isolated from each grape cultivar may be related to the fermentation time of each sample; spontaneous fermentation time was lower (on average 7-8 days) for the must of the varieties Tempranillo, Verdejo and Sauvignon Blanc than for the varieties Cabernet Sauvignon and Grenache (an average of 13-15 days). The grape's yeast microbiota depends on a variety of factors, including grape variety and the vineyard's age

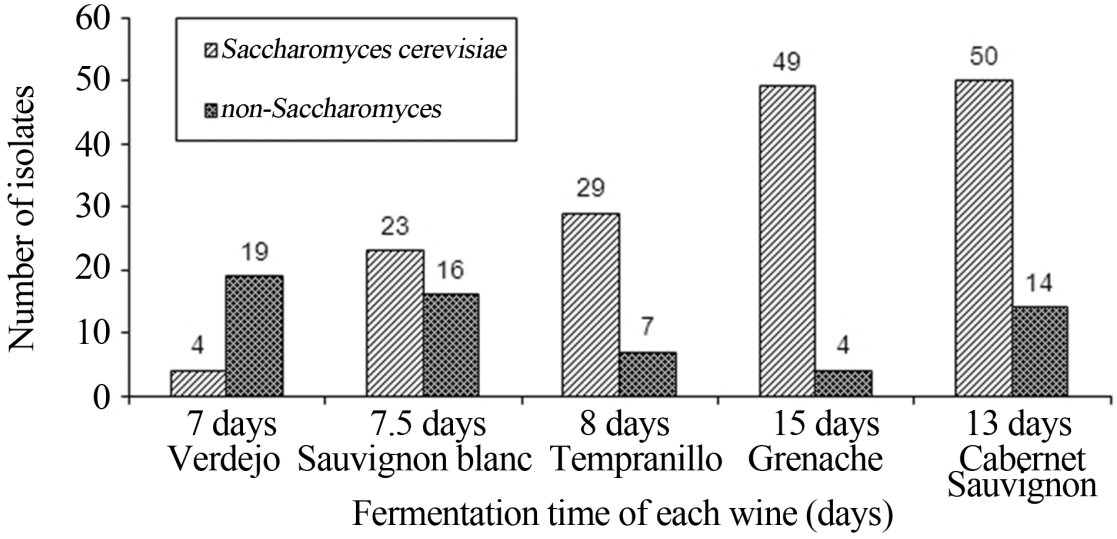

Figure 1 - Number of isolates of *Saccharomyces cerevisiae* and non-*Saccharomyces* yeasts obtained from fermented must and fermentation time of different grapes (*Vitis vinifera* L.) of the São Francisco Valley.

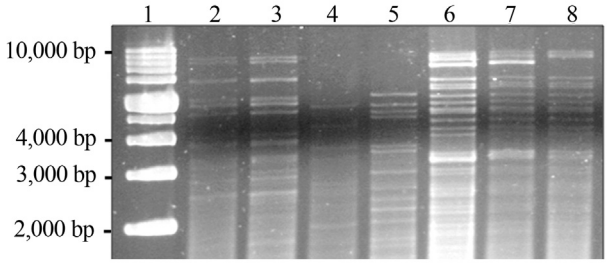

Figure 2 - Patterns generated by mitochondrial DNA-RFLP with *Hinf*I restriction endonuclease of indigenous *Saccharomyces cerevisiae* isolates from fermented grape must (*Vitis vinifera* L.). Lanes: 1, 1 Kb plus DNA Ladder; 2, profile P5 (strain LMA-V65); 3, profile P5 (commercial *S. cerevisiae* E); 4, profile P2 (strain LMA-V80); 5, profile P3 (strain LMA-V148); 6, profile P1 (strain LMA-V68); 7, profile P1 (strain LMA-V132); 8, profile P4 (strain LMA-V152).

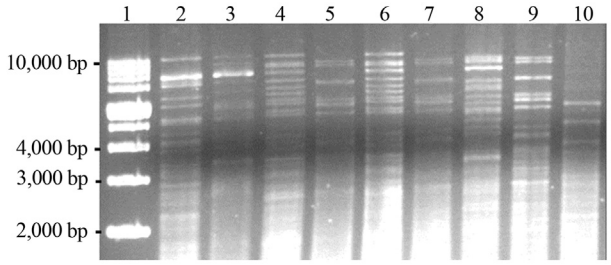

Figure 4 - Patterns generated by mitochondrial DNA-RFLP with *Hinf*I restriction endonuclease of indigenous *Saccharomyces cerevisiae* and commercial strains. Lanes: 1, 1 Kb plus DNA Ladder; 2, commercial yeast *S. cerevisiae* A, profile P6; 3, commercial *S. cerevisiae* B, profile P7; 4, commercial *S. cerevisiae* C, profile P8; 5, commercial *S. cerevisiae* D, profile P5; 6, commercial *S. bayanus* F, profile P8; 7, commercial *S. cerevisiae* E, profile P5; 8, strain LMA-V68, profile P1; 9, strain LMA-V65, profile P5; 10, strain LMA-V80, profile P2.

