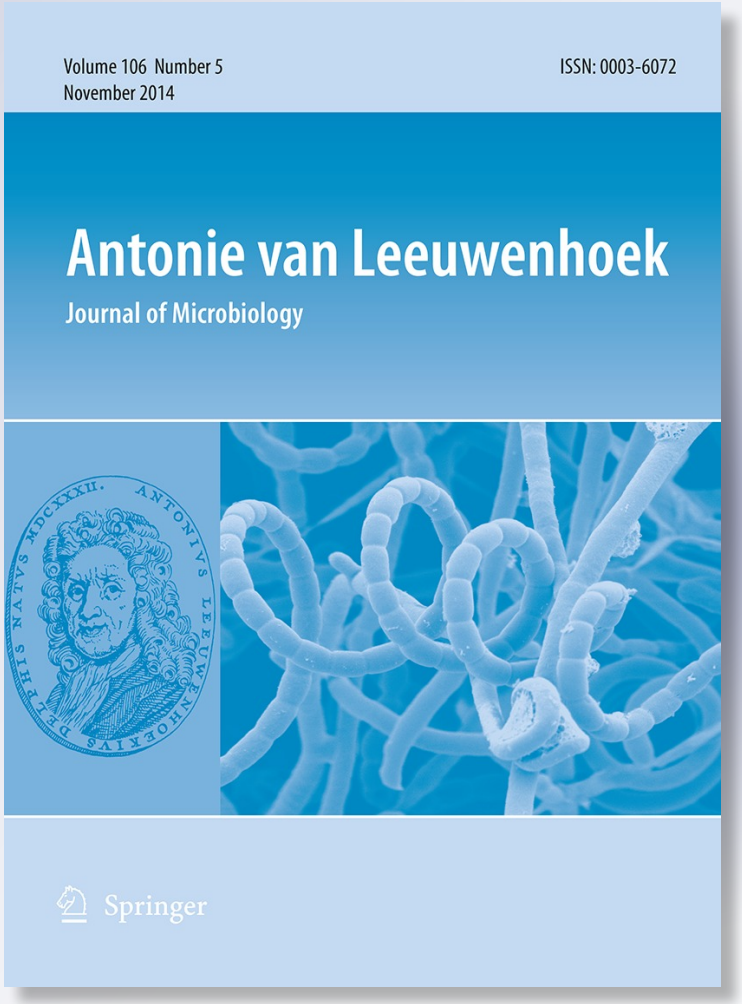
Killer activity of Saccharomyces cerevisiae *strains: partial characterization and strategies to improve the biocontrol efficacy in winemaking*

Miguel Fernández de Ullivarri, Lucía M. Mendoza & Raúl R. Raya

Antonie van Leeuwenhoek
Journal of Microbiology

ISSN 0003-6072
Volume 106
Number 5

Antonie van Leeuwenhoek (2014)
106:865-878
DOI 10.1007/s10482-014-0256-7



Springer

Antonie van Leeuwenhoek (2014) 106:865–878
DOI 10.1007/s10482-014-0256-7

ORIGINAL PAPER

Killer activity of *Saccharomyces cerevisiae* strains: partial characterization and strategies to improve the biocontrol efficacy in winemaking

Miguel Fernández de Ullivarri · Lucía M. Mendoza · Raúl R. Raya

Received: 27 May 2014 / Accepted: 8 August 2014 / Published online: 22 August 2014

Abstract Killer yeasts are considered potential biocontrol agents to avoid or reduce wine spoilage by undesirable species. In this study two *Saccharomyces cerevisiae* strains (Cf8 and M12) producing killer toxin were partially characterized and new strategies to improve their activity in winemaking were evaluated. Killer toxins were characterized by biochemical tests and growth inhibition of sensitive yeasts. Also genes encoding killer toxin were detected in the chromosomes of both strains by PCR. Both toxins showed optimal activity and production at conditions used during the wine-making process (pH 3.5 and temperatures of 15–25 °C). In addition, production of both toxins was higher when a nitrogen source was added. To improve killer activity different strategies of inoculation were studied, with the sequential inoculation of killer strains the best combination to control the growth of undesired yeasts. Sequential inoculation of Cf8–M12 showed a 45 % increase of killer activity on sensitive *S. cerevisiae* and spoilage yeasts. In the presence of ethanol (5–12 %) and SO_2 (50 mg/L) the killer activity of both toxins was increased, especially for toxin Cf8. Characteristics of both killer strains support their future application as starter cultures and biocontrol agents to produce wines of controlled quality.

M. F. de Ullivarri · L. M. Mendoza (✉) · R. R. Raya
Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 Tucumán, Argentina
e-mail: lmendoza@cerela.org.ar

Keywords Killer toxin · Autochthonous starter culture · Regional wine

Introduction

In the winemaking process, alcoholic fermentations are currently conducted using starters of selected strains of *Saccharomyces cerevisiae*. However, during fermentation other microorganisms are also present and could influence on the quality of the fermented product. Different kinds of microbial interactions have been studied to understand how a species may dominate or decline in a microbial ecosystem (Ciani et al. 2004; Perrone et al. 2013). One of the biological mechanisms for the regulation of population dynamics in several microbial ecosystems is the production of toxins capable of kill or inhibit other microorganisms, taxonomically related or not to the producing strains. The toxins synthesized by yeasts, known as killer factor, are proteins or glycoproteins whose action is mediated by specific receptors in the cell wall of the sensitive microorganism (Magliani et al. 1997). The killer character, first reported on the decade of the 1960s in a *S. cerevisiae* strain, is well distributed among other yeast genera as *Candida, Hansenula, Pichia, Debaryomyces, Ustilago, Cryptococcus, Metschnikowia, Williopsis, Kluyveromyces* and *Zygosaccharomyces* (Pfeiffer et al. 2004; Schmitt and Breinig 2002). There are three well characterized *S. cerevisiae*

killer toxins: K1, K2 and K28, encoded in cytoplasmatically inherited M dsRNAs (satellite virus) coexisting with the helper virus L-A dsRNA (Magliani et al. 1997); and the recently discovered *Saccharomyces* dsRNA-encoded toxin Klus (Rodríguez-Cousiño et al. 2011). There are also two chromosomal encoded toxins called killer of heat resistant (KHR) and killer of heat sensitive (KHS) (Goto et al. 1990).

In industrial fermentations, microbial spoilage is a frequent problem and its occurrence means high economic losses. In winemaking industry, this spoilage process is often carried out by yeast species of the genera *Dekkera/Brettanomyces*, *Pichia, Zygosaccharomyces* and *Candida*, usually isolated from wines with aroma defects (Loureiro and Malfeito-Ferreira 2003). Also, there are different bacterial groups involved in wine spoilage. Several species of lactic acid bacteria belonging to *Lactobacillus, Pediococcus* and *Leuconostoc* genera are often found in wine producing off-flavors, acrolein, hydrogen peroxide, biogenic amines (Arena and Manca de Nadra 2001; Bauer et al. 2010; Pasteris and Strasser de Saad 2009). Acetic acid bacteria are also considered spoilage microorganisms in winemaking to oxidize ethanol to acetic acid in a process known as acetification (Du Toit and Pretorius 2002). Interaction between bacteria and yeasts has been studied by some authors who reported bacterial inhibition by killer yeasts in alcoholic fermentations (Meneghin et al. 2010; Oliva-Neto et al. 2004). Additional studies on this subject are necessary to establish biocontrol activity of killer yeasts on wine bacteria. In order to avoid or reduce the spoilage by these microorganisms, sulphur dioxide (SO_2) is chemically added to grape musts previous the alcoholic fermentation as an antioxidant and antimicrobial additive. However, due to the current trends to reduce the use of chemical additives, many authors have proposed the utilization of starter cultures producers of killer toxins to avoid the contamination of the substrate by spoilage yeasts (Izgü et al. 2006; Lopes et al. 2007; Zagorc et al. 2001). The inclusion of a killer strain with appropriate fermentation properties, or a killer strain as an adjunct starter of a *S. cerevisiae* strain are usual strategies proposed in the literature (Santos et al. 2011).

