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Killer Toxins of the Yeasts; *Candida utilis* 22 and *Kluyveromyces marxianus* and their Potential Applications as Biocontrol Agents

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ABSTRACT

Objectives of the present work were to investigate optimal conditions for production and characterization of the killer toxins produced by *Kluyveromyces marxianus* and *Candida utilis* 22, as the most killer yeasts, against *Candida famata* var. *famata* and *Cryptococcus albidus* as the most sensitive strains and to conduct a primary investigation on the potential usefulness of these toxins in the biological control and biotechnological applications. Toxin secretion patterns followed the growth curves and reached their maximum activities at the early stationary phase. At pH 6, optimal killing activities were observed at 25°C in the presence of glucose as the best carbon source besides yeast extract and peptone together as the best nitrogen sources in case of *C. utilis* 22 strain, and ammonium sulphate in case of *K. marxianus.* Toxin activity decreased significantly with increasing the NaCl concentrations in culture medium. High stability of the toxins was observed at different temperatures up to 100°C for 1h, pH at a broad range (2.5-7) for 1 h and at salt concentration up to 3 and 4 M for 12 h for *K. marxianus* and *C. utilis* 22 strains, respectively. Low molecular weight was observed and chromosomal gene(s) might be responsible for both killer toxins. The toxins of both killer yeasts showed a wide range of antimicrobial activity against plant and human pathogens. These characteristics proved that such toxins are promising candidates for several biotechnological applications, such as biological control and medical purposes.

Key words: Yeast, *Candida utilis* 22, *Kluyveromyces marxianus,* Toxicity, Biocontrol agent.

INTRODUCTION

Microorganisms have evolved sophisticated strategies to gain selective advantages over their competitors. One of these is the secretion of toxic compounds that results in killing or arrest growth of other microorganisms. The antimicrobial activity of exotoxins mediated by specific cell wall receptors on susceptible microorganisms is relatively common phenomenon. Exotoxins (generally proteins or glycoproteins) that are able to kill susceptible strains have been defined as killer toxins (Santos *et al.*, 2004). The killer phenomenon in some strains of *Saccharomyces cerevisiae* was first discovered by Bevan and Makower (1963). Several other yeast species have been found to produce a toxic proteinaceous factor that kills sensitive strains (Baeza *et al.*, 2008), these species belong to the genera *Debaryomyces*, *Hanseniaspora*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Williopsis* and *Candida* (Schmitt and Breinig, 2002 and Xianghong *et al.*, 2007). Sensitive strains could be non-killer yeasts as well as yeasts of the opposite killer class, while the producing yeasts remain immune to their own toxin and to that produced by strains of the same killer group (Magliani *et al.*, 1997). Killer toxins kill the sensitive cells by different mechanisms; hydrolyze (İzgü *et al.*, 2004) or inhibit the synthesis of the major cell wall component β-1, 3-glucan (Dakshnamurthy *et al.*, 2006) or cause ion leakage by ion channel formation on cytoplasmic membrane (İzgü, 2006). In some cases, toxin blocks both the DNA synthesis and budding cycle (Schmitt and Breinig, 2002) or arrest the cells in G1 phase of the cell cycle (İzgü *et al.*, 1997).

The responsible genes for killer phenotype may be carried on a chromosome as in *S. cerevisiae* KHR, KHS and *Williopsis marakii* (Suzuki, 1999), on a double-stranded RNA (dsRNA) as in *S. cerevisiae* K1, K2 and K28 and *Ustilago maydis* (Wickner, 1996), or on linear dsDNA plasmids as in *Kluyveromyces lactis* (Schmitt and Breinig, 2002). Molecular weight of the killer toxins is strain-dependant. The molecular weight of the killer toxin of *S. cerevisiae* strain 28 (KT 28) was estimated to be 16 KDa (Wickner, 1996). Killer toxins from other yeast genera have molecular weights between 18 and 300 KDa (Marquina *et al.*, 2002).

