Occurrence of killer yeast strains in industrial and clinical yeast isolates

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ABSTRACT

The secretion of proteinaceous toxins is a widespread characteristic in environmental and laboratory yeast isolates, a phenomenon called "killer system". The killer phenotype (K^+) can be encoded by extrachromosomal genetic elements (EGEs) as double stranded DNA or RNA molecules (dsDNA, dsRNA) or in nuclear genes. The spectrum of action and the activity of killer toxins are influenced by temperature, salinity and pH of media. In the present work we determined the existence of K^+ in a collection of *S. cerevisiae* and *P. anomala* yeasts isolated from environmental, industrial and clinical sources. The assays were performed in strains belonging to three yeast genera used as sensitive cells and under a wide range of pH and temperatures. Approximately 51 % of isolates tested showed toxicity against at least one sensitive yeast strain under the conditions tested. The K^+ *P. anomala* isolates showed a wide spectrum of action and two of them had toxic activity against strains of the three yeast genera assayed, including *C. albicans* strains. In all *S. cerevisiae* K^+ isolates an extrachromosomal dsRNA molecule (4.2 Kb) was observed, contrary to *P. anomala* K^+ isolates, which do not possess any EGEs. The K^+ phenotype is produced by an exported protein factor and the kinetics of killer activity production was similar in all isolates with high activity in the log phase of growth, decaying in the stationary phase.

Key terms: killer system, mycotoxin, dsRNA.

INTRODUCTION

Many yeast synthesize and export proteins or glycoproteins with toxic effects against sensitive yeasts, a phenomenon called "killer system" (Young and Yagiu, 1978; Tipper and Bostian, 1984; Magliani et al., 1997; Marquina et al, 2002). Similar to that described in bacteriocins, it has been proposed that in natural habitats the production of these toxins confer to killer (K^+) yeast an advantage over sensitive microorganisms in the competition for nutrients (Lenski and Riley, 2002). The killer system was described for the first time in *S. cerevisiae* (Bevan and Makower, 1963) and soon after in many other yeast genera, such as *Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula, Kluyveromyces, Ustilago, Pichia*, etc. (Schmitt and Breinig, 2002). The killer activity of yeast is detectable only when it is assayed against proper yeast as sensitive, and is dependent on several factors, such as pH, salinity and temperature. Generally the killer toxins described are active at pH values from 3 to 5.5 (Golubev and Shabalin, 1994; Marquina et al., 2002). The genetic elements that encode for a killer phenotype may be double stranded RNA molecules (dsRNA) encapsulated in virus-like particles (VLPs), linear double stranded DNA plasmids (dsDNA) or nuclear genes (Schmitt and Breinig, 2002). The killer system of *S. cerevisiae* is the best studied model, corresponding to a genetically complex phenomenon because it depends both on cytoplasmic factors and approximately forty cellular genes (Wickner, 1976; Cartwright, 1992; van

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Vuuren, 1992; Vermut, 1994; Yasuyuki, 1995). The K^+ strains of *S. cerevisiae* have been classified in 3 groups (K1, K2 and K8) according to their toxin properties, mechanism of action, crossed immunity and genetic determinants (Magliani et al., 1997; Marquina et al., 2002). Two classes of dsRNA with different molecular sizes and functions are responsible for the killer phenotype in this yeast: L-dsRNA (4.6 kb) that encodes for a RNA polymerase and for capside proteins of VLPs; and M-dsRNA (1.6 - 1.8 kb) that encode for the toxin and confer immunity (Tipper and Bostian, 1984). The main mechanisms of action described for killer toxins are the formation of ionic channels in the cytoplasmic membrane and the inhibition of DNA synthesis (Magliani et al., 1997).

Investigations of killer systems have contributed important advances in basic and general aspects of eukaryotic cell biology, host-virus interaction and yeast virology. Furthermore, detailed analysis of toxin synthesis and structure has reinforced knowledge about the mechanisms of pre-protein processing and postraduccional modification in the eukaryotic secretion pathway. On the other hand, the possibility of finding killer toxins active against pathogens of medical importance is attractive for the treatment of fungal infections (Conti et al., 1998). An example is the killer system described in *Pichia anomala,* which shows toxic activity against a wide variety of nonrelated microorganisms, such as hyphomycetes and bacteria, including important opportunist pathogens, such as *Candida albicans* (Polonelli et al., 1986; Polonelli et al., 1989; Turchetti and Buzzini, 2003). In the biotechnological area, the use of killer strains to eliminate undesirable microorganisms in industrial fermentations or in food preservation has been suggested (Sulo and Michalcakova, 1992; Sulo et al., 1992; Lowes et al., 2000).

