

Killer yeasts as biocontrol agents of spoilage yeasts and bacteria isolated from wine

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Abstract. During the winemaking process *Saccharomyces cerevisiae* is the main yeast species but other yeasts called non-*Saccharomyces* as well as different species of lactic acid bacteria (LAB) are also present. Then, one strategy to prevent or reduce microbial contamination during the winemaking process is the use of killer yeasts. The aim of this study was to evaluate the killer activity (KA) of autochthonous yeasts from Northwest region of Argentine (*S. cerevisiae* Cf8 and *Wickerhamomyces anomalus* Cf20) on spoilage yeasts and in LAB of the wine. The KA was evaluated using cell-free supernatants obtained from pure and mixed cultures of strains Cf8-Cf20. *S. cerevisiae* Cf8 showed a growth reduction between 7 and 48% on *D. anomala* BDa15, *P. membranifaciens* BPm481 and *Z. bailii* Bzb317 while *W. anomalus* Cf20 exhibited KA of 20, 61, 91 and 92% against *B. bruxellensis* Ld1, *D. anomala* BDa15, *P. membranifaciens* BPm481 and *P. guilliermondii* Cd6, respectively. Killer mixed supernatants showed growth inhibition similar to strain Cf20. Screening against LAB showed that both killer toxins were able to inhibit the growth of *L. hilgardii* 5w as well as to reduce a 16–31% histamine production by this LAB strain. These results confirm the potential of autochthonous killer yeasts as biocontrol agents in winemaking process. The mixed culture *S. cerevisiae* Cf8-*W. anomalus* Cf20 presented a wide range of KA on spoilage yeasts as well as on *L. hilgardii*. Therefore, the use of killer yeasts as starter cultures would allow producing wines with controlled quality.

1. Introduction

During the winemaking process there is a predominance of the fermentative yeast *Saccharomyces cerevisiae*, which is used as starter culture of the alcoholic fermentation. However, during wine fermentation other yeasts called non-*Saccharomyces* as well as different species of lactic acid bacteria (LAB) are also present [1]. Yeasts of the genera *Dekkera/Brettanomyces* as well as *Pichia guilliermondii* and *P. membranifaciens* are generally considered wine contaminants during the fermentation stage or post-fermentation due to the production of phenolic aromas [2]. On the other hand, among wine LAB some species as *Lactobacillus hilgardii* and *Pediococcus pentosaceus* are also considered undesirable bacteria mainly for its ability to produce biogenic amines, compounds that affect the sanitary and sensory quality of wines [1]. One strategy to prevent or reduce microbial contamination during the winemaking process is the use of killer yeasts as starter cultures. These yeasts produce proteic toxins that inhibit the growth of unwanted yeasts and fungi, but its effect on wine LAB has not been reported yet [1,3,4]. The aim of this study was to evaluate the killer activity (KA) of autochthonous yeasts from Northwest region of Argentine, *S. cerevisiae* Cf8 and *Wickerhamomyces anomalus* Cf20 on different strains of spoilage yeasts and LAB of wine.

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2. Materials and methods

2.1. Strains and growth media

Yeasts and bacteria used in this study are listed in Tables 1 and 2, respectively. The autochthonous killer strains *S. cerevisiae* Cf8 and *W. anomalus* Cf20 were isolated from wineries of Northwestern region of Argentina. Spoilage yeasts were gently provided to us from Yeast collection of San Juan University (Argentina). LAB strains were obtained from Microbiology Institute of Tucuman National University (Argentina).

Depending on the experiments, the yeasts were cultured in: YPD broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) buffered at different pH with 0.1 M citric acid/dibasic sodium phosphate; YPD-MB agar (YPD supplemented with 30 mg/L methylene blue, 20 g/L agar). Depending on the experiments bacteria were cultured in MRS broth, MRS agar or basal medium supplemented with histidine 1% (BM-Histidine) described by Joosten and Northolt [5]. Media were sterilized by filtering or autoclaving at 121 °C for 20 min. Microorganisms were incubated at 28 °C.

2.2. Production of killer cell-free supernatants

Killer strains *S. cerevisiae* Cf8 and *W. anomalus* Cf20 were inoculated at 1×10^7 cells/mL in pure and mixed cultures into YPD broth pH 4.0 and incubated for 96 h at 20 °C. Yeast cells were separated from the supernatants by

Table 1. Killer and sensitive yeast strains used in this study.

Species	Strain	P*	Characteristics
<i>Saccharomyces cerevisiae</i>	Cf8	K+	Autochthonous ^a
<i>Wickerhamomyces anomalus</i>	Cf20	K+	Authochthonous ^a
<i>Pichia guilliermondii</i>	Cd6	K–	Authochthonous/spoilage ^a
<i>Zygosaccharomyces bailli</i>	BZb317	K–	Spoilage ^b
	Ld2	K–	Spoilage ^b
<i>Dekkera anomala</i>	BDA15	K–	Spoilage ^b
<i>Schizosaccharomyces pombe</i>	BSp399	K–	Spoilage ^b
<i>Pichia membranifaciens</i>	BPm481	K–	Spoilage ^b
<i>Dekkera bruxellensis</i>	Ld1	K–	Spoilage ^b

*Phenotype.