Interest in potential value of the killing property has been recently increased. In the agricultural field, killer toxins of both *Candida utilis* and *Saccharomyces cerevisiae* were found to suppress the growth of the phytopathogens *Aspergillus niger*, *A. oryza*, *Fusarium oxysporum* and *Pythium aphanidermatum* (Ozhovan *et al.*, 2002). In medical field, the killer toxins produced by *Hansenula*, *Pichia* and *Kluyveromyces* strains are demonstrated to have antifungal activity against pathogenic fungi (Magliani *et al.*, 2002). Some killer yeast strains have potential inhibitory activity against growth of Gram-positive pathogenic bacteria such as *Streptococcus pyrogenes*, *Bacillus subtilis*, and *Staphylococcus aureus* (İzgü *et al.*, 1997). In food industries, killer yeasts have been used to control spoilage yeasts (Baeza *et al.*, 2008).

Objectives of the present study were; investigating optimal conditions for production and stability of the killer toxins produced by *Kluyveromyces marxianus,*

Candida utilis 22 as the most killer yeasts, determining of location of the killer gene(s) and molecular weights of the killer toxins in addition conducting a primary investigation on the potential usefulness of these toxins in biological control and biotechnological applications.

MATERIALS AND METHODS

Organisms, growth media and culture conditions

Thirty eight yeast isolates obtained from different sources were used, 7 yeast strains, *Candida utilis* 22, *Kluyveromyces marxianus, Candida utilis* 28, *C. utilis* 41, *C. albicans, Saccharomyces cerevisiae* 43 and *S. cerevisiae* 44, were obtained from Microbiological Resources Centre (MIRCEN), Ain Shams University, Cairo, Egypt and 32 yeast isolates were obtained from natural sources; 11 were recovered from fruits (apple, strawberry, mango, date, guava, grape, apricot and peach) and 21 from salted foods (cheese, mesh, smoked fish, pickle) and sea water. All the yeasts were maintained in universal medium (UM), containing (g/l): glucose 10, peptone (Difco) 5, yeast extract (Difco) 3, malt extract (Difco) 3 and agar 20, at 4°C and/or at -80°C in 10% (w/v) glycerol. The yeast growth medium was yeast extract peptone dextrose (YEPD) composed of (g/l): glucose 20, peptone 20, and yeast extract 10 adjusted at pH 6. Killing activity was determined in killer medium (KM) containing (g/l): glucose 20, peptone 10, yeast extract 10, methylene blue (Sigma) 0.03 and agar 20 buffered with 100 mM citrate phosphate, pH 4.5. The yeast isolates were identified using the aid of API 20 C kit (API 20 C AUX, Bio Merieux) and yeast identification system (Barnett *et al.*, 2000).

Killer activity assay

Killer and sensitivity characteristics were evaluated by streak agar diffusion assay method (Kurtzman and Droby, 2001). A cell suspension (10^6 cell ml^{-1}) of the strain was mixed with 20 ml molten KM agar and poured onto sterile Petri plates to be tested for sensitivity to the killer toxin. Killer yeast cells of 24 h-old were streaked on the agar surface and the plates were incubated at 25°C for 48 h. Killing activity was evidenced as a clear zone of inhibition surrounding the streak, it was expressed in arbitrary units (Au) (Radler *et al.*, 1990), whereas one Au corresponded to that amount of killer toxin that caused an inhibition zone of 10 mm. All data of killer activity represented the average value of three separate replicates.

Improvement of killer activity

To improve the killer activity assay medium, different treatments were carried out as follows: (1) influence of temperature on killing activity was evaluated performing killer activity assay in KM agar (pH 4.5) at 15, 20, 25, 28, 30 and 35°C, (2) effect of pH was assessed preparing killer assay medium buffered with 10 mM citrate-phosphate at pH 4, 5, 6 and 7. Killer assays were performed at 25°C and (3) effect of salts on killing activity was tested adding 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 M NaCl (Merck) to the assay medium. Killer assays were performed in KM agar buffered at optimized pH 6 and incubated at optimized temperature 25°C. To improve KM medium buffered at pH 6, 11 carbon and 12 nitrogen sources were evaluated for maximum killer activity using killing activity assay at 25°C. The carbon sources were the D-fructose, galactose, mannose, D-arabinose, lactose, maltose, sucrose, D-xylose, starch, mannitol and glycerol replacing D-glucose in the recommended medium and the nitrogen sources were the yeast extract, peptone, tryptone, ammonium sulphate, ammonium phosphate, ammonium chloride, L-leucin, L-aspartic acid, sodium glutamate, sodium nitrate, L-glutamine and urea replacing yeast extract and peptone.