In the present work, we analyzed the existence of K^+ phenotype in a collection of *S. cerevisiae* strains obtained from industrial wine fermentation, and *P. anomala* strains isolated from environmental and clinical sources. Determinations of K^+ phenotype were performed using strains of the *Saccharomyces*, *Rhodotorula* and *Candida* genera as sensitive cells. The K^+ strains obtained were characterized in relation to the optimal temperature and pH for activity, and the kinetics of the production of killer activity. At a molecular level, the K^+ yeasts were analyzed in relation to the presence and chemical nature of extrachromosomal genetic elements.

MATERIALS AND METHODS

Yeast strains: Yeast isolates obtained from Chilean wine-producing areas (Martínez et al., 2004) were named V1, V2, V3, etc. The *P. anomala* strains isolated from environmental (A1, A2, A3, etc.) and clinical (P1, P2, P3, etc.) sources were described previously (Reyes et al., 2004). The strains AH22 (ATCC 38626) of *S. cerevisiae*, 1001 (ATCC 64385) and 5314 of *C. albicans,* and *Rhodotorula sloffiae* (CBS 7095) were used as killer-sensitive cells.

Culture media: Yeast cells were grown in YM medium containing 1% glucose, 0.3 % malt extract, 0.3 % yeast extract and 0.5 % peptone. YM-MB (YM containing 0.003 % methylene blue and 1.5 % agar) was used in assays for the killer phenotype.

Assay for mycocinogenic activity: Determinations were performed according to the method described previously (Salek et al., 1990). Sensitive lawns were made by mixing 200 µl of fresh culture of the sensitive strain with 20 ml of YM-MB (40 ºC), buffered with citrate-phosphate to obtain pH values ranging from 4.2 to 5.8 at intervals of 0.4 units, and poured onto Petri plates. The yeast isolates were seeded onto the sensitive lawns and the plates were incubated at 22, 30 or 37 ºC for 3 to 7 days. Positive killer activity was observed by a clear zone, surrounded by a blue precipitated halo, indicative of cellular death.

Determination of killer activity by the well test method: A volume of 100 µl of sample was inoculated into wells (10-mm diameter) cut into sensitive cell lawns and the diameters of the death zones were measured after incubation for 3 to 7 days at

22 or 30 ºC. Killer toxin activity was calculated according to the formula D = 5 logA x 10, where D is the diameter of death zone in mm, and A the activity in UA/ml (Schmitt and Tipper, 1990; Gulbiniene et al., 2004).

Determination of viable cells: Serial dilutions of culture samples were made and aliquots were seeded onto YM agar plate. After incubation at 22 ºC for 3-5 days, the colony forming units were determined.

Extraction of total nucleic acids: The nucleic acids were purified from protoplasts of yeasts. Cells were collected by centrifugation at 10,000 g for 10 min, resuspended in 5 ml of 0.9 M sorbitol, 0.1 M EDTA, 100 μg/ml zymoliase 100T and incubated for 25 min at 37ºC. After centrifugation at 4,000 g for 5 min, the cellular pellet was resuspended in 5 ml of 50 mM Tris-HCl, 20 mM EDTA, 1 % SDS and incubated at 65 ºC for 15 min. Then, 50 μl of proteinase K (20 mg/ml) was added, incubated at 55 ºC for 1 h and after the addition of 2 ml of cold 5 M potassium acetate, was incubated on ice for 10 min. One volume of saturated phenol pH 8.0 was added and both phases were mixed gently. The aqueous phase was recovered and washed twice with 1 volume of phenol:chloroform:isoamylic alcohol (25:24:21) and once with chloroform:isoamylic alcohol (24:1). The nucleic acids were precipitated with 2 volumes of ethanol at -20ºC.

Enzymatic treatments: Samples were treated with DNaseI, Nuclease S1, and RNaseH according to Sambroook et al. (1989), and Muthukrishnan and Shatkin (1975). Digestions with RNAseA were made in SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) at high (2 x SSC) and low (0.01 x SSC) ionic strength (Pryor and Boelen, 1987; Castillo and Cifuentes, 1994).

