

Saccharomyces cerevisiae CCM1 885 secretes peptides that inhibit the growth of some non-*Saccharomyces* wine-related strains

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Abstract The nature of the toxic compounds produced by *Saccharomyces cerevisiae* CCM1 885 that induce the early death of *Hanseniaspora guilliermondii* during mixed fermentations, as well as their ability to inhibit the growth of other non-*Saccharomyces* wine-related strains, was investigated. The killing effect of mixed supernatants towards *H. guilliermondii* was inactivated by protease treatments, thus revealing the proteinaceous nature of the toxic compounds. Analysis of the protein pattern of mixed supernatants on Tricine SDS-PAGE showed that this *S. cerevisiae* strain secretes peptides (<10 kDa), which were detected only when death of *H. guilliermondii* was already established. Death-inducing supernatants were ultrafiltrated by 10 and 2 kDa membranes, respectively, and the inhibitory effect of those permeates were tested in *H. guilliermondii* cultures. Results indicated that the (2–10) kDa protein fraction of those supernatants seemed to contain antimicrobial peptides active against *H. guilliermondii*. Thus, the (2–10) kDa protein fraction was concentrated and its inhibitory effect tested against strains of *Kluyveromyces marxianus*, *Kluyveromyces thermotolerans*, *Torulaspora delbrueckii* and *H. guilliermondii*. Under the growth conditions used for these tests, the (2–10) kDa protein fraction of *S. cerevisiae* CCM1 885 supernatants exhibited a fungistatic effect against all the strains and a fungicidal effect against *K. marxianus*.

Keyword Antimicrobial peptides · Yeast–yeast interactions · Wine fermentations · *Saccharomyces cerevisiae* · Non-*Saccharomyces* · Yeast population dynamic

Introduction

Hanseniaspora species have been widely reported as being the major non-*Saccharomyces* (NS) yeasts associated with grape microbiota and the initial stages of wine fermentations (Fleet and Heard 1993; Sabate et al. 2002; Capece et al. 2005). Certain strains of these apiculate yeasts have been found to enhance the wine flavour (Romano et al. 1997; Granchi et al. 2002; Romano et al. 2003). However, their contribution to the final composition of wine is limited since they begin to die off after the first few days of fermentation leaving way to the more fermentative *Saccharomyces cerevisiae* (Sc) strains to complete the vinification process (Egli et al. 1998; Lambrechts and Pretorius 2000; Fleet 2003).

The early disappearance of indigenous NS species, such as *Hanseniaspora uvarum* (Hu), *Hanseniaspora guilliermondii* (Hg), *Candida stellata* (Cst), *Kluyveromyces thermotolerans* (Kt), *Kluyveromyces marxianus* (Km) and *Torulaspora delbrueckii* (Td), from fermenting grape musts has been always ascribed to their weak capacity to withstand the increasingly adverse growth conditions as the fermentation progresses (Fleet and Heard 1993; Pretorius 2000). The selective pressure exerted by high levels of ethanol and organic acids, low pH values, scarce oxygen availability, depletion of certain nutrients, as well as other yeast–yeast interactions (e.g. killer toxins) have been referred as the main factors responsible for the fact that only few Sc strains can subsist until the end of wine fermentations (Bisson 1999; Bauer and Pretorius 2000; Hansen et al. 2001).

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The predominance of these classical factors on the yeast successions during wine fermentations has been recently questioned and other yeast–yeast interactions have been suggested by different authors (Ciani and Pepe 2002; Nissen and Arneborg 2003; Nissen et al. 2003; Arneborg et al. 2005; Pérez-Nevado et al. 2006). In a previous work, Pérez-Nevado et al. (2006) demonstrated that the early death of two NS wine-related strains (Hg and Hu) during mixed fermentations with Sc was not primarily induced by ethanol, nutrient or oxygen depletion, low pH values or classical killer toxins. It was further shown that Hg early death was induced by unknown toxic compounds produced during mixed fermentations with Sc (Pérez-Nevado et al. 2006).

During alcoholic fermentations yeasts produce besides ethanol other metabolites, such as short to medium-chain fatty acids (e.g. acetic, hexanoic, octanoic and decanoic acids) that can become inhibitory to other yeast species, including some strains of Sc (Viegas et al. 1989; Edwards et al. 1990; Bisson 1999; Fleet 2003). Production of these fatty acids varies significantly with yeast species and strain (Lema et al. 1996; Lambrechts and Pretorius 2000) but the impact of this factor on the sequential growth of yeasts during wine fermentations is still ill studied (Fleet 2003).