In a previous study we isolated and selected *S. cerevisiae* strains producers of killer toxins with optimal enological properties from wineries of northwestern region of Argentina (Fernández de Ullivarri et al. 2011). The use of these microorganisms as starter cultures in wine fermentations requires the characterization of killer phenotype in order to predict its technological and ecological impact.

In this work we evaluated the main characteristics of killer toxins produced by selected *S. cerevisiae* strains and we proposed new strategies regarding mixed inoculations to improve and to validate their future application as biocontrol agents in the winemaking process.

Materials and methods

Yeast strains and growth media

The yeast strains used in this study are listed in Table 1. The autochthonous killer strains of *S. cerevisiae* were isolated from wineries of Northwestern and Cuyo regions of Argentina. Also we used reference strains to evaluate killer activity (Sangorrín et al. 2002) whereas spoilage yeasts were gently provided to us from Yeast collection of San Juan 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) and pasteurized white grape juice. Media were sterilized by filtering or autoclaving at 121 °C for 20 min. Yeast strains were incubated at 28 °C. All strains were maintained in YPD supplemented with 20 % glycerol at −20 °C.

Production and partial purification of killer toxins

Killer strains Cf8 and M12 of *S. cerevisiae* were inoculated at 1×10^7 cells/mL into YPD broth and incubated during 96 h at 20 °C. Yeast cells were separated from the supernatants by centrifugation at $10{,}000 \times g$ for 10 min.

Killer activity was partially purified as follows: 1 liter of crude extract was concentrated with ultrafiltration units (MWCO, 30 kDa; Millipore) to a final volume of 35 mL. The concentrated fraction was then eluted through a Sephadex G-100 column of 10 × 135 mm with citric acid-phosphate pH 4.2 at a

Table 1 Killer and sensitive yeast strains used in this study

Species	Strain	Phenotype	Characteristic
Saccharomyces cerevisiae	Cf8	K+	Autochthonous[a]
	Cf13	K+	Autochthonous[a]
	M12	K+	Autochthonous[a]
	P351	K−	Ref. sensitive[b]
	Cb2	K−	Autochthonous[a]
	YAT 679 (K1 type)	K+	Ref. killer[b]
	NCYC 738 (K2 type)	K+	Ref. killer[b]
Candida glabrata	NCYC 388 (K4 type)	K+	Ref. killer[b]
Kluyveromyces marxianus	NCYC 587 (K6 type)	K+	Ref. killer[b]
Kluyveromyces drosophilarum	NCC 575 (K10 type)	K+	Ref. killer[b]
Zygosaccharomyces bailli	BZb317	K−	Spoilage[c]
	Ld2	K−	Spoilage[c]
Dekkera anomala	BDa15	K−	Spoilage[c]
Schizosaccharomyces pombe	BSp399	K−	Spoilage[c]
Pichia membranifaciens	BPm481	K−	Spoilage[c]
Dekkera bruxellensis	Ld1	K−	Spoilage[c]

[a] Strains isolated from Northwestern wineries (Fernández de Ullivarri et al. 2011). Strains of Yeast Collection of Tucuman National University (Argentina)

[b] Strains of Yeast Collection used and provided for Sangorrín et al. 2002

[c] Strains provided from Yeast Collection of San Juan University (Argentina)

flow of 0.9 mL/min. Protein concentration in the eluted fractions was measured by the Bradford method. The active fractions were pooled and concentrated 10× by vacuum at 28 °C for 12 h in a Speed Vac Savant SPD 121P (Thermo Scientific, Thermo Electron Corporation, Ohio, EEUU) and eluted again through Sephadex G-100 and concentrated as described above. The fractions were analyzed by SDS-PAGE 10 % at 65 V for 3.5 h. The protein bands were stained with Coomassie-blue G-250.

Evaluation of killer activity

Killer activity (KA) was quantified by the diffusion plate assay and expressed as arbitrary units (aU)/mL. The plate was seeded with the sensitive strain *S. cerevisiae* P351 (the most sensitive strain to Cf8 and M12 toxins) at a final concentration of 1×10^6 cells/mL in YPD-MB agar, pH 4.2. Then, 100 µL of supernatant or killer toxin-containing fraction were seeded on the agar; plates were incubated at 20 °C for 48 h. The diameter of the clear zones of inhibition was measured with a caliber. A regression analysis in Excel threw a linear relationship between the diameter and the logarithm of the aU of toxins was realized according to the next empirical formula: KA (aU/mL) $= 10^{(D + 5.64)/6.64}$ where D is the diameter of the inhibition zone in millimeters and 1 aU is the amount of toxin able to produce an inhibition zone of 1 mm.

Partial characterization of killer toxins Cf8 and M12

Determination of optimal pH and temperature for killer activity

To determine the optimal pH at which Cf8 and M12 toxins exert their killer activity, 50 µL of purified killer toxin were added to 950 µL of YPD broth (killer YPD, 2,700 aU/mL at pH 4.2), the pH was adjusted from 1.5 to 5.0 with HCl 1 M and NaOH 1 M and KA was quantified.

The optimal temperature of toxin was also assayed following the protocol described by Kabir et al. (2011). 1 ml of Killer YPD (5,000 aU/ml at pH 3.5) was incubated for 30 min with 300–400 cells/mL of *S. cerevisiae* P351 at 10, 15, 20, 25 and 30 °C and cell viability (V) of supernatants and control (fresh YPD) were measured. KA (%) at each temperature was calculated according to the next formula:

$$\text{KA } (\%) = 100 - V_{treat}/V_{ctrl} \times 100.$$

Determination of optimal conditions for production of killer toxins

Evaluation of optimal pH, temperature and incubation time

YPD broth buffered at pH 3.5 was inoculated with killer strains and incubated for 96 h at 20 °C. After incubation, cells were separated and KA was quantified. To evaluate pH effect the medium was buffered at pH range 2–6 whereas temperature effect was studied incubating at 10, 15, 20, 25 and 30 °C.

Nitrogen supplementation

Grape juice was supplemented with 0, 100, 300 and 500 mg N/L of ammonium sulphate. Killer producer yeasts were inoculated and incubated at 20 °C for 96 h. After incubation, dry weight of yeasts and KA were quantified to determine specific KA (aU mg).

Evaluation of biotype, cross immunity, sensitivity to proteolytic enzymes, pH stability, thermostability and hydrophobicity

Biotype was assayed by the plate technique. A lawn of killer strains at 1×10^6 cells/mL in YPD-MB agar pH 4.2 was seeded. 20 µL of reference killer toxins were spotted on the agar and incubated at 20 °C for 48 h. After incubation, sensitivity of the lawn strain was positive when a clear inhibition zone surrounded by a blue line was detected around the spot.

Cross-reactivity between Cf8 and M12 strains was evaluated by the diffusion plate technique as described above. Also, cross immunity was tested in liquid medium. Cf8 and M12 strains were inoculated in each other's killer YPD (5,000 aU/ml, pH 3.5) at a final concentration of 1×10^6 cells/mL in killer YPD. After 48 h incubation at 20 °C, A_{600} was measured and compared to a growth control culture of each killer yeast in fresh YPD broth, and KA was quantified.

Sensitivity of killer toxins to three proteolytic enzymes was tested adding 0.5 mL of aqueous solutions of trypsin, chymotrypsin, and pepsin (0.6 mg/mL) to killer medium; this mixture was incubated for 2 h at 20 °C. As growth control 0.5 mL of sterile distilled water were added. To quantify KA, *S. cerevisiae* P351 was inoculated in treated media.