Gel electrophoresis: The samples were separated electrophoretically in 1% agarose gels in TAE buffer containing ethidium bromide (0.5 μg/ml) and photographed under transilluminated UV light. The size of the bands was determined using the 1D Image Analysis Software version 2.0.1 (Kodak Scientific Image System) using as standard the λ-*Hind*III DNA marker (Fermentas), and corrected according to mobility difference between dsDNA and dsRNA molecules (Livshits et al., 1990).

PCR amplification: The primer pairs ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') were used to amplify the 5.8S rDNA and the adjacent ITS1 and ITS2 regions (Fujita and Hashimoto, 2001). The PCR reaction was performed in 25 μl final volume as follows: 10 ng of DNA, 2.5 μl of 10X PCR buffer, 0.5 μl of dNTP's mixture (10 mM of each), 2 μl of ITS1 and ITS2 primer mix (25 μM of each), 1 μl of $MgCl_2$ (50 mM) and 0.2 μl (1 U) of Taq polymerase (New England Biolabs). The final volume was adjusted with nuclease free water. Amplification was performed in a GeneAmp PCR system 2700 thermal cycler (Applied Biosystem) as follows: initial denaturation at 94 ºC for 5 min; 30 cycles of denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s and synthesis at 72 ºC for 1 min; and a final extension step at 72 ºC for 10 min. Control reactions without DNA were carried out simultaneously. The amplified products were separated electrophoretically in 1% agarose gels in TAE buffer containing ethidium bromide (0.5 μg/ml) and photographed under transilluminated UV light. The amplicons were purified from the agarose gels by an alternative to the glassmilk method (Boyle and Lew, 1995).

Automated DNA sequencing and data analysis: Nucleotide sequencing was performed using the DNA Sequencing Kit Dynamic Termination Cycle (Amersham Biosciences Limited), according to the manufacturer's instructions, and the Genetic analyzer 3100 Avant automatic sequencer (Applied Biosystem). The sequence data was analyzed using the Vector NTI 10.1 (Invitrogen Corporation).

Toxin crude extract preparations: Cell culture samples were centrifuged at 7,000 g for 5 min at 4 ºC. The supernatant was filtered through sterile 0.22-μm pore size polivinyliden fluoride membrane (Millipore).

Protein precipitation: A volume of ethanol was added to the cell-free supernatant to achieve a final concentration of 70 % v/v, incubated at 4 º C for 1 h and

centrifuged at 16,000 g for 40 min. The pellet was dried and resuspended in 1 - 2 ml phosphate/citrate buffer pH 4.6. Samples were maintained at -20 ºC.

RESULTS

The existence of a killer phenotype was investigated in 16 *P. anomala* strains of environmental and clinical origin (Reyes et al., 2004), and in 35 yeast isolates obtained from industrial wine fermentations. For the identification of industrial yeast isolates, a region spanning the internal transcribed spacer ITS1 and ITS2 (including 5.8S rDNA gene) was amplified using ITS1 and ITS4 primers (Fujita and Hashimoto, 2001). The PCR products were separated by agarose gel electrophoresis, the amplicons obtained were purified from the gel and both DNA strands were sequenced. From the analysis and comparison of sequences against the data base, all yeast isolates were identified as *S. cerevisiae* with an average identity of 96 %.

Determination of killer activity at different temperatures and pH values

A preliminary screening of K^+ yeast was performed on *S. cerevisiae* AH22 lawns (commonly used as killer-sensitive strain) buffered at pH 4.6, value in which are active the most killer yeast reported. Yeast isolates were seeded onto these lawns and incubated at 22, 30 and 37 ºC. The K^+ strains were identified by the presence of a death halo (precipitate of methylen blue) of the sensitive cells, as is shown in figure 1. All K^+ yeasts showed more activity at 22 (fig 1B) rather than 30 ºC (fig 1A), according to the diameter of the death zone, and no K^+ strains were observed at 37 ºC (not shown). Similar results were obtained using *R. sloffiae* as sensitive cells. When the assays were performed using *C. albicans* lawns, K^+ yeast were observed only on *C. albicans* A5314 lawns at 30 ºC (not shown). Therefore, the determinations of the optimal pH for killer activity were performed at 22 ºC for *S. cerevisiae* and *R. sloffiae*, and at 30 ºC for *C. albicans* lawns. The pH values of the cell lawns were adjusted from 4.2 to 5.8 with phosphate/citrate buffer. The yeasts analyzed were streaked onto the cell lawns and the plates were incubated for 3-7 days. The results obtained for K^+ yeast isolates are summarized in table I. Fifteen yeast isolates showed K^+ phenotype on *S. cerevisiae* AH22 lawns, corresponding to 12 *S. cerevisiae* and 3 *P. anomala* isolates, in the pH range of 4.2 to 5.4. When the assay was performed on *R. sloffiae* lawns, 16 yeast isolates showed