Other inhibitory mechanism that can occur in wine fermentations is the so-called yeast killer activity (Shimizu 1993), which consists of the production of specific extracellular glycoproteins able to induce death of other yeast strains (Woods and Bevan 1968; Marquina et al. 2002; Schmitt and Breinig 2002). Several NS wine-related strains have been identified as killer toxin producers. However, the killer toxins produced by Sc that were identified and characterized up till now (K1, K2 and K28) do not act against other yeast species but only against sensitive strains of the same species (Chen et al. 2000; Yap et al. 2000; Ciani and Faticenti 2001). Although the relation between killer activity and the early disappearance of NS yeasts from wine fermentations has not been well established, the involvement of killer-like toxins in the yeast–yeast, as well as yeast–bacteria, interactions during wine fermentations has been mentioned by some authors (Pretorius 2000). Indeed, recent studies showed that certain Sc wine strains produce proteinaceous compounds that are active against the growth of malolactic bacteria (Comitini et al. 2005; Osborne and Edwards 2007). Moreover, synthetic bioactive peptides have been reported to exhibit fungistatic and fungicidal activity against some wine spoilage yeasts (Enrique et al. 2007).

The aim of the present work was to investigate the nature of the toxic compounds produced by Sc strain CCMI 885 and to determine their inhibitory effect against other NS wine-related strains.

Material and methods

Microorganisms

Four NS strains and one Sc strain were used in the present work: Hg NCYC 2380; Kt PYCC 2908; Km PYCC 2671; Td PYCC 4487; Sc CCMI 885. PYCC is the abbreviation of the Portuguese Yeast Culture Collection (New University of Lisbon, Portugal) and CCMI of the Culture Collection of Industrial Microorganisms (INETI, Portugal). Strain Sc CCMI 885 was previously tested and classified as killer negative regarding classical Sc killer toxins (K1, K2 and K28) (Pérez-Nevado et al. 2006). Yeast strains were maintained on YEPD-agar slants (20 g l⁻¹ glucose, 5 g l⁻¹ yeast extract, 10 g l⁻¹ peptone, 3 g l⁻¹ malt extract, 20 g l⁻¹ agar), stored at 4°C.

Growth media and inoculum conditions

Inoculum cultures were performed in YEPD medium (10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone and 20 g l⁻¹ glucose). Single and mixed fermentations were performed in synthetic grape juice media (SGJ), prepared by mixing three solutions (A, B and C) as described by Pérez-Nevado et al. (2006). The final composition of the SGJ is (per litre): (from solution A) D-glucose, 110 g; D-fructose, 110 g; (from solution B) L-(1)-tartaric acid, 6.0 g; L-(2)-malic acid, 3.0 g; citric acid, 0.5 g; (from solution C) YNB (yeast nitrogen base; Difco), 1.7 g; CAA (vitamin-free Casamino Acids; Difco), 2.0 g; CaCl₂, 0.2 g; arginine-HCl, 0.8 g; L-(2)-proline, 1.0 g; L-(2)-tryptophan, 0.1 g. Solutions B and C were buffered at pH 3.5 with NH₄OH and H₃PO₄, respectively. The inhibitory tests were performed using a modified SGJ (mod-SGJ) that contained the same composition of the SGJ but just half of the sugars (i.e. 55 g l⁻¹ of glucose and 55 g l⁻¹ fructose) and 50 g l⁻¹ of ethanol.

Inoculums of all yeast strains used in the present work were obtained by transferring one YEPD-agar slant of each strain (pre-grown at 30°C for 48 h) into 50 ml of YEPD medium and incubating cultures at 30°C with 150 rpm of agitation during 16 h.

Mixed and single fermentations of Sc and Hg

Single fermentations (SF) of Sc and Hg and mixed fermentations (MF) of Sc/Hg were performed in 2 l flasks containing 1.5 l of SGJ. MF was inoculated with Hg and Sc inoculum cultures to attain an initial cell density of 1 × 10⁶ colony forming units (cfu) ml⁻¹ for each strain and SF of Hg and Sc with 1 × 10⁶ cfu ml⁻¹ of each strain, respectively. All fermentations were conducted at 20°C, without agitation and all experiments were done in duplicate. Samples

from each culture were taken daily to quantify cell viability by plate counts and determine sugars (glucose and fructose) and ethanol concentrations.

Cultivation of Hg in single (Sc and Hg) and in mixed (Hg and Sc) supernatants

Cell-free supernatants from previous MF (Sc/Hg) and SF (Sc and Hg) were obtained by filtration of 200 ml of culture medium after 4 days for the MF and SF of Sc and 5 days in the case of SF of Hg. Each cell-free supernatant was supplemented with 5 g l^{-1} of yeast extract (to avoid nitrogen limitations) and the ethanol concentration adjusted to 50 g l^{-1} in all cases. Those supernatants were inoculated with 10^6 cfu ml^{-1} of Hg and incubated at 20°C , without agitation. The cell viability of each culture was determined by plate counts.

