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ORIGINAL RESEARCH PAPER

Killer phenotype of indigenous yeasts isolated from Argentinian wine cellars and their potential starter cultures for winemaking

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Abstract Of 31 yeasts, from different surfaces of two cellars from the northwest region of Argentina, 11 expressed killer activity against the sensitive strain *Saccharomyces cerevisiae* P351. Five of these killer yeasts were identified as *S. cerevisiae* by phenotypic tests and PCR-RFLP analysis. Two *S. cerevisiae* killer strains, Cf5 and Cf8, were selected based on their excellent kinetic and enological properties as potential autochthonous mixed starter cultures to be used during wine fermentation. They could dominate the natural microbiota in fermentation vats and keep the typical sensorial characteristics of the wine of this region.

Keywords Autochthonous yeast · Killer biotype · *Saccharomyces cerevisiae* · Wine making · Wineries

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Introduction

Wine fermentation is a complex microbial process where yeasts represent a key parameter since they are responsible of the flavor compounds production and alcoholic fermentation, the main reaction involved in the transformation of grape must into wine. Alcoholic fermentation can be classified as spontaneous or induced. In spontaneous fermentations, yeasts of the genera *Candida*, *Hanseniaspora*, *Pichia*, *Torulaspora* and sometimes *Hansenula*, present on the grapes and winery equipment, grow during the early stages of fermentation. After three or 4 days, with the increasing ethanol concentration, these genera are replaced by elliptic yeasts as *Saccharomyces cerevisiae* that becomes the dominant yeast species (Fleet 2008). In induced fermentations it is common to use starter cultures of selected strains of *S. cerevisiae* in order to ensure not only a proper start of alcoholic fermentation, but also the appropriate endpoint of fermentation (Ribèreau-Gayon et al. 2006).

In recent years, the selection and use of autochthonous strains of *S. cerevisiae* as starter cultures has increased (Lopes et al. 2007; Martini 2003) since these yeasts are better adapted to the micro-environmental conditions of a specific wine-producing region and also they are endowed with exceptional enological properties and capable of producing an assortment of volatile compounds apparently contributing to the specific bouquet of locally produced wines (Martini 2003).

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Some yeast strains are capable to synthesize proteinaceous or glycoproteinaceous exotoxins that can kill or inhibit other microorganisms. There is a growing interest in using killer yeasts as starter cultures for wine production, related to their potential predominance during wine fermentation. This could ensure the production of wines with controlled quality due to the fact that fermentation process would be "self-protected". Several studies have shown that killer yeasts are distributed differently in various wine-producing areas (Buzzini et al. 2007). Sangorrín et al. (2001) and Lopes et al. (2007) studied the occurrence of killer yeasts in the North Patagonian region of Argentina and selected an indigenous *S. cerevisiae* yeast isolate in order to develop red wine starter culture for this area. The northwest region of Argentina has also an important industrial production of wine and thus, the selection of the appropriate yeasts of this region would assure the production of wines maintaining its differential properties and the natural biodiversity of the area.

The aim of this work was to isolate, identify and select killer strains of *S. cerevisiae* from different cellar equipment surfaces from northwest region of Argentina according to its enological properties to obtain potential starter cultures for wine production better adapted to the environmental conditions of this region.

Materials and methods

Sampling and isolation of yeast from cellar equipment surfaces

Samples were taken before the beginning of the winemaking season from different equipment surfaces of two wineries (Cf and Cb), belonging to the cities of Cafayate and Animaná, respectively, both located in the northwest of Argentina. Bothwineries use commercial starters. Sampling was based on standardized protocols (Sangorrín et al. 2007) and performed as follows: sterile wet cotton swabs were streaked on different surfaces of cellar equipment (internal and external areas of 5 cm^2) and immediately placed into YEPD agar medium: 20 g yeast extract l^{-1}, 20 g peptone l^{-1}, 10 g dextrose l^{-1}, 7 g agar l^{-1}; pH 4.5 supplemented with 100 mg chloramphenicol l^{-1} (Rontag, Buenos Aires, Argentina) to inhibit bacterial growth. The tubes were then incubated for 24–48 h at 28°C with vigorous shaking (120 rpm) and was plated on YEPD agar and incubated for 48 h at 28°C. Yeast colonies were isolated from streaked YEPD plates according to their macroscopic features (texture, surface, margin, elevation and color).