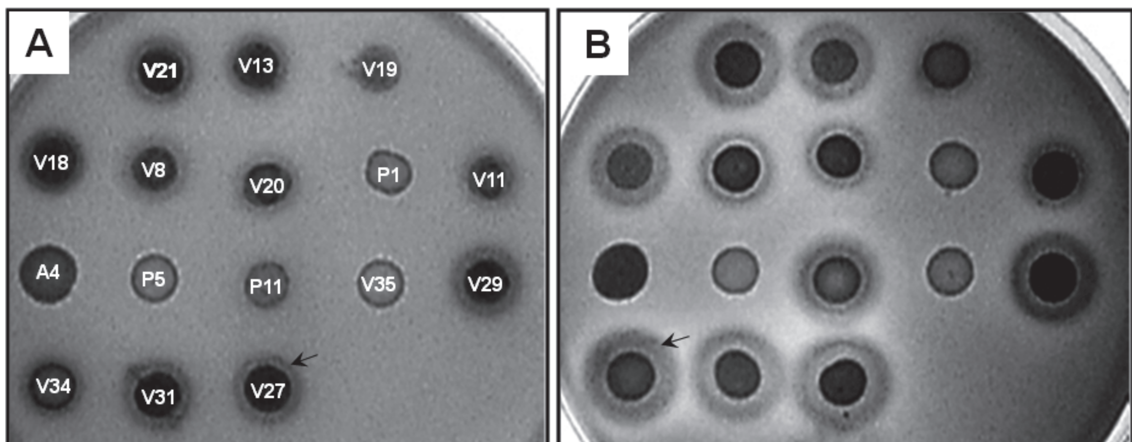

Figure 1: Agar plate assay for killer activity: A cells lawn of *S. cerevisiae* AH22 was made in YM-MB agar (pH 4.6) and 10 µl of yeast cultures were deposited onto this lawn. The plates were incubated at 30 (A) and 22 ºC (B). The number position represents the same strain in each panel. Arrow indicates the death zone (precipitate of methylen blue).

TABLE I

Determination of killer activity of different yeast isolates

Sensitive Strain	K+ strain	pH				
		4.2	4.6	5.0	5.4	5.8
S. cerevisiae AH22						
	A5	+++	-	-	-	-
	A6	-	+	-	-	-
	P11	±	++	+++	+	-
	V8	+++	++	++	-	-
	V11	++	+++	++	-	-
	V13	-	++++	++++	+	-
	V18	++++	++++	++++	+	-
	V19	++	++++	++	±	-
	V20	-	+++	++	-	-
	V21	+++	++++	++++	+	-
	V23	-	+	-	-	-
	V27	+	++	+	-	-
	V29	++	++++	+++	+	-
	V30	+++	++++	+++	+	-
	V34	±	++	++	+	-
R. sloffiae						
	A1	±	+	++	+	±
	A2	±	+	++	+	±
	A4	-	+	++	+	±
	A5	±	+	++	+	±
	A6	±	+	++	+	-
	P1		±	++	+	±
	P2	±	+	++	+	±
	P5	-	+	++	+	-
	P7	-	-	+	+	±
	P11	±	+	++	+	±
	P12	±	+	++	+	-
	P16	-	-	+	+	-
	P18	±	+	++	+	±
	P21	±	+	++	+	±
	V13	±	+	++	-	-
	V34	±	+	++	+	±
C. albicans 5314*						
	A1	++	++	-	-	-
	A4	-	++	-	-	-
	A5	++	++	-	-	-
	P5	++	++	-	-	-
	P11	++	-	-	-	-
	P16	++	++	-	-	-

The plate killer assay was performed as described in Materials and Methods (see fig. 1). *, results obtained after incubation at 30 ºC. –, no sensitivity; + to ++++, low to extensive sensitivity; ±, uncertain sensitivity.

