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Characterization of autochthonous wine yeasts isolated in vineyards of the State of Paraná, Brazil

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ABSTRACT. The transformation of grape must into wine is a complex microbiological process and is the product of the combined action of several genera and species of yeasts, dominated in the intermediate and final stages of fermentation by an alcohol-tolerant *Saccharomyces* sp. Current assay characterizes 42 autochthonous yeasts, isolated from the state of Paraná, southern Brazil, according to the following oenological properties: H_2S production, fermentation rate, flocculation capacity, and killer phenotype (killer, sensitive and neutral characteristics). Current analysis is the first to evaluate killer phenotype in yeasts isolated from the State of Paraná, Brazil. With regard to their oenological traits, the yeasts evaluated were not suitable for winemaking and suggested that, depending on the harvest, the winemakers may face problems during the spontaneous wine production process.

Keywords: *Saccharomyces cerevisiae*, killer phenotype, oenological properties.

Caracterização de leveduras vínicas autóctones isoladas em vinhedos do Estado do Paraná, Brasil

RESUMO. A transformação do mosto de uva em vinho é um processo microbiológico complexo, resultado da ação combinada de diferentes gêneros e espécies de leveduras, no qual, entretanto, prevalece uma levedura, *Saccharomyces* sp. álcool-tolerante nos estágios intermediário e final da fermentação alcoólica. O objetivo deste trabalho foi caracterizar 42 leveduras autóctones isoladas da região sul do Brasil (Estado do Paraná) de acordo com as seguintes características enológicas: produção de H_2S, taxa de fermentação, capacidade de floculação e fenótipo *killer* (características *killer*, sensível e neutra). Este estudo é o primeiro a avaliar fenótipo *killer* em leveduras isoladas do estado do Paraná. Com relação às características enológicas, as leveduras avaliadas não se mostraram promissoras para vinificação, sugerindo que, conforme a safra, o vinicultor pode enfrentar problemas durante o processo de fermentação espontânea das uvas para a elaboração de vinho.

Palavras-chave: *Saccharomyces cerevisiae*, fenótipo *killer*, propriedades enológicas.

Introduction

Brazil ranks 17th amongst world wine producers and 2010 data show that 43% of grapes cultivated in Brazil are used for juice, winemaking and its derivatives. Actually, there is a high potential for this activity in the country (MELLO, 2011a). The south region of Brazil produces 75% of total grapes in the country, with the state of Paraná ranking 4th in vine areas in Brazil (MELLO, 2011b). Current assay focuses on the municipalities of Colombo, Almirante Tamandaré and Campo Largo, all in the State of Paraná. Colombo is the main wine producer, with planted area corresponding to 130 ha and a manufacture of 800,000 liters of wine year^{-1}, largely done by descendants of Italian immigrants who settled in the area and kept the tradition of wine manufacturing. Many still employ the spontaneous fermentation process (GUIMARÃES et al., 2006).

The transformation of grape must into wine is the result of the combined action of several genera and species of yeasts. The yeast *Saccharomyces cerevisiae* is alcohol-tolerant and dominates the intermediate and the final stage of wine fermentation (CLAVIJO et al., 2010). The selection of *S. cerevisiae* yeasts facilitates the control of fermentation and reduces differences in wine quality from one harvest to another. Further, employing autochthonous yeasts are even more advantageous since they are better adapted to climate conditions and cultural practices of the region and have intrinsic properties that may introduce diversity and

regional character to the winemaking process (LOPES et al., 2007; FLEET, 2008). The desired basic oenological characteristics comprise flocculation and high fermentation capacity, low hydrogen sulfide production (H_2S) and neutrality concerning the killer factor (SILVA, 1996; NIKOLAOU et al., 2006; ORTIZ et al., 2013).

The use of flocculating strains facilitates the winemaking process: since decantation time is reduced, the filtration and centrifugation process may be eliminated (HAMERSVELD et al., 1996; GOVENDER et al., 2011).

The H_2S is a volatile compound that confers off-odors not easily removed from wine (LINDERHOLM et al., 2010).

The killer toxin may be lethal to other yeasts and guarantees the environmental prevalence of its producer (MATURANO et al., 2012). The use of commercial yeast strains harboring the killer plasmid to eliminate undesirable sensitive strains has been suggested. However, Silva (1996) insists that neutral yeasts may protect sensitive cells against killer toxin and the use of killer yeasts may be disadvantageous as they give to the winemakers a false sense of security, neglecting subsequent monitoring of some important issues related to microbiological precautions, which may be detrimental to the wine quality.

Current assay characterizes 42 autochthonous yeasts isolated from grapes grown in the state of Paraná, Brazil, with regard to hydrogen sulfide production, fermentation rate, flocculation and killer phenotype.