Stability of killer toxins to different temperatures was assayed. Killer YPD broth was treated for 1 h at 4, 18, 25, 30, 37, 50, 72 and 98 °C. As growth control, fresh YPD broth was used. After treatment, P351 strain was inoculated and after 48 h incubation at 20 °C, A_{600} was measured to quantify relative KA (KAr) according to the next formula:

$$\text{KAr } (\%) = \text{KA}_{\text{treat}}/\text{KA}_{20^\circ\text{C}} \times 100.$$

Stability of killer toxins to different pH was also tested. Killer YPD pH was adjusted for 1 h from 1.0 to 7.0 with HCl 1 M or NaOH 1 M. After treatment, pH was readjusted at 3.5. KAr was measured as follow:

$$\text{KAr } (\%) = \text{KA}_{\text{treat}}/\text{KA}_{\text{pH3.5}} \times 100$$

Affinity of killer toxins to hexadecane, toluene and xylene was assayed to determinate their hydrophobicity based on protein biphasic partition (Yamashiro 2013). Briefly, killer YPD broth was added with 1:6 volumes of organic solvent. The mixture was shaken in Vortex for 1 min, and after 15 min when the two phases were clearly defined, 500 µL of the aqueous phase were used to quantify relative KA (KAr) by the plate diffusion assay according to the next formula:

$$\text{KAr } (\%) = (\text{AK}_{\text{ctrl}} - \text{AK}_{\text{treat}}) \times 100/\text{AK}_{\text{ctrl}}.$$

Identification of genetic determinants of killer toxin

Presence of dsRNA molecules

We followed the protocol described by Rodriguez-Cousiño et al. (2011). As positive and negative control we used *S. cerevisiae* YAT 679 and P351, respectively. Samples were analyzed by electrophoresis in agarose gels 1.0 % in 1× TAE buffer, at 70 V for 1 h and stained with GelRed.

Curing of killer phenotype

A plasmid-curing protocol was used to search for mutants with no killer phenotype and genotype (K−) (Amberg et al. 2005). Temperature (37 °C), cycloheximide (0.5−75 mg/L) and acridine orange (0.1−200 mg/L) were the curing agents used in this assay. The protocol was carried out for *S. cerevisiae* Cf8, M12 and YAT 679 (K1, dsRNA, positive control). Overnight cultures of each strain at 25 °C were serially diluted (1:10) with YPD broth

supplemented with the curing agent (acridine orange or cycloheximide) and each dilution was incubated at 25 °C (for cycloheximide and acridine orange) or 37 °C (temperature). The maximum dilutions that formed a dense culture after incubation were seeded on YPD broth at 25 °C. After 24 h of incubation, serial dilutions of cultures were made with physiological solution and 300–400 cells were seeded on YPD agar and incubated at 25 °C for 48 h. 200 colonies were randomly isolated and transplanted on a lawn of *S. cerevisiae* P351 in YPD-MB agar pH 4.0 to test their killer phenotype after 48 h of incubation at 20 °C.

Study of KHR–KHS genes

Chromosomal DNA was isolated following the protocol described by Esteve-Zarzoso et al. (1998). PCR reactions were run using the following primers: KHR-f (5′-TCATGGGCCACTTAGCGATCCTTT-3′), KHR-r (5′-AAACAAGGGTCGGCCAATTGTGAC-3′), KHS-f (5′-TTTCTTGAACAATGAGCGCGGAGC-3′), KHS-r (5′-GAAGCGCTGTGGGCAAATTCTGTA-3′). PCR conditions were: initial denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were visualized on agarose gels 1.5 % in 1× TAE buffer, at 80 V for 1 h and stained with GelRed. Amplicons were purified and sequenced to compare them with data base genes using BLAST and CLUSTAL Omega.

The KHR toxin gene partial sequences were submitted to GenBank database available at NCBI under accession numbers: KM246831 (Cf8 killer toxin) and KM246832 (M12 killer toxin).

Evaluation of strategies to improve killer activity

Combination of killer supernatants

As shown in Fig. 1 three combinations of killer supernatants were assayed in order to evaluate if the inhibitory activity of *S. cerevisiae* killer toxins could increase compared to single culture supernatants. The mixed supernatants were obtained from cultures produced by simultaneous inoculation (coculture) or sequential inoculation (sequential) of the autochthonous killer strains *S. cerevisiae* Cf8, M12 and Cf13, or by mixing the supernatants (ratio 1:1) produced by single cultures (combined) of the above mentioned strains. In the sequential protocol, each culture was incubated for 96 h at 20 °C. Then, supernatants were supplemented with YPD components, sterilized by filtration (0.22 µm pore-size membranes, Millipore), and inoculated with a different yeast strain. Strain Cf8 was included in all combinations because it was selected in previous studies due to the early inhibition of its supernatant against *S. cerevisiae* P351 (Fernández de Ullivarri et al. 2011). Experiments with *S. cerevisiae* Cf8 and M12 mixed supernatants were carried out to further evaluate their inhibitory activity on selected 6 wine spoilage yeasts isolated from Argentinean wineries (Table 1). The supernatants were inoculated with a sensitive strain (*S. cerevisiae* P351 or non-*Saccharomyces* spoilage yeast) at 1×10^6 cells/mL, incubated for 48 h at 20 °C and A_{600} was measured. The KA was expressed as % reduction of absorbance of a culture compared with growth control according to the following formula:

$$KA = 100 - A_x/A_{GC} \times 100,$$

where A_x is the absorbance at 600 nm (A_{600}) of each filtrate, and A_{GC} is the Absorbance at 600 nm of the GC. To quantify the KA, the following controls were designed: non fermented medium as growth control (GC); sequentially fermented medium by *S. cerevisiae* Cb2, a non-killer strain (double fermented control DFC); sequentially fermented medium by Cf8 and Cb2 (DFC Cf8); sequentially fermented medium by Cb2 and Cf8 (DFC Cb2) and fermented medium by each strain as killer strain control (SKS). Sequential cultures were compared against all controls, while combined and co-culture combinations were not compared against DFC, DFC Cf8 and DFC Cb2.

Effect of ethanol and SO_2 on killer activity

To evaluate the effect of ethanol and SO_2 on the killer activities of Cf8 and M12 toxins, several concentrations of both compounds related to different fermentation stages (initial, intermediate and final) were assayed. Killer supernatants of *S. cerevisiae* Cf8 and M12 were supplemented with 0, 5 and 12 % of ethanol and 0, 50, 100 and 150 mg/L of SO_2 (as sodium metabisulphite) in every combination of these additives. As control, killer supernatants were treated at 121 °C for 15 min, and KA was tested to verify the lack of inhibitory activity. Then, the yeasts *S. cerevisiae* P351 or *D. anomala* BDa15 were inoculated

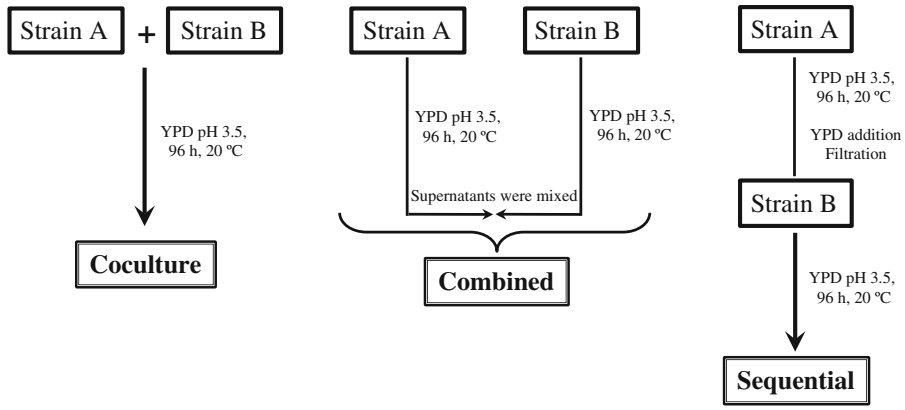


Fig. 1 Flow chart of inoculation strategies of killer strains

1×10^6 cells/mL in the supernatants and the absorbance at 600 nm (A_{600}) was measured for 48 h to quantify KA as % reduction of absorbance.