killer activity at pH values of between 4.2 and 5.8, 14 of them corresponding to *P. anomala* isolates. Seven strains showed toxicity against *C. albicans* 5314 in pH range from 4.2 and 4.6, all belonging to *P. anomala* isolates, while no K^+ yeast on *C. albicans* 1001 lawns was observed at 22 and 30 ºC in the pH range from 4.2 to 5.8. According to the spectrum of action, the K^+ isolates can be divided into 5 groups: Group1, active only against *S. cerevisiae* AH22 (V8, V11, V18, V19, V20, V21, V23, V27, V29, V30); Group 2, killer activity only against *R. sloffiae* (A2, P1, P2, P7, P18, P21); Group 3, killer activity against *S. cerevisiae* AH22 and *R. sloffiae* (A6, V13, V34); Group 4, killer activity against *R. sloffiae* and *C. albicans* 5314 (A1, A4, P5, P12, P16); and Group 5, represented by P11 and A5 isolates of *P. anomala* that display killer activity against all three *S. cerevisiae* AH22, *R. sloffiae* and *C. albicans* 5314 strains.

Extraction and electrophoresis of nucleic acids

To determine the existence of exctrachromosomal genetic elements (EGEs) in K^+ yeast, the total nucleic acids were purified from a culture of each isolate and analyzed by agarose gel electrophoresis. None of the K^+ isolates of *P. anomala* showed the presence of any extrachromosomal band of nucleic acids. The *S. cerevisiae* K^+ isolates showed the presence of one extrachromosomal band of nucleic acids of about 4.2 kb, with the exception of the isolate V34. For the determination of the chemical nature of these EGEs, the samples were treated with different DNases and RNases, and resolved in agarose gels. As is shown in figure 2A and 2B, these molecules were not digested by treatment with RNaseH and DNaseI, indicating that they were not hybrid DNA/ RNA or DNA molecules, respectively. When the samples were treated with RNaseA at different ionic strength, they were degraded only under low ionic strength conditions (figure 2C, 2D), indicative that these extrachromosomal elements are molecules of dsRNA.

Kinetics of the killer activity production

To determine if toxic activity of K^+ yeast isolates is produced by an exported factor, aliquots of 100 µl of cell-free crude extract obtained from yeast cultures was seeded

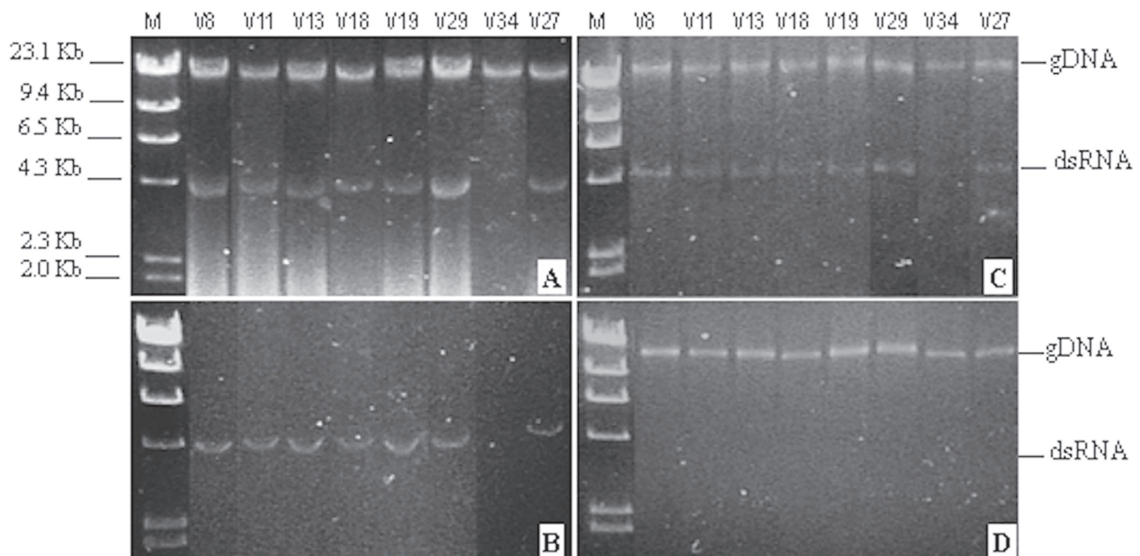

Figure 2: Agarose gel electrophoresis of total nucleic acids isolated from K^+ yeasts: The samples were treated with RNaseH (A), DNaseI (B), and with RNaseA at high (C) and low (D) ionic strength. M, λ-*Hind*III DNA marker. gDNA, genomic DNA. dsRNA, double stranded RNA.