Determination of killer activity and byotype

Killer activity (production) of all isolates was tested against two sensitive reference strains: *S. cerevisiae* P351 (PROIMI yeast collection). The sensitive reference yeast strain was plated as a lawn at approx. 10^5 cell ml^{-1} onto YEPD-MB agar (10 g yeast extract l^{-1}, 20 g peptone l^{-1}, 20 g dextrose l^{-1}, 20 g agar l^{-1}, 0.003 g Methylene Blue l^{-1}, buffered at pH 4.5 with 0.1 M citrate/phosphate). The plate was then spotted with 14 h old cultures of the isolates and incubated at 18°C for 5 days. The isolates were designated as killer (K+) when the spot grew surrounded by a clear zone of inhibition. The assays were replicated three times.

To test for killer-sensitive traits (biotype), YEPD-MB pH 4.5 plates were seeded with the isolates to be tested and overlaid with five reference killer strains: *S. cerevisiae* YAT 679 (K1 type), *S. cerevisiae* NCYC 738 (K2 type), *C. glabrata* NCYC 388 (K4 type), *Kluyveromyces marxianus* NCYC 587 (K6 type) and *K. lactis* var. *drosofilarum* NCYC 575 (K10 type). The plates were incubated at 18°C for 5 days. When the spot grew surrounded by a clear zone of inhibition, the isolate was designated as sensitive (R−). Each sensitivity pattern was identified with an Arabic numbers each of one refers to the toxin type with lethal action on the indigenous isolates (Sangorrín et al. 2002). The assays were replicated three times.

Quantification of killer activity of *S. cerevisiae* strains

For quantification of killer activity of saccharomycetic yeasts, cultures were made in YEPD broth (20 g yeast extract l^{-1}, 20 g peptone l^{-1}, 10 g dextrose l^{-1}, pH 4.5) for 4 days at 18°C. The culture supernatants were sterilized by filtration and the sensitive yeast *S. cerevisiae* P351 was then inoculated at 5% v/v in each supernatant and in a non-fermented medium used as control. Cultures were incubated at 18°C and growth was followed from the OD_{600} values, cell dry

weight (g l^{-1}) and evaluation of the cell viability by plate count method in YEPD agar medium.

Taxonomic identification of killer yeasts

All yeasts that showed killer activity were phenotypically identified at species level following the methods and keys proposed by Kurtzman and Fell (1998). Also, molecular identification was performed by PCR-RFLP analysis of the ITS1-5.8S-ITS2 region from the nuclear rDNA gene complex. PCR reaction was carried out according to protocols described by Esteve-Zarzoso et al. (1999) using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR products were digested with *Cfo*I, *Hin*fI and *Hae*III following the supplier's instructions. Amplified products and their restriction fragments were separated on 1.4 and 3% agarose gels, respectively, with 1× TAE buffer (Tris/acetic acid/EDTA, pH 8). Electrophoresis gels were stained with SYBR Safe (0.1 µl ml^{-1}) and visualized under UV. A 100 bp DNA ladder (Promega) marker served as size standard.

Characterization of kinetic and enological properties of *S. cerevisiae* strains

Cultures preparation and sampling during yeast growth

For the evaluation of growth and enological properties of selected yeasts, these strains were inoculated at 10^6 cells ml^{-1} in natural grape juice, and incubated at 25°C. Samples were taken to evaluate growth (as OD_{600}) and by counting viable cells. Cell-free samples were collected by centrifugation and kept at −20°C for subsequent analytical determinations.