IC_{50} of the different combinations of toxin, ethanol and SO_2 were calculated to evaluate the FIC and FIC index. The interaction was defined as synergistic if the FIC index was <1, additive if the FIC index was 1.0, subadditive if the FIC index was between 1.0 and 2 and antagonistic if the FIC index was >2 (Perea et al. 2002).

Results and discussion

Different killer yeast species and strains have been reported in winemaking (Comitini et al. 2004; Santos et al. 2011); however the application of the killer toxins in commercial fermentations is still a topic of discussion (Gutiérrez et al. 2001; Maqueda et al. 2012). Further research on the influence of killer yeasts in wine-making process is required to validate their efficacy as biocontrol agents. In this study we evaluated the characteristics of two toxins produced by *S. cerevisiae* strains to know their behavior in conditions related to vinification.

Purification of killer toxins

Killer toxins were concentrated by ultrafiltration and partially purified by exclusion chromatography with a Sephadex G-100 column (Fig. 2). As shown in Table 2, the KA of the partially purified toxins increased 189 and 239 fold, and the yield was 82 and 81 % for Cf8 and M12, respectively. The partially purified toxins were used to characterize their main properties.

Characterization of killer strains and its toxins

Optimal conditions for activity and production of killer toxins

Table 3 shows that both killer toxins were active at pH values between 2.5 and 5.0 with an optimal activity at 3.5. These toxins showed stronger KA at wine pH than other toxins produced by *S. cerevisiae* such as K2 (4.0–4.3), Klus (4.0–4.7) and KHR (5.7) (Dignard et al. 1991; Goto et al. 1990; Rodríguez-Cousiño et al. 2011). Optimal temperature for Cf8 and M12 toxins was 10–20 and 20 °C, respectively (Table 3). These results are in accordance with previous studies of others killer toxins (Maqueda et al. 2012; Santos et al. 2011). When KA was tested in grape juice we observed the same behavior for both toxins (data not shown).

When the optimal conditions of production were studied, the maximum production of killer toxin (4,000 aU/mL) was reached at pH 3.5 for both strains. Regarding temperature for killer toxins production, both strains showed an optimal temperature of production (3,300 aU/mL) between 15–20 °C. In addition, killer toxin production was coupled to the exponential growth of both strains, although Cf8 reached the highest toxin concentration at the end of the log phase (48 h of incubation) while M12 reached it at the early stationary phase (96 h of incubation).

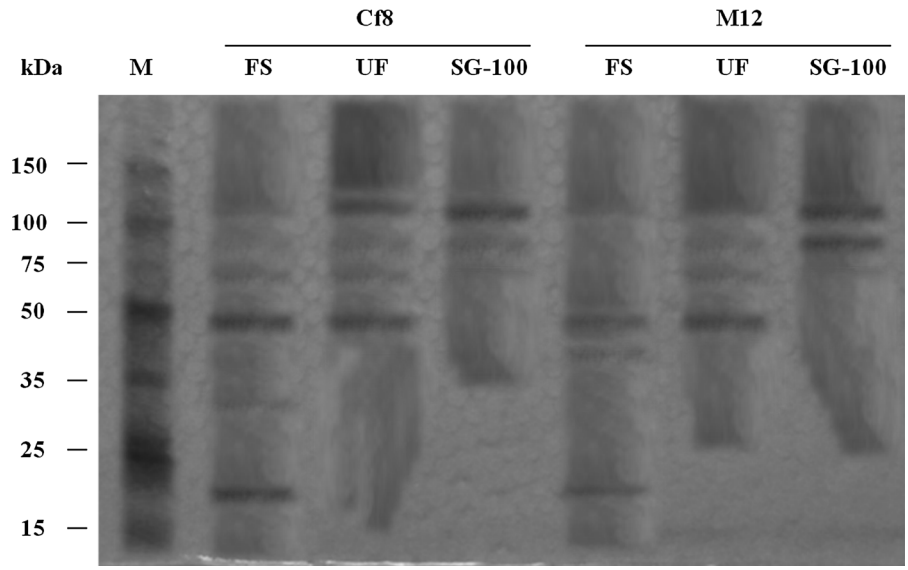


Fig. 2 SDS-PAGE gel of partially purified Cf8 and M12 killer toxins. *FS* fermented supernatant, *UF* ultrafiltered supernatant and SG-100: fraction eluted from column of Sephadex G-100

Table 2 Purification of killer toxins Cf8 and M12

Step	Total Volume (ml)		Total Protein (mg)		Total act. (10^6aU)		Specific act. (aU/mg)		Purification (fold)		Yield (%)	
	Cf8	M12	Cf8	M12	Cf8	M12	Cf8	M12	Cf8	M12	Cf8	M12
Fermented supernatant	1,000	1,000	9,733	10,667	6.5	6.7	668	628	1	1	100	100
Ultrafiltration (MWCO 30 kDa)	35	38	1,085	1,022	6.3	6.4	5,806	6,262	9	10	97	96
Sephadex G-100/savant	3.5	3.8	123	115	5.7	6	46,341	52,174	69	83	88	90
Sephadex G-100/savant	1	1	42	36	5.3	5.4	126,190	150,000	189	239	82	81

Table 3 Properties of killer toxin of *S. cerevisiae* Cf8 and M12

Killer toxin	Activity pH		Activity temperature		Production pH		Production temperature	
	Range	Optimal	Range	Optimal	Range	Optimal	Range	Optimal
Cf8	2.5–5.0	3.5	10–25 °C	10–20 °C	2.5–5.0	3.5	10–30 °C	15–20 °C
M12	2.5–5.0	3.5	10–25 °C	20 °C	2.5–5.0	3.5	10–30 °C	20 °C

On the other hand, Cf8 and M12 strains were able to increase the specific KA when ammonium sulphate was added to the medium as nitrogen source. The highest specific KA for both strains was reached with an addition of 500 mg N/L (Fig. 3) showing an increase of 150–220 %. In winemaking, the addition of a nitrogen source as ammonium phosphate, ammonium sulphate, ammonia, or aminoacids is a common practice in winemaking process. The literature reported that its greater benefits, besides to lead to a proper alcoholic fermentation, are the lower amount of H_2S produced by the yeasts nitrogen metabolism, the increase of the fermentation speed and the higher production of esters (Barbosa et al. 2009; Marks et al. 2003; Styger et al. 2011). The results of activity and production of killer toxins Cf8 and M12 showed that their characteristics may be due an adaptation of the yeast strains and their killer phenotype to the winery

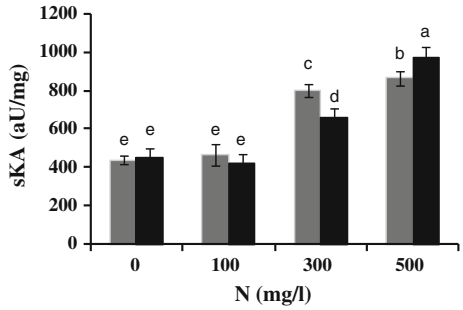


Fig. 3 Specific killer activity (aU/mg cells) for Cf8 (■) and M12 (■) strains in grape juice with different nitrogen concentrations. Values are the means of two experiments. *Vertical bars* represent standard deviation. Tukey test ($p < 0.05$)

environment. Killer toxins, as other proteins and enzymes, seem to have environmental adaptations to maintain an active performance in different conditions determined by the environment (Buzdar et al. 2011; Buzzini et al. 2012; Hua et al. 2010).