into wells made in lawns of *S. cerevisiae* AH22. An example of these assays is shown in figure 3, where a death halo surrounding the wells seeded with extract from V20, V21 and V34 yeast cultures can be observed, contrary to *S. cerevisiae* AH22 crude extract. These results strongly suggest that an exported factor is responsible for the toxic activity in K^+ isolates. The kinetics of killer activity production was determined along the growth curve of six K^+ yeast isolates grown in YM media at pH 4.6 and 22 ºC. As is shown in figure 4, a similar kinetics was observed in all yeast cultures analyzed with an increase in killer activity in the log phase, followed by a decay of activity parallel to the decrease of cell viability (death phase). The exception was the V18 isolate which showed killer activity over a long period of time (160 h) even when the culture was in late stationary phase. Two of *S. cerevisiae* and *P. anomala* K^+ isolates were selected and their kinetics of activity production were determined, this once with extracellular proteins obtained by ethanol precipitation. The activity of the protein extract was determined on *S. cerevisiae* AH22 and *C. albicans* 5314 lawns for *S. cerevisiae* and *P. anomala* K^+ isolates, respectively. The kinetics of killer activity production by cultures of *S. cerevisiae* K^+ showed an increase in the log phase followed by a decay in the stationary phase (fig 5 A). The toxic activity against *C. albicans* of P11 isolate showed an increment in the log phase of growth, decaying at the end of this phase. The A5 isolate showed an increment of activity in the log phase, which was maintained even in the stationary phase (fig 5 B).

DISCUSSION

To evaluate the existence of a killer phenotype in a collection of *S. cerevisiae* and *P. anomala* isolates we used strains of three yeast genera as sensitive cells: *S. cerevisiae* AH22 ("universal" sensible strain); *R. sloffiae* (environmental isolate); and *C. albicans* 1001 and 5314 strains, with potential clinical interest. The assays were performed in a wide range of temperature and pH, parameters with a strong influence in the determination of killer phenotype.

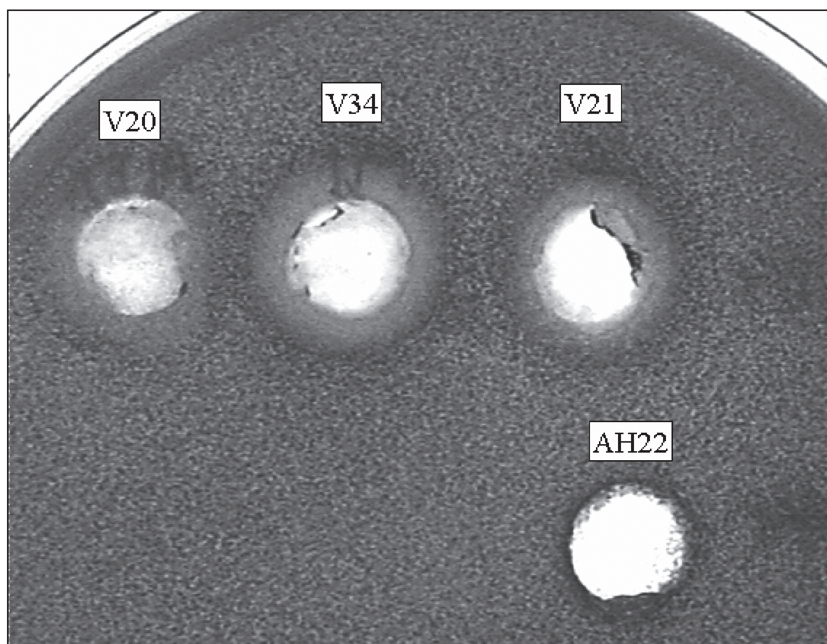

Figure 3: Well test assay of supernatant of K^+ yeast cultures: A 100 µl sample of culture filtrate was added to wells (10-mm diameter) made in a lawn of *S. cerevisiae* AH22.