Protease treatments

A cell-free supernatant obtained from a 4-day-old MF (Sc/Hg) was subjected to protease treatments. The following proteases, pepsin and a mixture of trypsin plus alkaline protease (all supplied by Sigma, USA), were added to 100 ml of mixed supernatant (MS) at a final concentration of 2 g l^{-1} . The pH of each protease-added supernatant was set to a final value according to optimal protease activity (pH 2.0 for pepsin and pH 7.5 for trypsin + alkaline protease mixture). Flasks containing the MS, added and non-added with the above-mentioned proteases, were incubated at 37°C for 72 h. Afterwards, all flasks were autoclaved (121°C for 15 min) for protease inactivation, supplemented with 5 g l^{-1} of yeast extract and pH values readjusted to 3.5. Each untreated and protease-treated supernatants were then inoculated with Hg at an initial cell density of 10^6 cfu ml^{-1} . All cultures were incubated at the same growth conditions used for SF and MF and experiments performed in duplicates. Cell viability was monitored throughout cultivation by plate counts.

Protein electrophoresis

The protein pattern of a MS obtained from 1, 2, 4 and 7-day-old MF (Sc/Hg) and of the (2–10) kDa protein fraction of a 4-day-old MS were analysed by Tricine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schägger and Von Jagow (1987). The protein content of 15 ml of each supernatant was precipitated using the same volume of trichloroacetic acid (20% w/v). Protein precipitates were then resuspended in Tricine sample buffer (Invitrogen) and loaded into a 10–20% gradient Tris-Tricine Novex® PreCast Gels. Electrophoresis was carried out using a Xcell SureLock™ Mini-Cell apparatus (Invitrogen) and the gels were silver stained.

Partial purification of supernatant proteins by membrane ultrafiltration

A 4-day-old MS was ultrafiltered through centrifugal filter units (Vivaspin 15R, Sartorius, Germany) equipped with 10 and 2 kDa cut-off membranes and two ultrafiltered MS were obtained: one containing $<10 \text{ kDa}$ proteins and other $<2 \text{ kDa}$ proteins. A concentrated (5-fold; 2–10) kDa protein fraction was obtained by first passing the MS through the 10 kDa centrifugal unit and then concentrating (5-fold) this permeate in the 2 kDa centrifugal unit.

Inhibitory tests of the different protein fractions

The ultrafiltered MS (<2 and $<10 \text{ kDa}$, respectively) were inoculated with 10^6 cfu ml^{-1} of Hg and incubated at 20°C , without agitation. The cell viability of these cultures was evaluated by plate cell counts during several days and compared with the viable cell profile of Hg in a non-ultrafiltered MS. All ultrafiltered and non-ultrafiltered supernatants were supplemented with 5 g l^{-1} of yeast extract.

To evaluate the growth inhibitory effect of the (2–10) kDa peptidic fraction, one strain of four different yeast species (Hg, Km, Kt and Td) were cultivated in mod-SGJ medium (110 g l^{-1} of total sugars and 50 g l^{-1} of ethanol) with and without addition of that protein fraction. Cultures were performed in 150-ml flasks containing 75 ml (final volume) of mod-SGJ to which the (2–10) kDa fraction (5-fold concentrated) was added. Each flask was inoculated with $1 \times 10^6 \text{ cfu ml}^{-1}$ of each NS strain and incubated at 20°C , without agitation, for several days. The growth profile of each culture was analysed by viable cells counts and absorbance measurements at 600 nm. All experiments were carried out in duplicates.

Analysis of growth

Yeast growth was analysed by viable cells enumeration using the classical plate count method. Samples, taken aseptically throughout the fermentations, were inoculated onto YEPD-agar plates after appropriate dilution in sterile water. In the MF of Sc/Hg, enumeration of Hg was obtained by cell counts on 0.01% cycloheximide YEPD-agar plates and the number of Sc viable cells determined as the difference between the Hg cell counts and the total number of colonies on the YEPD-agar plates without cycloheximide. All plates were incubated at 30°C for 2–4 days and viable cells enumeration carried out after no increase on the cfu was observed.

Analytical assays

Glucose, fructose and ethanol concentrations of sterile samples (after filtration through 0.45 μm Millipore membranes) were determined by HPLC (Merck Hitachi, Darmstadt, Germany) using a Sugar-PakTM column (Waters, Milford, USA) connected to a RI detector (L-7490, Merck Hitachi, Darmstadt, Germany). The column was eluted at 90°C with degassed aqueous mobile phase containing 50 mg l⁻¹ CaEDTA, at a flow rate of 0.5 ml min⁻¹. All samples were analysed in duplicate.