Main characteristics of killer toxins

When sensitivity of killer strains to reference toxins was assayed, Cf8 was sensitive to K6 and K10 toxins, while M12 was sensitive to K1 and K2 toxins. In addition, the cross-reactivity test revealed that both strains are not sensitive to each other toxin. The differences found in the sensitivity to reference killer strains and the lack of resistance of both strains to each other's killer toxin suggests that Cf8 and M12 toxins are not the same type, since killer strains are able to kill yeasts of some different killer types, while the producing yeast remains immune to its own toxin type (Magliani et al. 1997; Melvydas et al. 2007).

On the other hand, to determine if activity of Cf8 and M12 toxins was due to a protein or glycoprotein as for all other killer toxins, we tested the effect of three proteolytic enzymes (Comitini et al. 2004; Santos and Marquina 2004; Santos et al. 2009). Treatment with trypsin, chymotrypsin and pepsin led to a loss of 25, 85 and 45 % of KA on M12 toxin, respectively, while Cf8 toxin showed a loss of KA of 100 % after the treatments confirming their proteinaceous nature.

Stability of both toxins was assayed at 98 °C for 1 h. Cf8 and M12 killer toxins lost 10 and 30 % of KA in an aqueous medium, respectively. These results indicate that both killer toxins are thermostable (Fig. 4a) compared to other *S. cerevisiae* toxins such

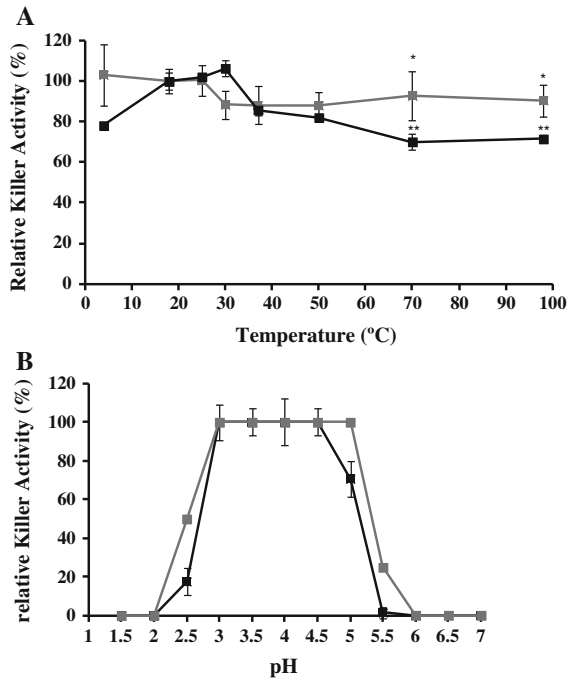


Fig. 4 Stability of killer toxins Cf8 (■) and M12 (■) after a 1 h treatment at 98 °C (**a**) and different pH values (**b**). Values are the means of three experiments. *Vertical bars* represent standard deviation. Tukey test ($p < 0.05$)

as K1, K2 and K28 which are sensitive to temperatures above 30 °C, whereas KHR losses stability near 40 °C (Goto et al. 1990; Magliani et al. 1997). Toxins stability after a 1 h treatment at different pH, between 1.0 and 7.0, was also evaluated, Cf8 was stable from pH 3.0 to 5.0, while M12 from 3.0 to 4.5 (Fig. 4b). Killer toxins of Cf8 and M12 strains showed narrow pH stability compared to other *S. cerevisiae* toxins. K1 and K2 toxins have a pH stability ranging from 2.0 to 5.0, and KHR remains stable between 3.0 and 8.0 (Goto et al. 1990; Magliani et al. 1997).

In addition, studies of hydrophobicity revealed that affinity of killer toxins to organic solvents decreased as a function of the polarity of the solvent, showing a high affinity to xylene. These results indicate the low hydrophobicity of the proteins. Further research should be done to correlate this characteristic to their killing mechanisms.

Genetic determinant of killer toxins

Most of wine *S. cerevisiae* strains produce killer toxins that are encoded by a double-stranded RNA virus

(dsRNA) (Rodríguez-Cousiño et al. 2011; Schmitt and Breinig 2006). However, Cf8 and M12 strains of *S. cerevisiae* did not show these dsRNA molecules (data not shown). To identified the genetic determinants of killer toxins, different curing agents often used to cure DNA and RNA plasmids such as temperature (37 °C), cycloheximide and acridine orange (Amberg et al. 2005), were tested. Results showed that none of the agents assayed could cure the killer phenotype of the 300 colonies of Cf8 and M12 strains assayed after treatment while the 100 % of the K1 assayed were K−. These findings suggest that genetic determinant of the killer phenotype could be chromosomally encoded.

Finally, we evaluated the presence of the KHR and KHS chromosomal genes using specific primers. To our knowledge, KHR and KHS toxins are the only chromosomally-encoded toxins. For both strains KHR primers amplified a product of approximately 580 bp, a PCR product whose size is in agreement with the expected for strain EC1118 (Goto et al. 1990), while the expected KHS products were not found. Further sequencing of the KHR amplicons revealed identity of 99 and 100 % with EC1118 KHR gene for Cf8 and M12 toxin, respectively. Goto et al. (1990) found that the KHR toxin of *S. cerevisiae* EC1118 is a 20 kDa protein, with thermostability below 40 °C for 30 min, pH stability between 3.0 and 8.0, an optimal pH of 5.6 for killer activity, self-toxicity to the producing cell, while the Cf8 and M12 toxins present completely different features: a molecular mass higher than 30 kDa (determined by ultrafiltration membranes cut-off), thermostability of 100 °C for 1 h, pH stability between 3.0 and 5.0, optimal pH of 3.5 and no toxicity to the producing cells. These findings suggest that Cf8 and M12 toxins may be similar but not identical to EC1118 KHR toxin, or that Cf8 and M12 cells produced more than one killer toxin.

Evaluation of strategies to improve killer activity

Inhibitory activity of combined killer supernatants

We tested if different combined supernatants of Cf8 with M12 or Cf13 may increase the inhibitory activity on *S. cerevisiae* P351 compared to single-strain supernatants (Fig. 5). In previous studies, these strains showed remarkable starter properties for wine-making process (Fernández de Ullivarri et al. 2011). None of the three mixed supernatants assayed between Cf8 and

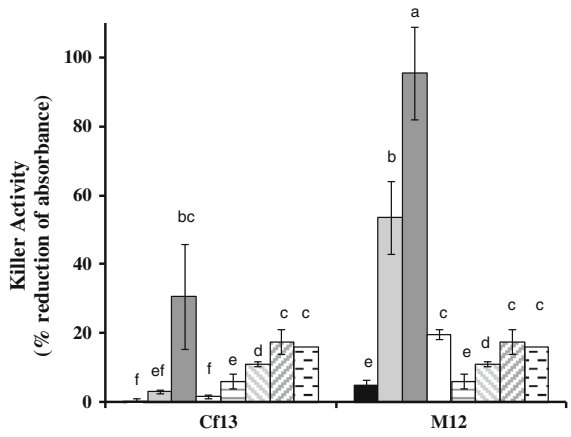


Fig. 5 Killer activity of different combinations of *S. cerevisiae* Cf8 with others killer strains against the sensitive strain *S. cerevisiae* P351. Coculture (■), monoculture (▒), sequential (▓), SKS (□), Cf8 (▤), DFC (▧), DFC Cf8 (▨) and DFC Cb2 (▦).Values are the means of three experiments. *Vertical bars* represent standard deviation. Tukey test ($p < 0.05$)

Cf13 strains reached a higher inhibition compared to the controls ($p < 0.05$). Regarding Cf8-M12, co-culture combination exhibited a lower KA than controls, probably due to the antagonistic effect of each killer toxin on the other strain, as reported above. Combined and sequential combination showed a significant increase of the KA of 280 and 500 % compared to the controls ($p < 0.05$), respectively. As Cf8-M12 sequential combination was the most efficient, we tested if the order of inoculation affects the KA, inoculating M12 first and then Cf8. Results showed that there were no differences in the KA for Cf8-M12 and M12-Cf8 sequential combination (data not shown). These findings allow us to postulate that the second strain inoculated could produce a higher amount or different inhibitory compounds in response to the presence of one or more stress factors present in the medium, produced by the first strain inoculated. It is important to note that in co-culture combination the strains growth simultaneously in the same medium and possibly the competition for nutrients and activity of each killer toxin on the other strain may affect the production of the inhibitory factors by either both strains, or one of them.