^aStrains isolated from Northwestern wineries [7].

^bStrains provided from Yeast Collection of San Juan University.

Table 2. Inhibitory effect of Cf8 and Cf20 killer supernatants on wine lactic acid bacteria.

<i>L.hilgardii</i>	Cf8	Cf20	<i>P.pentosaceus</i>	Cf8	Cf20
5w	+	+	E2p	–	–
6F	–	–	10p	–	–
6D	–	–	Xp	–	–
X1B	–	–	E5	–	–
N4L	+	+	9p	–	–
5A	–	–	13p	–	–
N7L	–	–	X2p	–	–
5S	–	–	12p	–	–

centrifugation at 10,000 g for 10 min. Control supernatant was obtained treating Cf20 cell-free supernatant at 100 °C for 1 h to denature killer toxin.

2.3. Inhibitory effect on wine spoilage yeasts

Spoilage yeasts were inoculated at 1×10^6 cell/mL in the killer- and control- cell-free supernatants. Cultures were incubated at 20 °C for 48 h and absorbance at 600 nm (A_{600}) was measured. Killer activity (KA) was expressed as % reduction of absorbance according to the following formula:

$$KA = 100 - A_x/AC \times 100. \quad (1)$$

Where A_x is the absorbance of cultures in killer supernatants, and A_C is the absorbance of cultures in control supernatants.

2.4. Inhibitory screening assay on wine lactic acid bacteria (LAB)

LAB strains (Table 2) were cultured as a lawn in MRS agar plates and 50 μ L of killer cell-free supernatants concentrated 10 \times by ultrafiltration (30 kDa, Amicon) were spotted on the agar. Control supernatants (heat treated) were also spotted on the agar. Plates were incubated at 20 °C for 5–7 days. Inhibitory activity was considered positive when a clear zone with no growth was detected in the agar.

2.5. Effect of killer supernatants on growth and histamine production of *Lactobacillus hilgardii* 5w

Cultures of *L. hilgardii* 5w in MB-histidine supplemented with 10 \times killer- and control-cell-free supernatants (final 1 \times) were carried out and incubated at 20 °C for 48 h. After incubation, cultures were centrifuged and filtrated to separate bacterial cells. Supernatants were deproteinized with trichloroacetic acid (TCA) 100% w/v and stored at –20 °C. Histamine concentration was quantified by RP-HPLC (C18 Novapack column, 60 Å, 4 μ m (Phenomenex, Torrance, USA) with previous derivatization with O-phthaldialdehyde [6].

3. Results and discussion

3.1. Inhibitory effect on wine spoilage yeasts

S. cerevisiae Cf8 supernatant produced a growth reduction between 7 and 48% on *D. anomala* BDA15, *P. membranifaciens* BPm481 and *Z. baillii* Bzb317 (Fig. 1A) while *W. anomalus* Cf20 exhibited KA of 20, 61, 91 and 92% against *B. bruxellensis* Ld1, *D. anomala* BDA15, *P. membranifaciens* BPm481 and *P. guilliermondii* Cd6, respectively (Fig. 1B). Killer mixed supernatants showed growth inhibition similar to strain Cf20 (data not shown). These results demonstrate the biocontrol potential of these killer strains on spoilage yeasts, noting that *P. guilliermondii* Cd6 is an autochthonous strain from northwestern region of Argentina as Cf8 and Cf20 strains. These findings are in concordance with the literature, which express that killer toxins from *S. cerevisiae* have narrow inhibition spectra, while those belonging from *W. anomalus* often present broad inhibition spectra [8–10].

Bibliographic reports frequently make reference to the inhibitory effect of one killer strain or its toxin acting on sensitive or spoilage yeasts [11–13]. This is the first report about the effect of combined killer strains and its toxins in the inhibitory activity against spoilage yeasts as a strategy to increase the inhibitory capacity of a starter culture.

3.2. Inhibitory effect on wine LAB

As shown in Table 2, screening assay of concentrated killer cell-free supernatants (> 30kDa) against LAB showed that Cf8 and Cf20 supernatants were able to inhibit