Determination of enological properties

The enological parameters considered were: glucose and fructose consumption; ethanol production expressed as fermentative power (FP, % v/v) and ethanol yield (Yp/s, % w/w) evaluated as the ratio between maximum amount of ethanol produced and the amount of sugars consumed at each time; glycerol production (g glycerol l^{-1}) and volatile acidity, expressed as concentration of g acetic acid l^{-1}. Glucose, fructose, ethanol, glycerol and acetic acid were analyzed with enzymatic test kits (R-Biopharm AG, Darmstadt, Germany). Titratable acidity was measured by acid–base titration with standardized 0.1 M NaOH and expressed as concentration of g tartaric acid l^{-1} (Ribèreau-Gayon et al. 2006). Also, hydrogen sulfide production was evaluated by the method of lead acetate (Spiropoulos et al. 2000). The resistance to SO_2 was evaluated by adding 200 mg sodium metabisulphite l^{-1} to natural grape juice medium and assaying cell viability of yeasts.

Statistical analysis

Experiments were performed in duplicated. One-way analysis of variance (ANOVA) was applied to the experimental data and Tukey's test was used for multiple mean comparisons. All statistical analyses were performed with Statistica software version 7.

Results and discussion

Sampling, determination of killer behavior and identification of killer yeasts

Thirty one yeasts were isolated from different surfaces of two cellars from the northwest region of Argentina based on their macroscopic colony features. 21 were from the Cf cellar and 10 from the Cb cellar (data not shown).

As shown in Table 1, 11 isolates from cellar Cf exhibited killer activity against the indicator strain *S. cerevisiae* P351 and were further characterized. No killer yeasts were isolated from the Cb cellar. The killer yeasts were identified by phenotypic and molecular tests as *S. cerevisiae* (five strains) and as non-*Saccharomyces* yeasts of the genera *Pichia* and *Candida* (six strains). Their killer sensitivity pattern (KSP) towards a panel of selected killer toxins was then determined to establish their yeast killer biotype (Buzzini et al. 2007). The results showed a high diversity of the killer biotype among the isolates from cellar Cf. Although three different biotypes were determined between the *S. cerevisiae* strains, the killer activity and biotype of yeasts Cf5, Cf13 and Cf21 suggest the same strain of *S. cerevisiae* has been isolated repeatedly. However, it could be inferred that the other strains were different based on their killer behavior. Previous works reported intraspecific

Table 1 Killer behavior and identification of autochthonous yeasts isolated from the northwest region of Argentina

Isolate	Killer activity[a]	Killer biotype[b]	Killer phenotype[c]	Species[d]
Cf1	+	6, 10	K+ R−	*Candida intermedia*
Cf3	+	2, 6, 10	K+ R−	*Pichia burtoni*
Cf4	+	6, 10	K+ R−	*Candida intermedia*
Cf5	++	1	K+ R−	*Saccharomyces cerevisiae*
Cf8	+	6, 10	K+ R−	*Saccharomyces cerevisiae*
Cf9	+	4, 6, 10	K+ R−	*Pichia farinosa*
Cf11	+	1, 2, 6, 10	K+ R−	*Pichia farinosa*
Cf13	++	1	K+ R−	*Saccharomyces cerevisiae*
Cf19	++	1, 6, 10	K+ R−	*Saccharomyces cerevisiae*
Cf20	+++	6, 10	K+ R−	*Pichia anomala*
Cf21	++	1	K+ R−	*Saccharomyces cerevisiae*

[a] Killer activity evaluated against reference sensitive strains *Saccharomyces cerevisiae* P351: low (+), halo between 0.1 and 0.2 cm; medium (++), halo from 0.2 to 0.4 cm; and high (+++), halo bigger than 0.4 cm. All measures were taken from the edge of the streak to the edge of the inhibition zone

[b] Numbers indicate the type of killer toxin to which the isolated yeasts are sensitive

[c] Phenotypes: killer/sensitive (K+ R−)

[d] Identified by phenotypic and molecular tests

diversity in *S. cerevisiae* isolates using the killer biotype (Santamaría et al. 2005; Torija et al. 2001). Lopes et al. (2006) detected three *S. cerevisiae* killer sensitivity patterns among the yeast biota associated with an Argentinean Patagonia winery, suggesting that these killer biotypes could represent "stable" resident biotypes of this cellar.