Thus, we selected Cf8 and M12 strains to continue evaluating the KA of the mixed supernatants against wine-spoilage strains due the higher inhibitory effect obtained with this combination. As shown in Fig. 6,

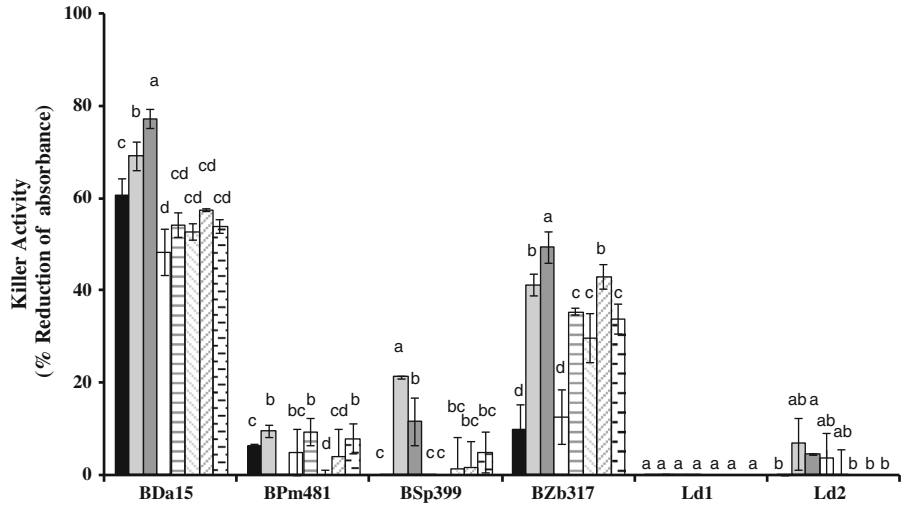


Fig. 6 Killer activity of different combinations of *S. cerevisiae* Cf8 with strain M12 against the spoilages strains *D. anomala* BDa15, *P. membranifaciens* BPm481, *Sch. pombe* BSp399, *Z. bailli* BZb317, *D. bruxellensis* Ld1 and *Z. bailli* Ld2. Coculture (■), monoculture (■) sequential (■), SKS (□), Cf8 (⊟), DFC (⊠), DFC Cf8 (⊡) and DFC Cb2 (⊡). Values are the means of three experiments. *Vertical bars* represent standard deviation. Tukey test ($p < 0.05$)

three out of six spoilage strains were significantly sensitive to the mixed supernatants of Cf8 and M12 strains. The most sensitive strains were *D. anomala* BDa15 and *Z. bailli* BZb317 showing a growth reduction of 78 and 50 % for sequential combination, respectively. In accordance with results for *S. cerevisiae* P351, co-culture supernatants were less inhibitory than combined and sequential supernatants. The inhibitory effect was strain-dependant as it can be observed for *Z. bailli* BZb317 that was very sensitive, while strain Ld2 showed virtually no growth inhibition in the combined supernatants.

These results suggest that sequential inoculation of Cf8 and M12 strains is the most effective combination to be used for biocontrol of *S. cerevisiae* and wine spoilage yeasts.

Previous works of other authors regarded the inhibitory effect of single killer strain or its toxin acting on sensitive or spoilage yeasts (Muccilli et al. 2013; Santos et al. 2009, 2011). To our knowledge, this is the first report about the effect of combined killer strains and its toxins on the inhibitory activity against spoilage yeasts, in an effort to increase the inhibition capacity of a starter culture to avoid the spoilage contamination in wine fermentations. The literature has extensively reported studies about the use of mixed simultaneous and sequential starters of *Saccharomyces* and non-*Saccharomyces* strains to improve the wine flavor and to avoid high production of aroma compounds (Bely et al. 2008; Ciani et al. 2006; Mendoza et al. 2011). In contrast, we studied and preliminarily propose the use of mixed *S. cerevisiae* strains sequentially inoculated to improve their inhibitory activity against undesired yeasts. The proposal of sequential inoculation of killer yeasts is a challenge to wine fermentations of industrial scale. However, taking in account potential benefits on the application of sequential inoculation to control spoilage microorganisms in wine industry this strategy could be considered in the future.

Effect of ethanol and SO_2 on killer activity

We evaluated the effect of ethanol and SO_2 on the killer activities of Cf8 and M12 toxins. *S. cerevisiae* P351 and *D. anomala* BDa15 were used as sensitive strains to evaluate the KA in wine fermentation conditions.

The KA of Cf8 toxin showed a maximal inhibition of 60 % at the highest concentrations of both additives (12 % ethanol and 150 mg/L SO_2). Nevertheless, in presence of 50 mg/L of SO_2 the Cf8 toxin showed an inhibition of 20 and 37 %, for ethanol 5 and 12 %,

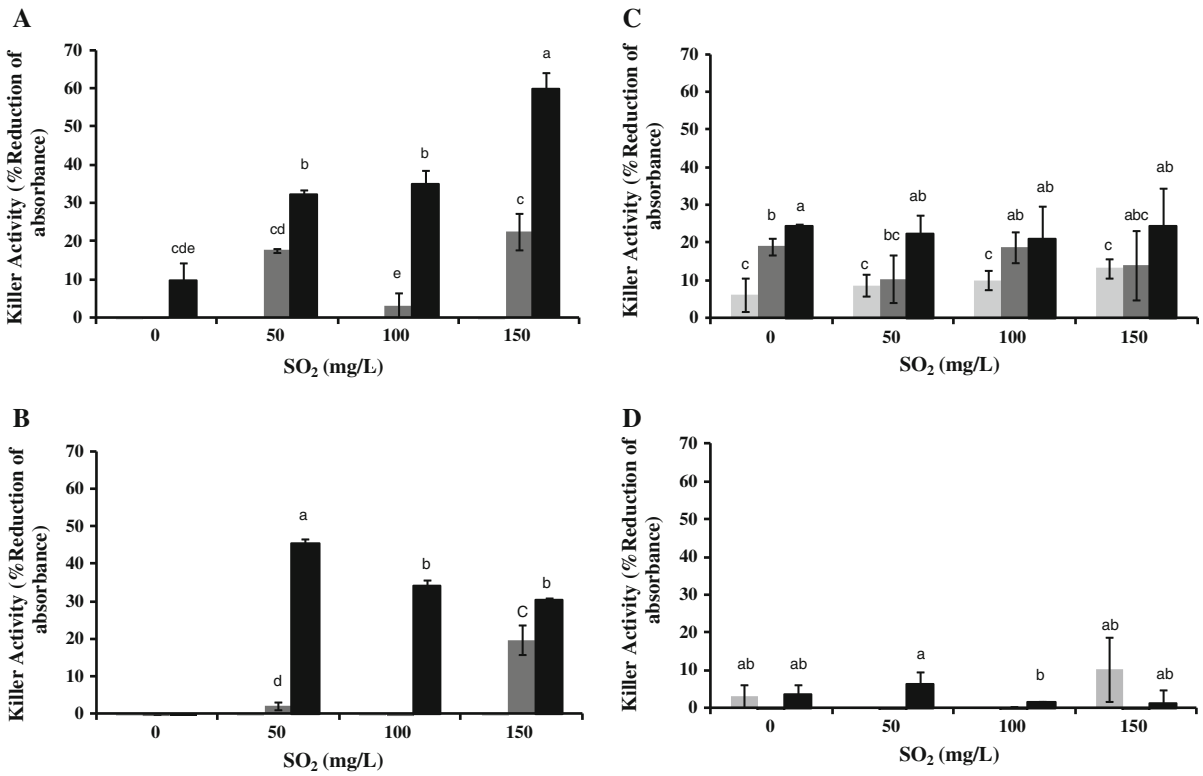


Fig. 7 Effect of ethanol at 0 (▒), 5 (■) and 12 % (■) and SO_2 concentration on the inhibitory activity of Cf8 (**a, c**) and M12 (**b, d**) toxins against *S. cerevisiae* P351 (**a, b**) and *D. anomala* BDa15(**c, d**). Values are the means of two experiments. *Vertical bars* represent standard deviation. Tukey test ($p < 0.05$)