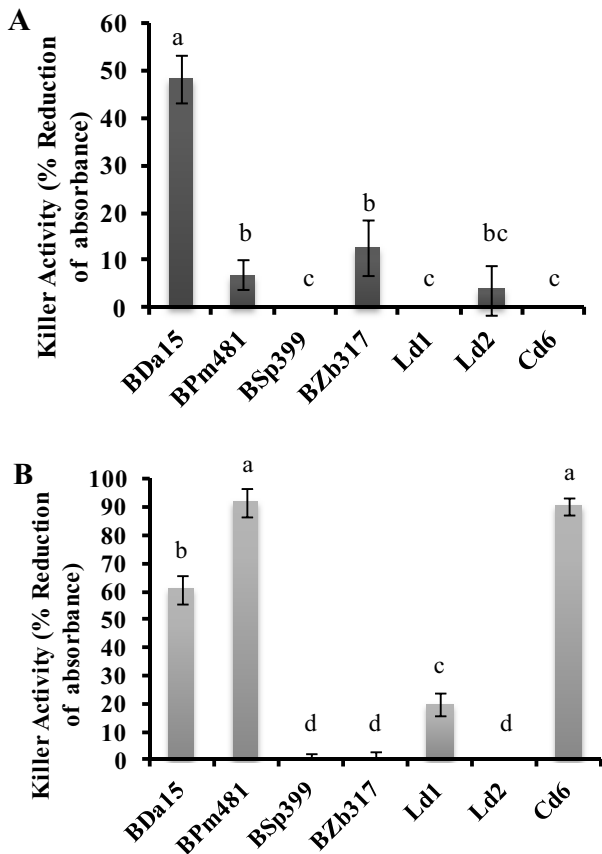


Figure 1. Killer activity of the cell-free supernatants from *S. cerevisiae* Cf8 (A) and *W. anomalus* Cf20 (B) against the wine spoilage yeast strains *D. anomala* BDa15, *P. membranifaciens* BPm481, *Sch. pombe* BSp399, *Z. bailli* BZb317, *B. bruxellensis* Ld1, *Z. bailli* Ld2 and *P. guilliermondii* Cd6.

the growth of *L. hilgardii* N4L and 5w, the latter a histamine-producing strain. When the inhibitory effect of Cf8 and Cf20 supernatants was evaluated on *L. hilgardii* 5w in liquid cultures, it was observed a longer lag phase and lower final growth (Fig. 2A) as well as a 16–31% decrease in biogenic amine production, being higher the inhibitory effect in the coculture supernatant (Fig. 2B). Several authors have reported the inhibitory effect of non-*Saccharomyces* killer yeast supernatants on different bacterial groups, mainly gram positive bacteria [14–16]. No studies about the mechanism of action of the killer toxins on prokaryotic cells have been performed. Although the toxic mechanisms remain unknown, the decrease on histamine production may be due to the growth inhibition of *L. hilgardii*. This is the first report of the effect of killer yeasts on wine-relevant LAB and the first report of the inhibitory effect of a *S. cerevisiae* killer strain on bacteria.

The literature has extensively reported studies about the use of simultaneous and sequential starter cultures of *Saccharomyces* and non-*Saccharomyces* strains in order to improve the wine flavor and avoid the excessive production of aroma compounds [8,17,18]. In contrast, this work propose the use a combination of a *S. cerevisiae* killer strain with remarkable enological properties as Cf8 [7] and a potent non-*Saccharomyces* killer strain to optimize

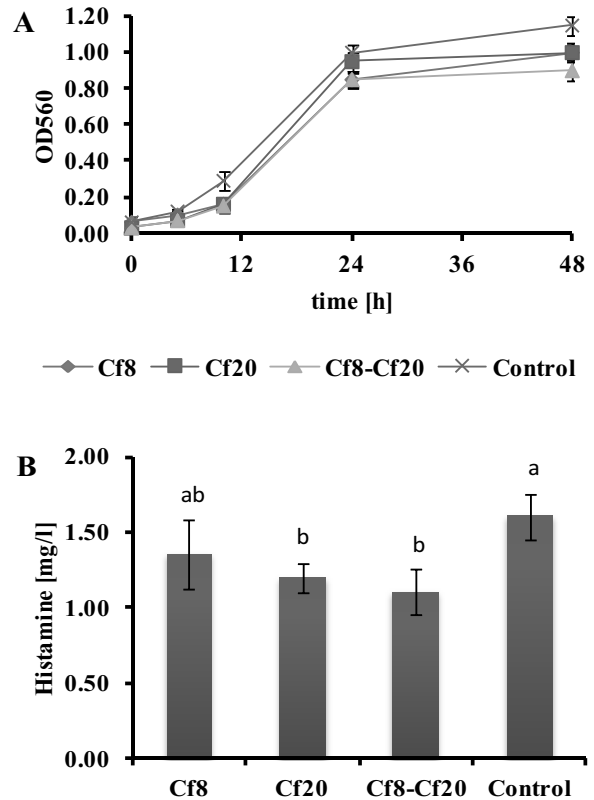


Figure 2. Effect of killer supernatants of pure and mixed cultures of *S. cerevisiae* Cf8 and *W. anomalus* Cf20 on growth (A) and histamine production (B) of *L. hilgardii* 5w.

their inhibitory activity against wine relevant spoilage microorganisms during the fermentation.

The results of this work confirm the potential of autochthonous killer yeasts as biocontrol agents in winemaking process. The mixed culture *S. cerevisiae* Cf8-*W. anomalus* Cf20 presented a wide range of KA on spoilage yeasts as well as on *L. hilgardii*. Therefore, the use of killer yeasts as starter cultures would permit to produce wines with controlled quality.

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