It is important to note the relevance of isolating killer strains of *S. cerevisiae* from cellar surfaces. This makes the selection of autochthonous and enological important strains possible. Several ecological studies about the yeast-like flora associated to cellar environments report the presence of *S. cerevisiae* as main species (Fleet 2008), although other yeast species like *Pichia anomala*, *Rhodotorula mucilaginosa* and *Dekkera anomala*, also may be found on cellar surfaces. Sangorrín et al. (2007) studied the diversity and killer behavior of the yeast biota associated with surfaces of four Patagonian wineries, indicating that the wineries using spontaneous fermentation showed a major percentage of *S. cerevisiae* and a minor percentage of *Kloeckera apiculata.*

Determination of main enological parameters of killer *S. cerevisiae* strains

Considering the potential application on wine production of the isolated native strains of *S. cerevisiae* selected by their killer behavior, their main kinetic and enological characteristics were evaluated. Lopes et al. (2007) selected a *S. cerevisiae* MMf9 isolate according to physiological, technological and ecological characteristics. Red wines with differential volatile profiles and interesting enological features were obtained at laboratory scale by using this selected indigenous strain of Patagonian region of Argentina.

Figure 1 shows the growth profiles of five *Saccharomyces* killer yeasts. All *Saccharomyces* strains reached a high cell population at 3 days of fermentation and exhibited high biomass at the end of fermentation (10 days) with a population of 10^8 cells ml^{-1}. Growth rate and yields of the analyzed strains were not significantly different (Table 2).

These results indicated that the selected strains would allow achieving a better control of a fermentative process. Zagorc et al. (2001) studied the kinetic behavior of a killer *Saccharomyces* strain SS12/10 inoculated in pasteurized musts and then they compared it with the observed during inoculated fermentations with commercial starters. The authors indicate that local killer strain shows a higher biomass at the end of fermentation compared to the commercial strains.

Regarding the enological characteristics, all *Saccharomyces* strains studied rapidly consumed

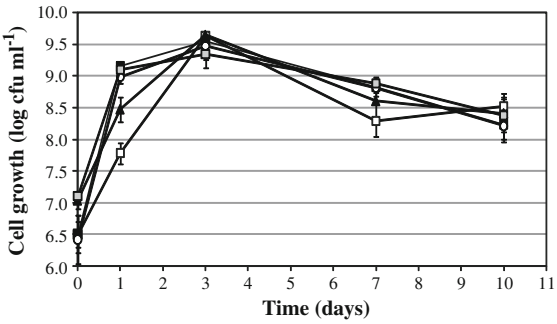

Fig. 1 Growth profiles at 28°C of autochthonous *S. cerevisiae* killer strains Cf5 (□), Cf8 (■), Cf13 (▲), Cf19 (○) and Cf21 (■) in natural grape juice medium

Table 2 Growth kinetic parameters of *S. cerevisiae* killer strains

Strain	μ^a (h^{-1})	$Y_{X/S}{}^b$
Cf5	0.14 ± 0.02	1.08 ± 0.07
Cf8	0.14 ± 0.04	0.77 ± 0.02
Cf13	0.10 ± 0.01	0.82 ± 0.05
Cf19	0.14 ± 0.02	0.77 ± 0.03
Cf21	0.12 ± 0.01	0.77 ± 0.06

One way ANOVA was performed using Tukey's test with a confidence level of 99%. Data are mean values of three experiments ± standard deviation

[a] μ, maximum specific growth rate

[b] $Y_{X/S}$, biomass yield coefficient, expressed as % w/w of the ratio between biomass produced (g) after 10 days of growth in YEPD and sugars consumed (g)

glucose and fructose of the medium (data not shown). One of the main criteria for selection of starter strains is the fermentation time, because yeasts that exhibit prolonged times do not ferment completely the sugars, producing defective wines with low ethanol grade and microbiologic stability (Ribèreau-Gayon et al. 2006). Table 3 shows that the fermentative power (FP) and ethanol yield of the killer strains were adequate to the ethanol content of regional wines, being Cf5 the strain that reached the highest ethanol grade, while Cf19 was the most efficient converting sugars to this compound, with the highest yield (Yp/s = 48.6).