Table 4 Determination the FIC index for killer toxins, ethanol and SO_2

SO_2 (mg/L)	Cf8			M12		
	0 % ethanol	5 % ethanol	12 % ethanol	0 % ethanol	5 % ethanol	12 % ethanol
S. cerevisiae P351						
0	–	3.35 (A)	1.90 (sub)	–	3.25 (A)	18.67 (A)
50	3.00 (A)	0.19 (Sy)	0.58 (Sy)	3.00 (A)	1.56 (Sub)	0.41 (Sy)
100	3.00 (A)	1.11 (sub)	0.53 (Sy)	3.00 (A)	3.25 (A)	0.55 (Sy)
150	3.00 (A)	0.15 (Sy)	0.31 (Sy)	3.00 (A)	0.17 (Sy)	0.61 (Sy)
D. anomala BDa15						
0	–	0.19 (Sy)	0.22 (Sy)	–	3.70 (A)	1.66 (sub)
50	0.35 (Sy)	0.35 (Sy)	0.24 (Sy)	3.09 (A)	3.76 (A)	0.98 (Sy)
100	0.31 (Sy)	0.19 (Sy)	0.26 (Sy)	3.14 (A)	3.81 (A)	3.86 (A)
150	0.23 (Sy)	0.26 (Sy)	0.22 (Sy)	0.28 (Sy)	3.50 (A)	5.82 (A)

A Antagonistic, *Sub* sub-additive, *Sy* synergistic

respectively. The KA was similar when 50 and 150 mg/L of SO_2 were used in presence of 5 % ethanol (Fig. 7a). The FIC index analysis revealed that Cf8 toxin showed synergistic interactions with SO_2 at ethanol 5 and 12 % being antagonistic in absence of ethanol (Table 4).

Meanwhile, M12 toxin exhibited the highest inhibition (45 %) with a 50 mg/L of SO_2 and ethanol 12 %, conditions corresponding to final or post-fermentation stages (Fig. 7b). Synergistic behavior was only observed at the highest concentrations of ethanol and/or SO_2 (Table 4).

On the other hand, the highest inhibition exerted by Cf8 toxin on *D. anomala* BDa15 was 25 % at ethanol 12 %. The toxin also had a high activity of 19 % at ethanol 5 %. However, SO_2 concentration had no effect on the inhibitory activity of the toxin on *D. anomala* BDa15. There were no significant differences ($p < 0.05$) among the inhibitory activities reached for each concentration (Fig. 7c). The FIC index indicated synergistic interactions of Cf8 toxin with ethanol and SO_2 at all assayed concentrations (Table 4).

M12 toxin had only little inhibitory effect (10 %) in the studied conditions (Fig. 7d). This spoilage strain seems to be less sensitive to this toxin than it is for Cf8 toxin. Regarding to the FIC index we found antagonistic interactions for this toxin in most cases (Table 4).

Our results showed that it is possible to reduce the concentration of sodium metabisulphite as a SO_2 source when killer toxins are present in the medium. Several authors have reported the use of non-*Saccharomyces* killer strains to inhibit the growth of spoilage yeasts and moulds in winemaking conditions with high inhibition results in order to reduce the use of SO_2 as an antimicrobial additive (Ciani and Fatichenti 2001; Comitini et al. 2004; Santos and Marquina 2004; Santos et al. 2009, 2011). Based on our studies we propose an alternative to reduce the concentration of SO_2 often used in wine-making practices (150 mg/L), using autochthonous *S. cerevisiae* killer strains to reduce the growth of undesired *S. cerevisiae* and *D. anomala* cells in the presence of a SO_2 concentration of 50 mg/L to reach a significant inhibition.

In conclusion, the results found in this study demonstrated that the killer toxins produced by two strains of *S. cerevisiae* (Cf8 and M12) showed appropriate characteristics, suggesting their potential and feasible application in wine fermentation. Among these characteristics, both toxins were active and stable under pH, temperature and nutritional conditions used in wine-making process. Also, these toxins were able to inhibit *S. cerevisiae* as well as spoilage yeast species using reduced SO_2 concentrations (from 150 to 50 mg/L). Furthermore, to our best knowledge this is the first report of a sequential inoculation of two *Saccharomyces* killer strains as a new strategy to improve the biocontrol against undesired yeasts in wine-making. Future studies will determine the performance of these killer strains in grape must during winemaking process and further molecular research will help to understand the killer mechanisms of these toxins.

Acknowledgments This work was supported by grants of Agencia Nacional de Promoción Científica y.Tecnológica (PICT 2010 N° 0847) and Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 0320), Argentina.

References

Amberg DC, Burke DJ, Strathern JN (2005) Methods in Yeast Genetics: a Cold Spring Harbor Laboratory Course Manual. Cold Spring, Harbor New york

Arena ME, Manca de Nadra MC (2001) Biogenic amine production by *Lactobacillus*. J Appl Microbiol 90:158–162

Barbosa C, Falco V, Mendes-Faia A, Mendes-Ferreira A (2009) Nitrogen addition influences formation of aroma compounds, volatile acidity and ethanol in nitrogen deficient media fermented by *Saccharomyces cerevisiae* wine strains. J Biosci Bioeng 108:99–104. doi:10.1016/j.jbiosc.2009.02.017

Bauer R, Cowan DA, Crouch A (2010) Acrolein in wine: importance of 3-hydroxypropionaldehyde and derivatives in production and detection. J Agr Food Chem 58:3243–3250. doi:10.1021/jf9041112

Bely M, Stoeckle P, Masneuf-Pomarède I, Dubourdieu D (2008) Impact of mixed *Torulaspora delbrueckii–Saccharomyces cerevisiae* culture on high-sugar fermentation. Int J Food Microbiol 122:312–320. doi:10.1016/j.ijfoodmicro.2007.12.023

Buzdar MA, Chi Z, Wang Q, Hua MX, Chi ZM (2011) Production, purification and characterization of a novel killer toxin from *Kluyveromyces siamensis* against a pathogenic yeast in crab. Appl Microbiol Biotechnol 91:1571–1579. doi:10.1007/s00253-011-3220-8

Buzzini P, Branda E, Goretti M, Turchetti B (2012) Psychrophilic yeasts from worldwide glacial habitats: diversity, adaptation strategies and biotechnological potential. FEMS Microbiol Ecol 82:217–241. doi:10.1111/j.1574-6941.2012.01348

Ciani M, Fatichenti F (2001) Killer toxin of *Kluyveromyces phaffii* DBVPG 6076 as a biopreservative agent to control apiculate wine yeasts. Appl Environ Microbiol 67:3058–3063

Ciani M, Mannazzu I, Marinangeli P, Clementi F, Martini A (2004) Contribution of winery-resident *Saccharomyces cerevisiae* strains to spontaneous grape must fermentation. Antonie Van Leeuwenhoek 85:159–164

Ciani M, Beco L, Comitini F (2006) Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. Int J Food Microbiol 108:239–245