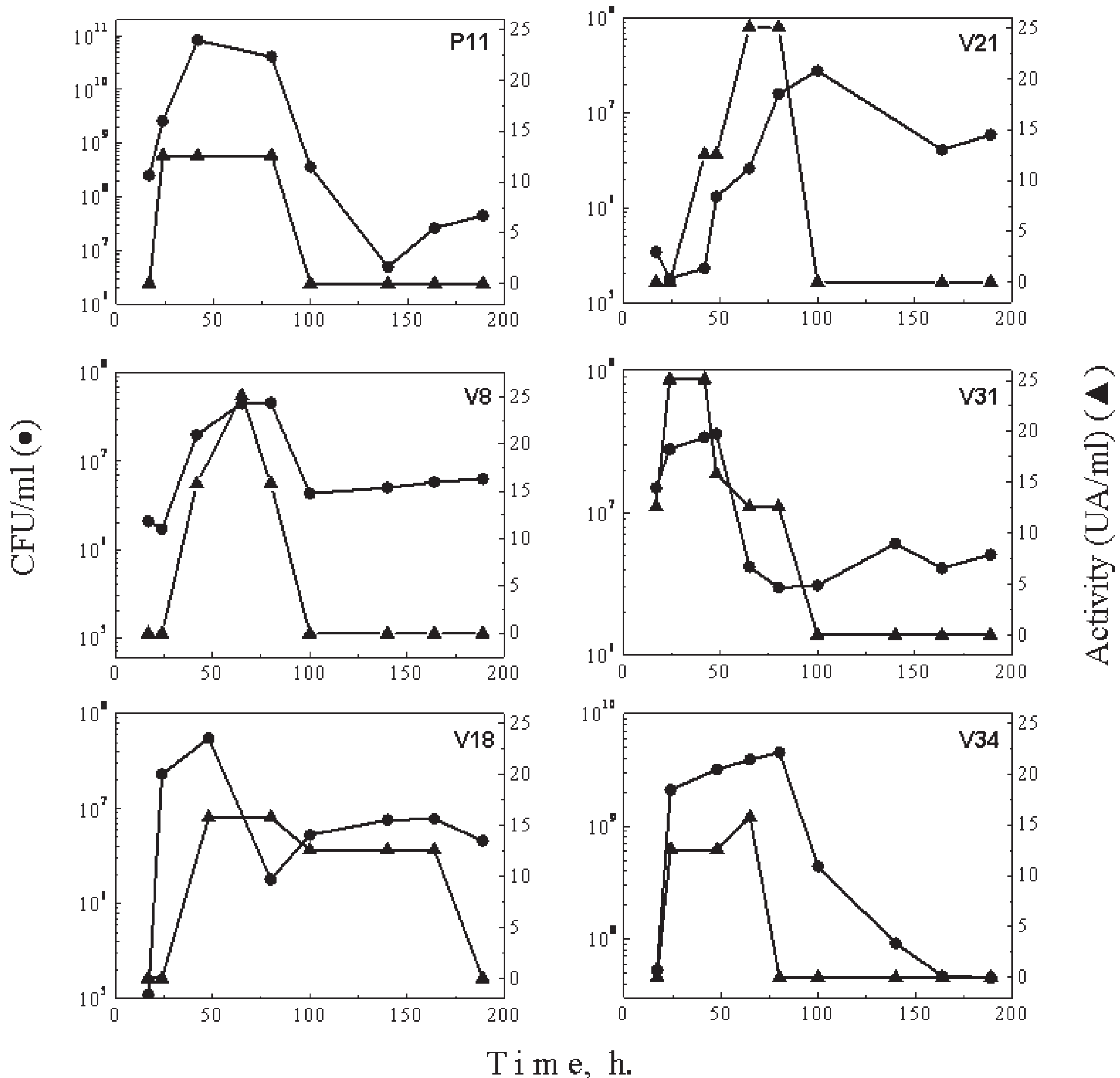

Figure 4: Kinetics of toxin production of K+ yeast isolates: Yeasts were grown in YM broth at pH 4.6 and 22 ºC with constant shaking. A 100 µl sample of culture filtrate was added to wells (10-mm diameter) in pour plates of *S. cerevisiae* AH22 lawn.

All K+ isolates showed activity at pH values below 5.4, which agrees with most of the killer yeasts described in the literature. A relation between the sensitive strain and optimal pH for killer activity was found. An example is the isolate A5 of *P. anomala* whose optimal pH was 4.2 on *S. cerevisiae*, 5.0 on *R. sloffiae* and 4.2 - 4.6 on *C. albicans*. Differences in the spectrum of activity among *S. cerevisiae* isolated from wine fermentation suggest the existence of different strains that can be grouped in: i) active only against *S. cerevisiae* AH22 (10 isolates); ii) active against *S. cerevisiae* AH22 and *R. sloffiae* (2 isolates); and iii) no killer strains. These results would complement molecular methods used for the differentiation of yeast strains of the same or different origins (Martínez et al., 2004). Likewise, the clinical isolates of *P. anomala* can be grouped by their activity against: i) *R. sloffiae* (6 isolates); ii) *S. cerevisiae* AH22 and *R. sloffiae* (1 isolate); iii) *R. sloffiae* and *C. albicans* (5 islotates); and iv) *S. cerevisiae, R. sloffiae* and *C. albicans* (2 isolates). The strains that have a