Yeasts, in general are not just agents responsible of conducing alcoholic fermentation, but also, through their metabolism, they affect significantly the body, viscosity, and flavor of wines (Ribèreau-Gayon et al. 2006). The ability of the yeasts for producing glycerol and volatile acidity (as acetic acid), and to modify titratable acidity (as tartaric acid) in grape must was evaluated. The concentrations of glycerol produced by these strains are too low (4.4–5.1 g l^{-1}) to affect the body of wine; however these levels may enhance the flavor and taste of the wine (Ribèreau-Gayon et al. 2006). All strains produced levels of acetic acid that did not exceed 0.8 g l^{-1} a concentration above which a vinegar flavor can be perceived. In addition, the concentration reached helps improve the complexity of flavor compounds, such as esters (Romano et al. 2003). Furthermore, all growth media fermented by selected *S. cerevisiae* killer strains presented concentrations of tartaric acid about 0.4%. At this concentration, tartaric acid and its salts play an important role not only contributing to wine total acidity, but also to the maintaining of buffer systems of juice and wine. As result of its activity, juice and wine are buffered into a pH relatively low, which enhance microbiologic stability and color of wine, in addition to the product taste (Ribèreau-Gayon et al. 2006).

Resistance to SO_2 is an important criterion for selection of wine fermentation starters because it is a compound commonly added into must and used as antimicrobial and antioxidant (Aranda et al. 2006). Due to this, starter cultures must be resistant to this compound. Table 3 shows that SO_2 addition affected the growth of all strains, being Cf5 the most resistant strain. Mendoza et al. (2009) reported no effectiveness of SO_2 regarding control of a *Saccharomyces* strain from Argentina wine, even if the theoretical concentration of molecular SO_2 would be enough to produce the desired antimicrobial effect. Also, Henick-Kling et al. (1998) found that *S. cerevisiae* cultures showed a slight diminution of cell population in presence de 50 mg l^{-1} of SO_2.

The presence of H_2S in wine is considered a sensorial defect because it gives a rotten-egg odor to the product. It is generally produced by yeasts in nitrogen-source deficient media, where the sulfured amino acids are catabolized, releasing H_2S (Swiegers and Pretorius 2007). Therefore, a qualitative analysis of H_2S production by selected killer yeasts was performed. All strains produced this compound; Cf5, Cf8 and Cf21 had the lowest production. These results are in agreement with those reported by other authors (Lopes et al. 2007) who observed that all the yeasts including commercial starters have capability

Table 3 Evaluation of main enological properties of selected killer strains

Killer strains	FP*	Yp/s**	Glycerol (g l^{-1})	Volatile acidity[#]	Titratable acidity[^]	Production of H_2S[‡]	Resistance to SO_2
Cf5	12.7 ± 0.23^{c}	45.5 ± 0.84^{c}	$4.5 \pm 0.13^{a,b}$	0.4 ± 0.04^{a}	$4.2 \pm 0.04^{b,c}$	+	++
Cf8	10.9 ± 0.07^{a}	39.3 ± 0.25^{a}	5.1 ± 0.16^{b}	0.46 ± 0.02^{a}	4.5 ± 0.06^{c}	+	+
Cf13	12.9 ± 0.10^{c}	$46.,2 \pm 0.35^{c}$	$4.9 \pm 0.23^{a,b}$	0.43 ± 0.07^{a}	3.7 ± 0.06^{a}	++	+
Cf19	12.2 ± 0.24^{b}	48.6 ± 0.96^{d}	4.4 ± 0.10^{a}	0.68 ± 0.06^{b}	4.1 ± 0.06^{b}	+++	+
Cf21	10.3 ± 0.04^{a}	42.9 ± 0.13^{b}	$4.7 \pm 0.18^{a,b}$	0.38 ± 0.06^{a}	4.3 ± 0.01^{c}	+	+