Material and methods

Microorganisms

Forty-two yeast strains were isolated from grapes grown in the municipalities of Colombo, Almirante Tamandaré and Campo Largo by the Enzymology and Fermentation Technology Laboratory, Universidade Federal do Parana (Curitiba, Brazil) in 2012. The standard strain K1 was purchased from Lallemand Inc. (Montreal, Canada) and standard strains 91B, 26B, 1B and 1vvt/97 were obtained from the Culture Collection of the Centro Nacional de Pesquisa de Uva e Vinho (CNPUV-EMBRAPA, Bento Gonçalves, Rio Grande do Sul State, Brazil). Yeasts were maintained at 4ºC on must agar slants (MA) and sub-cultured every 6 months.

Hydrogen sulfide production

The production of H_2S was verified using 6.0 cm x 0.5 cm filter paper impregnated with a 3.0% lead acetate solution. The filter paper was placed inside the lid of test-tubes containing 9 mL of grape must, 0.1% anhydrous sodium sulfite, 1% triptone, and 1 mL of a 10^7 cells mL^{-1} test-yeast suspension. The tubes were kept at 24ºC and the evaluation of the H_2S formation was monitored during 96 hours with a 6-18 hours interval. Tubes were also used to evaluate the fermentation rate and flocculation. *S. cerevisiae* strains Embrapa 1vvt/97 and commercial K1 were the negative and positive controls, respectively, with regard to H_2S production. The assays were performed in triplicate. After 96 hours, the qualitative evaluation of the H_2S production was evidenced by the brown color on the filter paper. The results were expressed as negative or positive.

Fermentation rate

The fermentative rate was monitored by gravimetry during 96 hours with a 6-18 hours interval and compared to *S. cerevisiae* standard strains Embrapa 1vvt/97 and commercial K1. Assays were performed in triplicate.

Flocculation

Spontaneous flocculation capacity was evaluated visually by the occurrence or absence of precipitation in the test tubes during 4 days. Degree of flocculation was determined subjectively by applying a scale from 0 (absence of flocculation) to 3 (high flocculation). The *S. cerevisiae* strain Embrapa 1vvt/97 was used as reference of high flocculation. Assays were performed in triplicate.

Killer phenotype

Killer, sensitive and neutral phenotype assays were performed in the medium must Lorena, following Silva and Almeida (2006). Briefly, 20 mL yeast extract, 0.003 g methylene blue and 1 g Bacto agar were added to 80 mL Lorena grape must. Final pH was adjusted to 4.5. The neutral/sensitive assay consisted of plating suspended cells as a lawn (approximately 10^7 cells mL^{-1}) onto the medium. Punctual mass of the standard killer strains 1B, 91B and K1 were streaked over the lawn in triplicate. In the killer assay the sensitive strain 26B was plated as a lawn and each of the 42 strains were inoculate in duplicate. Inoculated plates were incubated at 24ºC during 48 to 72 hours. If the punctual masses were surrounded by a clear zone inhibition, the yeast plated as a lawn was sensitive and the yeast streaked over it was considered killer.

Results and discussion

Thirty-six out of the 42 isolated yeasts produced H_2S. All strains (219) isolated from the Douro region in Portugal produced H_2S when cultured in

BiGGY agar medium (Difco) (NETO; MENDES-FERREIRA, 2005). Alternatively, in Spain, Ortiz et al. (2013) reported that 18 out of the 39 strains studied formed H_2S during the fermentation process. H_2S production may be related to several factors such as the demand for sulfur amino acids by yeast, intracellular nitrogen content, environmental and nutritional factors, and genetic characteristic of the yeast strain (MENDES-FERREIRA et al., 2009). Further, the yeasts isolated from grapes of different regions around the world are affected by factors such as altitude and aspect of the vineyard, geographical location, climatic conditions, age of vineyard, grape variety and viticulture practices (use of fungicides or elemental sulfur) (JOLLY et al., 2006). Therefore, the differences concerning H_2S production cannot be assigned to only one factor.

Regarding to other enological characteristic evaluated such as the fermentation rate, only one yeast strain (CB103171, isolated from grapes cultivated in Colombo, Paraná State, Brazil) showed high fermentation rate (figure 1) similar to K1 *S. cerevisiae* standard strain. The freshly grape juice presents several yeast species, mainly non-*Sacchromyces* which initiate spontaneous alcoholic fermentation of the juice, but are very soon overtaken by the growth of *S. cerevisiae* that dominates the mid - final stages of the process (FLEET, 2008). Settanni et al. (2012) found non-homogeneous distribution of *S. cerevisiae* yeasts isolated from vineyards in some regions of Sicily, Italy. The fermentation power of 14 *S. cerevisiae* isolated yeasts was evaluated and the fermentation rate ranged between 2.20 and 3.29 CO_2 day^{-1} after 72 hours of fermentation. Comitini et al. (2011) observed that 31 non-*Saccharomyces* yeasts showed lower fermentation rates (0 to 1.07 g CO_2 day^{-1}) when compared to *S. cerevisiae* control strains (1.33 to 2.51 g CO_2 day^{-1}). The higher fermentation rates presented by *S. cerevisiae* strains used in these studies, when compared to the CB103171 isolated yeast and K1 *S. cerevisiae* strain, may be explained by the different composition of grape must employed in the fermentations.