Results

Growth profile of Hg and Sc during single and mixed fermentations

Analysis of the cell density profiles of Sc and Hg during MF and SF performed in SGJ at enological growth conditions (Fig. 1a), showed that both strains grow at similar rates during the first day of fermentation reaching similar cell density values, of about 10⁷ cfu ml⁻¹. After the first day, the cell viability of Sc remained almost unchanged, either in MF or in SF, but the cell viability of Hg drastically changed between MF and SF (Fig. 1a). Cell density of Hg in the SF raised up to 10⁸ cfu ml⁻¹ and kept that value till day 5, while in the MF decreased from 2 × 10⁷ cfu ml⁻¹ at day 1 to 5 × 10² cfu ml⁻¹ at day 4. Sugar consumption and ethanol production rates were very similar between the MF and the SF of Sc but much slower in the SF of Hg (Fig. 1b). After 4 days of fermentation yeasts consumed *ca* 120 g l⁻¹ of sugars and produced *ca* 58 g l⁻¹ of ethanol in the MF and in the SF of Sc, while in the SF of Hg the same amount of sugars was consumed in 5 days producing 48 g l⁻¹ of ethanol.

Toxic compounds inactivation by protease treatments

Cell-free supernatants obtained from the previous MF and SF of Sc after 4 days of cultivation and from the SF of Hg after 5 days, were inoculated with 10⁶ cfu ml⁻¹ of Hg. Viable cells profiles of Hg on those cultures are represented on Fig. 2a and show total death of Hg within 4 days in the 4-day-old MS and similar death profile in the 4-day-old SS of Sc (1.2 × 10¹ cfu ml⁻¹ at day 4). However, in the 5-day-old Hg SS the cell viability of Hg remained at high values (above 10⁵), although a slight decrease occurred during the 7 days of cultivation (from 4 × 10⁶ to 4 × 10⁵ cfu ml⁻¹). Since all supernatants (single and mixed) contained similar amounts of ethanol (*ca.* 50 g l⁻¹) and were all supplemented with a nitrogen source, it can be stated that death of Hg was induced by toxic compounds produced by Sc, other than ethanol or nutrient depletion.

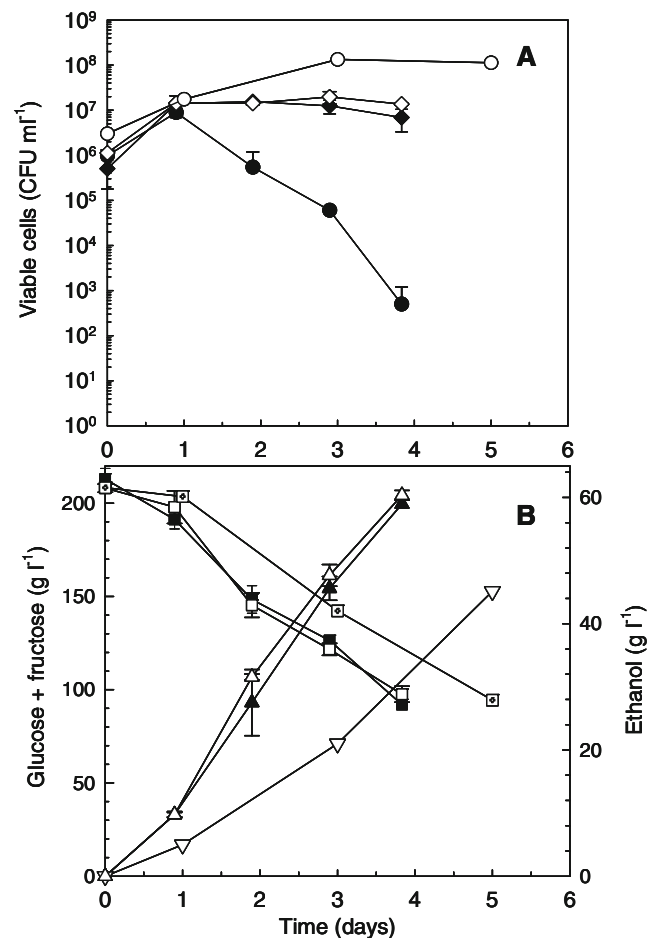


Fig. 1 Viable cells of Sc (filled diamonds and empty diamonds) and Hg (filled circles and empty circles) (a), sugars consumption (filled squares, empty squares and squared dot operators) and ethanol production (filled triangles, empty triangles and inverted empty triangles) (b) during a MF of Sc/Hg (filled diamonds, filled circles, filled squares and filled triangles), a SF of Sc (empty diamonds, empty squares and empty triangles) and SF of Hg (empty circles, squared dot operators and inverted empty triangles). Data presented are mean values of two independent experiments \pm standard deviation (error bars)