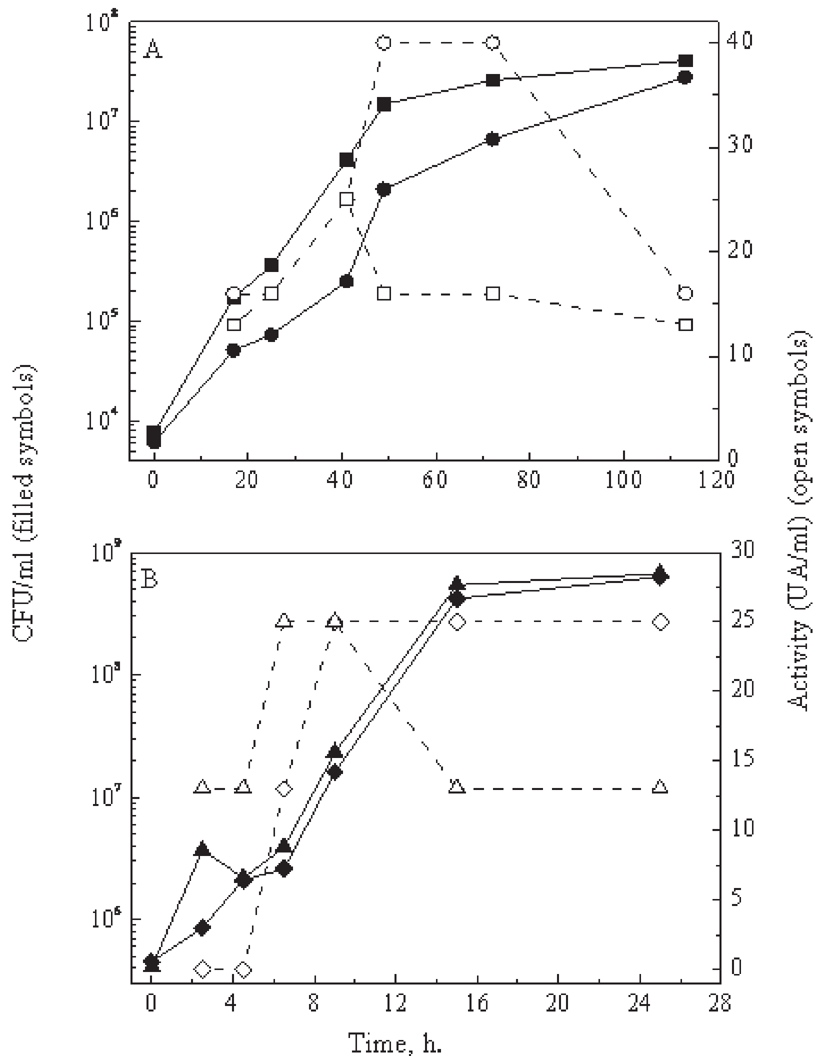

Figure 5: Kinetics of toxin production of K+ *S. cerevisiae* (A) and *P. anomala* (B) isolates: Yeasts were grown in YM broth at pH 4.6. In each point the protein from the cell-free supernatant was obtained by ethanol precipitation as described in materials and methods. Killer activity of samples was determined by the well test method on *S. cerevisiae* AH22 (A) and *C. albicans* 5314 (B) lawns. V18, circles; V21, squares; P11, triangles; A5, rhombs.

wide spectrum of action are interesting because they display toxic activity against yeasts of industrial and/or clinical interest. At a molecular level, all K+ isolates of *P. anomala* and the *S. cerevisiae* V34 do not have any EGEs, suggesting that the phenotype is encoded in the genome of the cells. In the other K+ isolates of *S. cerevisiae,* a dsRNA molecule of about 4.2 kb was observed and according to its molecular size it could correspond to the helper virus (L-dsRNA) of the killer system of *S. cerevisiae*. However in K+ *S. cerevisiae* strains the killer toxin is encoded by the satellite virus (M-dsRNA) with a molecular size of between 1.6 to 1.8 Kb. This does not agree with the presence only of the helper virus in all K+ isolates described in this work, suggesting that the toxin production is encoded in the genome. Similar results were obtained by other authors in yeast isolated from fruits and berry wine yeast populations (Gulbiniene et al., 2004).

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