Mean values with different superscript letters within the vertical column are significantly different according to the Tukey's test with a confidence level of 99%. Data are mean values of three experiments ± standard deviation

* Fermentative power: expressed as % v/v of ethanol

** Ethanol yield: expressed as % w/w of the ratio between maximum amount of ethanol produced (g) and sugars consumed (g)

[#] Expressed as g acetic acid l^{-1}

[^] Expressed as g tartaric acid l^{-1}

[‡] High (+++), medium (++) and low (+) sulphide producers

[¶] Growth in natural grape juice medium added with 200 mg SO_2 l^{-1}, respect to the control (+++) without the additive (10^6 cells ml^{-1})

to produce sulphide, showing an intermediate production.

Quantification of killer activity of *S. cerevisiae* strains

The killer activity of indigenous *S. cerevisiae* strains against the sensitive strain *S. cerevisiae* P351 in liquid medium was evaluated, comparing the cell viability as percentage of growth inhibition of the sensitive yeast growing in the presence and in the absence of killer toxins (Fig. 2). The growth profiles and inhibition levels show that effectively the presence of killer toxins in the supernatant affects the viability of sensitive cells. For all cases the inhibition reaches values of 1 logarithmic cycle (90% growth inhibition) at 48 h of incubation. However at 8 h incubation, there is a difference in the inhibition produced by the toxin produced by *S. cerevisiae* Cf8, which exhibited an inhibition of 86% at this time while the other yeasts display an inhibition that ranges between 1 and 24%. These early inhibitory effect of Cf8 strain could avoid the formation of detrimental compounds by other spoilage yeasts during the first hours of must fermentation.

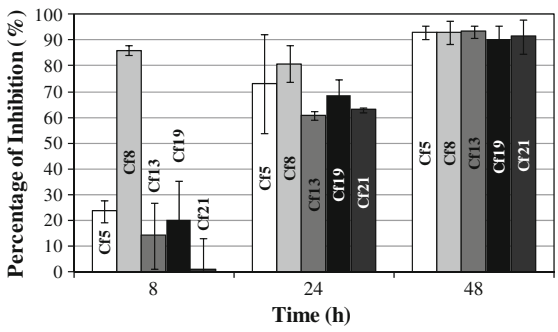

Fig. 2 Percentage of *S. cerevisiae* P351 growth inhibition at 18°C in presence of killer toxins produced by autochthonous *S. cerevisiae* strains Cf5 (□), Cf8 (▲), Cf13 (○), Cf19 (■) and Cf21 (■) compared with growth control. Values are the means of results obtained from two experiments. *Vertical bars* represent standard deviation

In conclusion, Cf5 strain displayed the best combination of killer behavior and enological parameters analyzed, such as a high FP and ethanol yield, low volatile acidity, low production of hydrogen sulfide, satisfactory levels of tartaric acid able to act as a buffer system providing stability to the product, and high resistance to SO_2. Also, considering the killer potential of the isolated yeasts, strain Cf8 presented the highest activity during the first 8 h of incubation. The use of this strain as starter could be crucial to inhibit the growth of unwanted yeasts since early stages of fermentation, and to avoid the presence of undesired compounds in the product.

Strains Cf5 and Cf8 showed the best characteristics to be used as starter cultures to ferment natural grape juice to produce wines of high and controlled quality. We are aware that many enological and biocontrol tests are still necessary to reach the

validation of these indigenous killer strains with positive characteristics, before they could have a great incidence and a great value for the regional wine industry of northwest of Argentina, an industry that keeps growing at present.

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