In order to investigate the nature of those toxic compounds, the 4-day-old MS was treated with different proteases (pepsin and a mixture of trypsin plus alkaline protease) and cell viability of Hg in those supernatants was compared with the untreated MS (Fig. 2b). Results showed that both protease-treated MS allowed the growth of Hg, while in the untreated MS a total loss of cell viability was observed within 3–4 days (Fig. 2b). These findings clearly demonstrated that the 4-day-old MS contained toxic compounds of proteinaceous nature that were inactivated by the proteases. Besides, death profile of Hg in the 4-day-old MS (without protease treatment) after autoclavation (121°C for 15 min) (Fig. 2b) was very similar to the one exhibited in the 4-day-old MS not subjected to the previous heat procedure (Fig. 2a), which means that these antimicrobial proteinaceous compound(s) are heat-resistant.

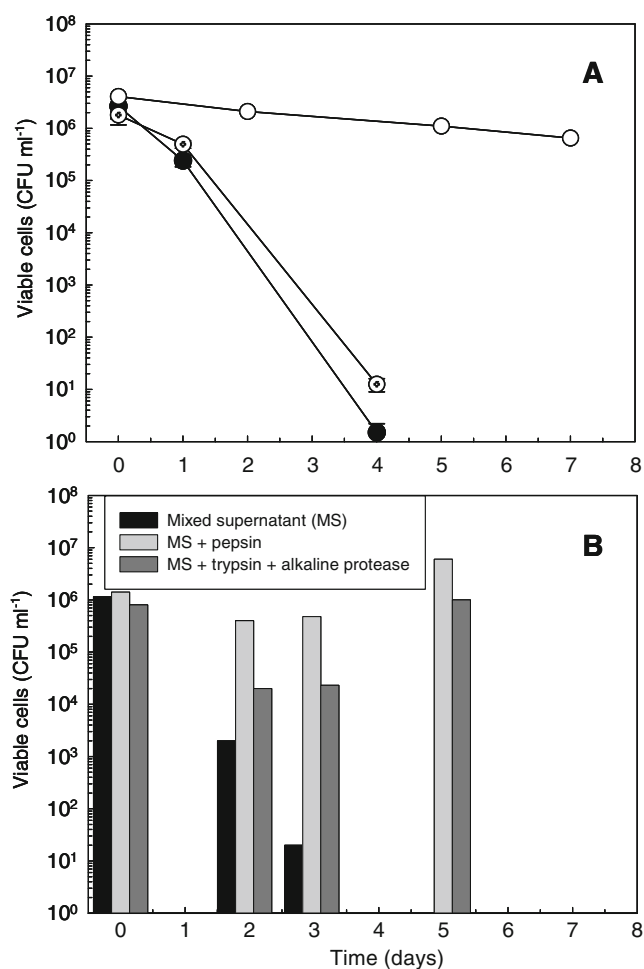


Fig. 2 Cell viability of Hg in a 4-day-old MS (Sc/Hg; filled circles), in a 4-day-old SS (Sc; circle dot operators) and in a 5-day-old SS (Hg; empty circles) (a); Cell viability of Hg in a 4-day-old MS (Sc/Hg) non-treated with proteases and treated with pepsin and with a mixture of trypsin plus alkaline protease (b). Data presented are mean values of two independent experiments \pm standard deviation (error bars)

Protein pattern during mixed fermentation (Sc/Hg)

The protein pattern of supernatants obtained after 1, 2, 4 and 7 days of (Sc/Hg) MF and also of the (2–10) kDa protein fraction of a 4-day-old MS were analysed on Tricine SDS-PAGE gel. Results showed the presence of three small bands, of apparent MW of about 6.0, 4.5 and 4.0 kDa, after the second day of MF (Fig. 3). The intensity of those small bands (<10 kDa) increased continuously from days 2 till day 7 in the MF (Fig. 3, lanes 1, 3 and 4). Besides, when supernatants obtained from an SF of Sc were analysed in SDS-PAGE gels the same small bands (<10 kDa) appeared at the same days of fermentation (data not shown). These results indicate that the peptides lower than 10 kDa are produced by Sc stain. Since death of Hg was observed either in the 4-day-old MS or in the 4-day-old (Sc) SS (Fig. 2a), these peptides (<10 kDa) might correspond to the toxic compounds responsible for death of Hg.