Comitini F, De Ingeniis J, Pepe L, Mannazzu I, Ciani M (2004) *Pichia anomala* and *Kluyveromyces wickerhamii* killer toxins as new tools against *Dekkera/Brettanomyces* spoilage yeasts. FEMS Microbiol Lett 238:235–240

Dignard D, Whiteway M, Germain D, Tessier D, Thomas DY (1991) Expression in yeast of a cDNA copy of the K2 killer toxin gene. Mol Gen Genet 227:127–136

Du Toit WJ, Pretorius IS (2002) The occurrence, control and esoteric effect of acetic acid bacteria in winemaking. Ann Microbiol 52:155–179

Esteve-Zarzoso B, Manzanares P, Ramón D, Querol A (1998) The role of non-*Saccharomyces* yeasts in industrial winemaking. Int Microbiol 1:143–148

Fernández de Ullivarri M, Mendoza LM, Raya R, Farías ME (2011) Killer phenotype of indigenous yeasts isolated from wine cellars: potential starter cultures in winemaking from northwest region of Argentina. Biotech Lett 33:2177–2183. doi:10.1007/s10529-011-0674-9

Goto K, Iwase T, Kichise K, Kitano K, Totuka A, Obata T, Hara S (1990) Isolation and properties of a chromosome-dependent KHR killer toxin in *Saccharomyces cerevisiae*. Agric Biol Chem 54:505–509

Gutiérrez AR, Epifanio S, Garijo P, López R, Santamaría P (2001) Killer yeasts: incidence in the ecology of spontaneous fermentation. Am J Enol Vitic 52:352–356

Hua MX, Chi Z, Liu GL, Buzdar MA, Chi ZM (2010) Production of a novel and cold-active killer toxin by *Mrakia frigida* 2E00797 isolated from sea sediment in Antarctica. Extremophiles 14:515–521. doi:10.1007/s00792-010-0331-6

Izgü F, Altınbay D, Acun T (2006) Killer toxin of Pichia anomala NCYC 432; purification, characterization and its exo-β-1,3-glucanase activity. Enzyme Microb Technol 39:669–676

Kabir ME, Karim N, Krishnaswamy S, Selvakumar D, Miyamoto M, Furuichi Y, Komiyama T (2011) Peptide derived from anti-idiotypic single-chain antibody is a potent antifungal agent compared to its parent fungicide HM-1 killer toxin peptide. Appl Microbiol Biotechnol 92:1151–1160. doi:10.1007/s00253-011-3412-2

Lopes CA, Rodríguez ME, Sangorrín MS, Querol A, Caballero AC (2007) Patagonian wines: the selection of an indigenous yeast starter. J Ind Microbiol Biotechnol 34:539–546

Loureiro V, Malfeito-Ferreira M (2003) Spoilage yeasts in the wine industry. Int J Food Microbiol 86:23–50

Magliani W, Conti S, Gerloni M, Bertolotti D, Polonelli L (1997) Yeast killer systems. Clin Microbiol Rev 10:369–400

Maqueda M, Zamora E, Álvarez ML, Ramírez M (2012) Characterization, ecological distribution, and population dynamics of *Saccharomyces* sensu stricto killer yeasts in the spontaneous grape must fermentations of southwestern Spain. Appl Environ Microbiol 78:735–743. doi:10.1128/AEM.06518-11

Marks VD, van der Merwe GK, van Vuuren Hennie JJ (2003) Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. FEMS Yeast Res 3:269–287

Melvydas V, Serviene E, Cernishova O, Petkuniene O (2007) A novel X factor secreted by yeast inhibits *Saccharomyces cerevisiae* K1, K2 and K28 killer toxins. Biologija 53:32–35

Mendoza LM, Merín MG, Morata VI, Farías ME (2011) Characterization of wines conducted by mixed culture of autochthonous yeasts and *Oenococcus oeni* from the Northwest region of Argentina. J Ind Microbiol Biot 38:1777–1785. doi:10.1007/s10295-011-0964-1

Meneghin MC, Reis VR, Ceccato-Antonini SR (2010) Inhibition of bacteria contaminating alcoholic fermentations by killer yeasts. Braz Arch Biol Technol 53:1043–1050

Muccilli S, Wemhoff S, Restuccia C, Meinhardt F (2013) Exoglucanase-encoding genes from three *Wickerhamomyces anomalus* killer strains isolated from olive brine. Yeast 30:33–43. doi:10.1002/yea.2935

Oliva-Neto P, Ferreira MA, Yokoya F (2004) Screening for yeast with antibacterial properties from an ethanol distillery. Biores Technol 92:1–6

Pasteris SE, Strasser de Saad AM (2009) Sugar-glycerol cofermentations by *Lactobacillus hilgardii* isolated from wine. J Agric Food Chem 57:3853–3858. doi:10.1021/jf803781k

Perea S, Gonzalez G, Fothergill AW, Kirkpatrick WR, Rinaldi MG, Patterson TF (2002) In vitro interaction of caspofungin acetate with voriconazole against clinical isolates of *Aspergillus* spp. Antimicrob Agents Ch 46:3039–3041

Perrone B, Giacosa S, Rolle L, Cocolin L, Rantsiou K (2013) Investigation of the dominance behavior of *Saccharomyces cerevisiae* strains during wine fermentation. Int J Food Microbiol 165:156–162. doi:10.1016/j.ijfoodmicro.2013.04.023

Pfeiffer I, Golubev WI, Farlas Z, Kucsera J, Golubev N (2004) Mycocin production in *Cryptoccocus aquaticus*. Anton Leeuw Int J G 86:369–375

Rodríguez-Cousiño N, Maqueda M, Ambrona J, Zamora E, Esteban R, Ramírez M (2011) A new wine *Saccharomyces cerevisiae* double-stranded RNA virus encoded killer toxin (Klus) with broad antifungal activity is evolutionarily related to a chromosomal host gene. Appl Environ Microbiol 77:1822–1832. doi:10.1128/AEM.02501-10

Sangorrín M, Zajonskovsky I, van Broock M, Caballero A (2002) The use of killer biotyping in an ecological survey of yeast in an old Patagonian winery. World J Microb Biotechnol 18:115–120

Santos A, Marquina D (2004) Killer toxin of *Pichia membranifaciens* and its possible use as a biopreservative agent to control grey mould disease of grapevine. Microbiology 150:2527–2534

Santos A, San Mauro M, Bravo E, Marquina D (2009) PMKT2, a new killer toxin from *Pichia membranifaciens*, and its promising biotechnological properties for control of the spoilage yeast *Brettanomyces bruxellensis*. Microbiology 155:624–634. doi:10.1099/mic.0.023663-0

Santos A, Navascués E, Bravo E, Marquina D (2011) *Ustilago maydis* killer toxin as a new tool for the biocontrol of the wine spoilage yeast *Brettanomyces bruxellensis*. Int J Food Microbiol 145:147–154. doi:10.1016/j.ijfoodmicro.2010.12.005

Schmitt MJ, Breinig F (2002) The viral killer system in yeast: from molecular biology to application. FEMS Microbiol Rev 26:257–276

Schmitt MJ, Breinig F (2006) Yeast viral killer toxins: lethality and self-protection. Nat Rev Microbiol 4:212–221

Styger G, Prior B, Bauer FF (2011) Wine flavor and aroma. J Ind Microbiol Biotechnol 38:1145–1159. doi:10.1007/s10295-011-1018-4

Yamashiro D (2013) Partition and partition chromatography of peptides and proteins. In: Li CH (ed) Hormonal proteins and peptides. Academic Press, London, pp 25–107

Zagorc T, Maraz A, Cadez N, Povhe Jemec K, Peter G, Resnik M, Nemanik J, Raspor P (2001) Indigenous wine killer yeasts and their application as a starter culture in wine fermentation. Food Microbiol 18:441–451