Further, 19% of isolated yeasts showed high flocculation when compared to standard *S. cerevisiae* strain Embrapa 1vvt/97. According to the most accepted theory, flocculation occurs in yeasts when there is an interaction between lectin and mannose in the cell wall of the yeasts (MIKI et al., 1982; LI et al., 2012). The absence of structural components in the cell surface may have kept the cell in suspension. Shinohara et al. (1997) have also shown low frequency of yeasts that flocculate, isolated from vineyard areas. The search for flocculating yeasts aims at improving the wine's sensorial quality perception, with decrease in costs and an increase in productivity.

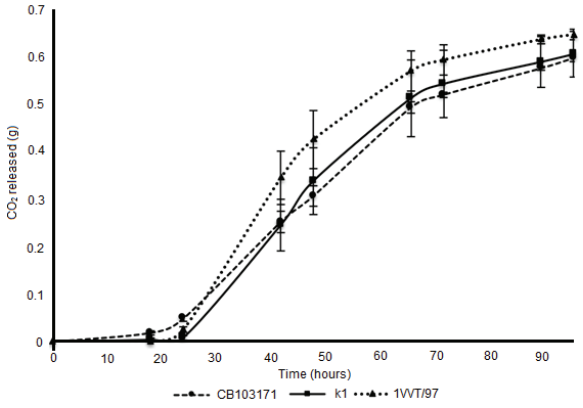

Figure 1. Fermentation rate of CB103171 strain. Yeasts K1 and 1VVT/97 were used as standards.

The frequency of killer yeasts is not uniform around the world. In current assay, 16.7% presented the killer phenotype. Figure 2 shows the inhibition zone formed by the killer yeast CB104126 isolated from grapes grown in the municipality of Colombo, Paraná State, Brazil. In four Patagonian wineries in Argentina, 35% of yeasts isolated from fermentation vat surfaces presented killer behavior, whereas 25% and 40% of yeasts were neutral and sensitive, respectively (SANGORRÍN et al., 2007). Two out of 86 yeasts isolated from spontaneously fermenting wines from four different regions in Hungary presented the killer phenotype, whereas 80 had the sensitive phenotype (CSOMA et al., 2010). Thus, killer yeasts are spread around the most vineyard regions of the world (MAQUEDA et al., 2012).

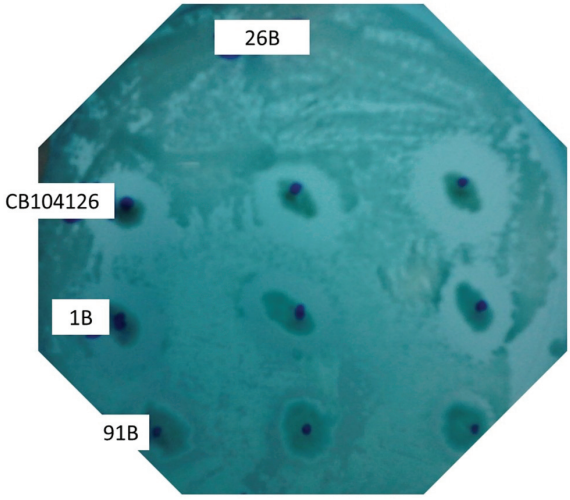

Figure 2. Inhibition zone by killer yeast CB104126; yeasts 1B and 91B are standard killer strains.

Among the yeasts evaluated, not one was sensitive to the killer factor and 83.3% were considered neutral. In the region of La Mancha, Spain, 64% of the yeasts were characterized as neutral and 38% as killer (ORTIZ et al., 2013). In a similar study on the same region, the reverse was reported. Most of the strains, or rather, 62%, showed the killer phenotype, while only 31% showed the neutral phenotype (NIKOLAOU et al., 2006). Beltran et al. (2002) followed the yeast population during spontaneous alcoholic fermentation in the region of Tarragona (Spain) between 1995 and 2000. The study revealed differences among the isolated strains according to the harvest. In 1999, the sanity of the grapes was exceptional, which resulted in low incidence of phytopathogenic microorganisms on the berries. Therefore, differences in grape microflora are also expected from one year to another related to incidence of killer, sensitive, and neutral strains.

Conclusion

Most yeasts isolated from grapes grown in the State of Paraná (Brazil), in a first killer phenotype evaluation, were neutral to the killer factor; however, among these yeasts none had other desired enological traits. It is important to bear in mind that it is not always possible to isolate a proper autochthonous strain to wine production. Winemakers from the State of Paraná, Brazil, still use the spontaneous fermentation process and, depending on the harvest, they may face problems related to wine quality.

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