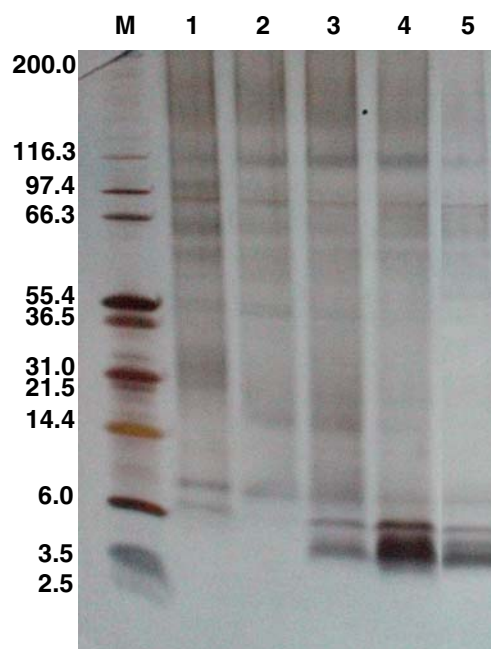


Fig. 3 Silver stained 10–20% gradient Tricine SDS-PAGE gel. *M* molecular weight standards (Invitrogen); Lane 1, 2-day-old MS; lane 2, 1-day-old MS; lane 3, 4-day-old MS; lane 4, 7-day-old MS; lane 5, (2–10) kDa protein fraction of the 4-day-old MS

Inhibitory effect of the small peptides

To test the previous hypothesis, we inoculated two ultrafiltrated MS, one <2 kDa and other <10 kDa, with 10^6 cfu ml⁻¹ of Hg and compared the cell viability of both cultures with the one exhibited in the non-ultrafiltrated MS. Results showed that ultrafiltration of the MS by 10 kDa membranes had no effect on the death profile of Hg by comparison with the non-ultrafiltrated MS (Fig. 4b), which demonstrated that proteins of MW above 10 kDa were not responsible for the death of Hg. Conversely, when proteins of MW above 2 kDa were removed from the MS, i.e. in the <2 kDa ultrafiltrated MS, a higher percentage of viable cells was observed throughout cultivation (Fig. 4a), showing that death-inducing capacity of the MS decreased after ultrafiltration. Despite this, the <2 kDa ultrafiltrated MS was still able to induce death of Hg, although at a slower rate. This could be due either to the existence of other toxic compounds besides the small proteins (<2 kDa) in the MS or to incomplete removal of the >2 kDa proteins from the MS during the ultrafiltration process. Nevertheless, the present results suggested that the (2–10) kDa protein fraction of the MS seemed to exert inhibitory effect against Hg.

Thus, a (2–10) kDa peptidic fraction of a MF (4-day-old) was obtained by fractioning and concentrating the MS through 10 and 2 kDa membranes and analysed on Tricine SDS-PAGE gels to confirm the presence of the three small peptides (4.0, 4.5 and 6.0 kDa) (Fig. 3, lane 5). Then, the (2–10) kDa peptidic fraction (5-fold concentrated) was

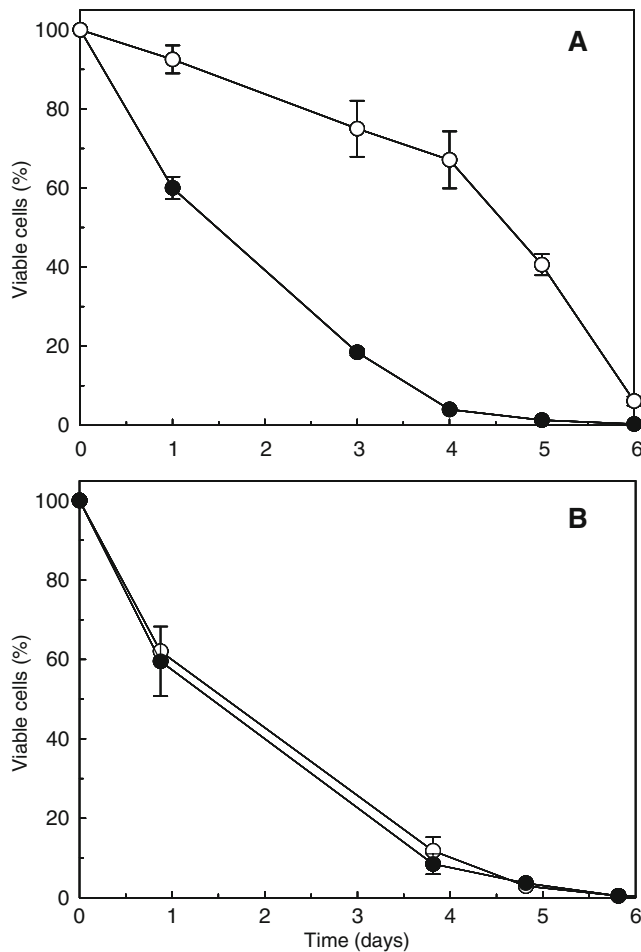


Fig. 4 Percentage of Hg viable cells in a 4-day-old MS, non-ultrafiltrated (*filled circle*), ultrafiltrated by 2 kDa membranes (*empty circle*) (**a**) and ultrafiltrated by 10 kDa membranes (*empty circle*) (**b**). Data presented are mean values of two independent experiments \pm standard deviation (*error bars*)