(Schuller *et al.*, 2005; Lederer *et al.*, 2013). *Saccharomyces cerevisiae* isolates produced five different mtDNA-RFLP molecular profiles (Figure 2). The mtDNA-RFLP analysis indicated a low genetic polymorphism of the *S. cerevisiae* populations associated with the grapes studied, and this result may be linked to the age of grape cultivars studied in the São Francisco Valley. These cultivars were approximately three years old during our study, and this time may not have been sufficient for the colonisation of the grapes by a greater number of indigenous strains of *S. cerevisiae*. Schuller *et al.* (2005) found different results in an ecological survey of *S. cerevisiae* strains from vineyards in the Vinho Verde Region of Portugal. A total of 1,620 yeast isolates were obtained from 54 spontaneous grape fermentations collected in 3 vineyards. A total of 297 different profiles were found by mtDNA-RFLP. It is possible these vineyards were already well established in the region, which would explain the large number of strains found with different patterns of mtDNA. The differences found could also be explained by the skin morphology of the grapes. For example, Sauvignon blanc presented high quantity of bloom in the thin skins, while in the grapes of Verdejo, the skins were very thick and presented less quantity of bloom, as compared with the skins from Sauvignon blanc. In our study, Sauvignon Blanc grape fermentation presented four different mtDNA patterns of *S. cerevisiae* while Verdejo only one.

Analyses of the restriction mtDNA profiles suggest that the commercial strains *S. cerevisiae* D and E (P5 molecular profile) are widespread in São Francisco Valley vineyards. One explanation for this is the use of wine production residuals as fertiliser in the region.

The results of molecular profiles shown in Figure 4 suggest that a single strain could be marketed by different companies with different names. The commercial yeast *S. cerevisiae* D and *S. cerevisiae* E showed the same restriction mtDNA profile. Commercial strain *S. bayanus* F and commercial strain *S. cerevisiae* C had also an identical mtDNA profile. Fernández-Espinar *et al.* (2001) showed that some commercial strains had identical molecular profiles, and several companies are commercialising the same strain under different names. Our results suggest that the same problem seems to be occurring with the commercial strains of *S. cerevisiae* in the São Francisco Valley.

Species belonging to the genera *Pichia*, *Candida*, *Meyerozyma*, *Rhodotorula* and *Kloeckera* isolated in our study were also frequently isolated in other studies of grape

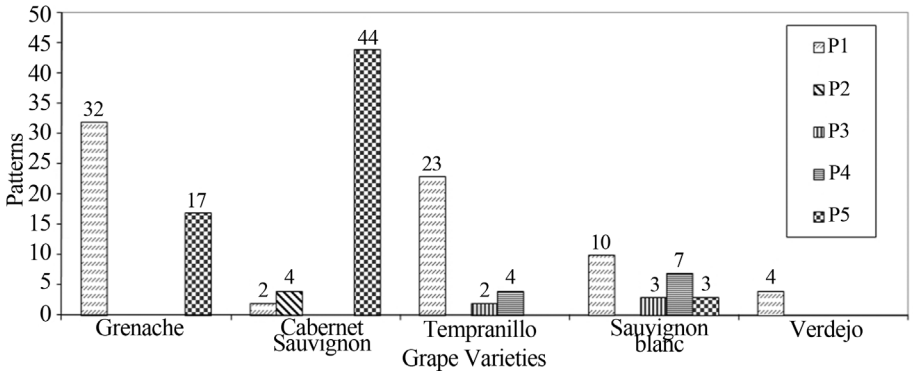

Figure 3 - Number of isolates of Saccharomyces cerevisiae yeasts for each pattern of mitochondrial DNA-RFLP with *Hinf*I restriction endonuclease obtained from different grape varieties studied in the São Francisco Valley region, northeastern Brazil.

fermentation for wine production (Esteve-Zarzoso *et al.*, 2000; Clemente-Jimenez *et al.*, 2004; González *et al.*, 2007; Urso *et al.*, 2008; Settanni *et al.*, 2012; Ortiz *et al.*, 2013; Wang and Liu, 2013), and they are a minor component of grape fermentation microbiota. *Rh. mucilaginosa* has been associated with the phylloplane (Fonseca and Inácio, 2006) and can be a coloniser of the surface of grapes. *Wickerhamomyces anomalus* and *M. guilliermondi* have been isolated from grape must and winemaking equipment (Barrajón *et al.*, 2009; Kurtzman *et al.*, 2011; Settanni *et al.*, 2012). Another species isolated in this work is *Candida zemplinina,* which has been isolated from botrytis-affected ("botrytised") wine fermentations in the Tokaj (Hungary) wine region (Sipiczki, 2003), botrytised grapes in California (Mills *et al.*, 2002; Lederer *et al.*, 2013), spontaneous fermentations of Austrian wines (Lopandic *et al.*, 2008) and from grapes, must and wines of different regions of Italy (Tofalo *et al.*, 2012). These findings suggest that this strain is related to fermented grape musts.

Indigenous strains of *S. cerevisiae* may contribute to the overall sensorial quality of wine because they are more competitive in their local environmental conditions than non-indigenous strains. Indigenous strains isolated from grapes of the São Francisco Valley can be further tested as potential starters for wine production. Fermenting yeast populations have never been characterized before in this region, and the knowledge about the occurrence of indigenous *S. cerevisiae* strains in São Francisco Valley represents an initial step for further studies for the development a regional wine start strain.

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