added to mod-SGJ and its inhibitory effect evaluated against strains of Km, Kt, Td and Hg. Each strain was inoculated in mod-SGJ without (control) and with the (2–10) kDa peptidic growth and growth followed by absorbance and viable cells counts (Fig. 5 a to d). Results showed that the (2–10) kDa protein fraction of the MS delayed the growth of all yeast strains and induced death of Km. These results pointed out that at least one of the three peptides (MW of 4.0, 4.5 and 6.0 kDa) produced by this Sc strain seems to exert a fungistatic and/or a fungicidal effect against these NS yeast strains.

Discussion

During spontaneous wine fermentations NS yeast strains begin to die off after the first days of alcoholic fermentation (Fleet and Heard 1993; Sabate et al. 2002). This behaviour has been attributed to weaker fermentative capacity of these

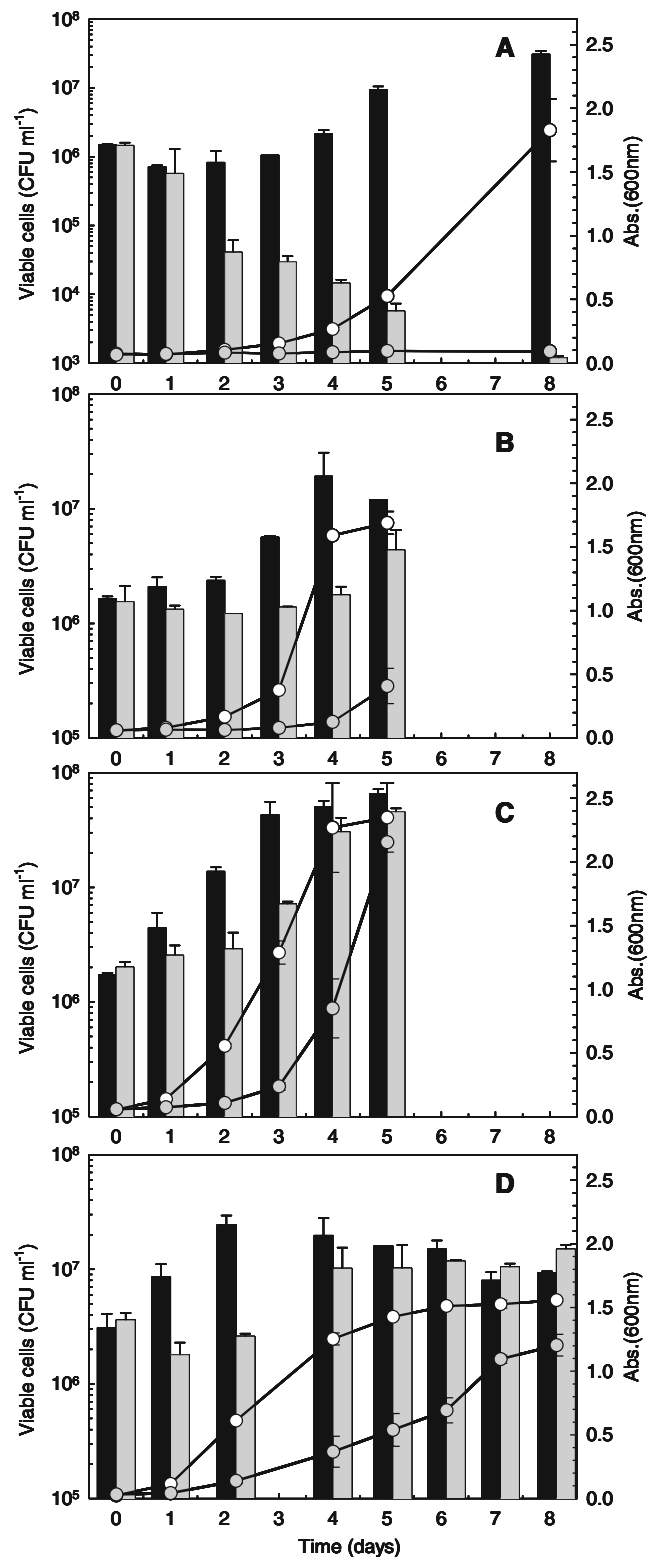


Fig. 5 Absorbance (*circles*) and viable cells (*bars*) of Km (**a**), Kt (**b**), Td (**c**) and Hg (**d**) cultures performed in mod-SGJ medium (*white circles and dark bars*) and in mod-SGJ with the (2–10) kDa peptidic fraction of MS (*grey circles and grey bars*)

yeast strains, lower ethanol tolerance and other adverse factors, such as low pH values, scarce oxygen availability or nutrient limitations (Fleet and Heard 1993; Pretorius 2000). However, previous work (Pérez-Nevado et al. 2006) carried out with two *Hanseniaspora* strains (Hg and Hu) showed that their early death during mixed fermentations with Sc was not primarily induced by ethanol, nutrient or oxygen depletion, low pH values or classical killer toxins. Conversely, results pointed out that other unknown toxic compounds should be responsible for that phenomenon. In the present study, we investigated the nature of the toxic compounds and studied their inhibitory effect on the growth of Hg.

Firstly, our data shows that Sc produces one or more proteinaceous compounds able to induce death of Hg, since protease treatments of death-inducing supernatants prevented death of Hg (Fig. 2). Besides, our study, which was carried out under enological growth conditions—low pH values, low oxygen availability and high sugars content—demonstrates that these wine-related NS strains (Km, Kt, Td and Hg) are not only able to withstand ethanol concentrations of about 50 g l⁻¹ but are even able to grow under these stressful conditions (Fig. 5 a to d). These results agree with those of Pina et al. (2004), showing that some NS strains withstand much higher ethanol concentrations than previously reported (Fleet and Heard 1993).

The present work had also shown that the (2–10) kDa protein fraction of Sc CCMI 885 supernatants exert a fungistatic effect against some NS wine-related strains and a fungicidal effect against Km (Fig. 5 a to d). The protein pattern of this (2–10) kDa fraction was analysed on SDS-PAGE (Fig. 3, lane 5) and results showed the presence of three bands (of about 4.0, 4.5 and 6.0 kDa) that correspond to peptides produced by this Sc strain during alcoholic fermentation. Thus, our results strongly suggest that one or more of those peptides might correspond to antimicrobial compounds produced by Sc that are active against some NS wine-related strains. The different sensitivity exhibited by each NS species towards this (2–10) kDa protein fraction suggests that this antimicrobial effect is species-dependent. Future work must be carried out in order to characterise the antimicrobial effect of these peptides against different yeast species and strains.

The existence of killer yeasts amongst the wine yeast microflora has long been recognised (Shimizu 1993; Vadasz et al. 2000; Fleet 2003). However, the classical killer toxins produced by Sc (K1, K2 and K28) are active only against strains of the same species and in a previous work (Pérez-Nevado et al. 2006) it was demonstrated that those killer toxins were not involved in the early death of this Hg strain. Moreover, conversely to most killer toxins that are heat-labile, these antimicrobial compounds(s) were not inactivated by heat (Fig. 2b). Indeed, the majority of the yeast killer

toxins are protease-sensitive, heat-labile glycoproteins that are stable and act only at acidic pH values (Marquina et al. 2002). Nevertheless, some killer toxins have been found to be much more stable, as those of *Hansenula mrakii*, stable at pH 2–11 and unaffected by heating at 60°C for 1 h, and of *H. saturnus*, stable at pH 3–11 and 75 % stable at 80°C for 1 h (Kimura et al. 1993; Takasuka et al. 1995). Some bacteriocins related with wine environments and comprised by small peptides had also shown to be heat-resistant (Navarro et al. 2000). In addition, the existence of synthetic and natural bioactive peptides able to act against wine spoilage yeasts have recently been reported (Enrique et al. 2007). Recent studies have also shown that certain Sc strains produce proteinaceous compounds able to inhibit the growth of *Oenococcus oeni* strains and malolactic fermentation (Comitini et al. 2005; Osborne and Edwards 2007). Although the proteinaceous compounds found either in the above-mentioned studies or in the present one, have not been identified at a molecular level they strongly suggest that antimicrobial peptides may be an important factor to consider when dealing with microbial population dynamics of wine fermentations. To the best of our knowledge, this is the first time that peptides produced by Sc are reported as being possible inhibitors of the growth of wine-related NS yeasts under conditions mimicking those occurring in wine fermentations.

Future work will be carried out in order to purify and sequence the putative bioactive peptides and to investigate their antimicrobial range of action, as well as their biochemical and inhibitory properties.

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