Killer toxin production

Killer yeast cells of 24 h-old were cultured in optimized KM broth without methylene blue buffered to pH 6 with 100 mM citrate phosphate for 48 h at 25°C and 120 rpm. After centrifugation (5000 g for 10 min at 4°C), the supernatant was filtered through 0.45 µm Millipore membranes and subsequently precipitated with ice-cold ethanol to a final concentration of 70% (v/v). After centrifugation, the pellet obtained was resuspended in 100 mM citrate-phosphate buffer, pH 6 (Santos and Marquina, 2004) and the killing activity was determined.

Stability of killer toxin at different temperatures, pH and salt concentrations

To assess thermal stability, samples of concentrated killer toxins were incubated at 4, 18, 25, 30, 37, 50, 70 and 100°C for 1 h. Aliquots (100 µl) were removed and assayed for killing activity against the selected sensitive strains. For stability to pH, killer toxin concentrates were adjusted with 100 mM citrate-phosphate buffer at a range of pH values between 2.5 to 7 (0.5 increments) and the solutions were incubated at 25°C for 1 h. Aliquots (100 µl) were removed and assayed for killing activity against the selected sensitive strains. Effect of ionic strength on killer toxin stability was assessed by preparing killer toxin samples in 100 mM citrate-phosphate buffer, pH 6 with different concentrations of NaCl (0.5: 4 M, 0.5 increments) and incubating the samples at 25°C for 12 h. Aliquots (100 µl) were removed and assayed for killing activity against the selected sensitive strains.

Estimation of killer toxin molecular weight

Molecular weight of the killer protein was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and a wide range molecular weight standard (Sigma) was used according

to Laemmli (1970). Gels were photographed using a Bio-Rad Gel Documentation System and the analysis of data was done by Bio-Rad Quantity One software (version 4.0.3).

Curing experiments

Cells of killer yeast strains were subjected to cure their killer phenotype by cultivation in YEPD broth containing 20 µg/ml ethidium bromide at 30°C for 72 h (Gunge and Sakaguchi, 1981) and/or at elevated temperature of 40°C for 72 h (Weinstein *et al.*, 1993). Samples were removed from the incubator every 24 hours in case of the elevated temperature. Several dilutions were plated onto YEPD to obtain separate single colonies. A total of 50 single colonies were randomly isolated and tested for the killer phenotype.

Assay of antimicrobial activity

Antagonistic effect of killer yeast strains tested against three phytopathogenic fungi; *Alternaria alternata, Fusarium* sp., *Aspergillus niger* and *C. albicans* as a human pathogen, which were obtained from the MIRCEN, were maintained in UM medium and three pathogenic bacterial strains, Gram-positive *Staphylococcus aureus ATCC 29213* and the Gram-negative, *Pseudomonas aeruginosa ATCC 27953,* and *Escherichia coli ATCC 25922* were obtained from American Type Culture Collection. The strains were maintained in nutrient agar. Antifungal activity assay in case of phytopathogens was performed according Hewedy and Ashour (1998). Discs (4mm diameter), obtained from 96 h-culture of the pathogenic fungi were placed in the middle of optimal KM agar plates. Two discs or loop-full of 24 h-culture of killer yeast strains were placed at a distance of 3 cm from the specified fungus. Control plates were prepared using agar plates inoculated with fungal discs only and incubated at 25°C for 72 h and then the diameter of the fungal growth was measured. Percentage of reduction in radial growth of the pathogens was determined using the following formula developed by Backman and Rodrigue-Kabana (1975):

$$X = [(G2 / G1) \times 100] - 100$$

Where; X = percent of mycelial growth reduction (PMGR), G1 = growth of pathogenic fungus in control plates (mm), G2 = growth of pathogenic fungus in treated plates (mm).

In the antibacterial activity assay, cells of the killer yeast strains were streaked on the bacterial cells grown at 37°C for 24 h on nutrient agar and/or *C. albicans* cells of 24 h-old to be tested for sensitivity, which was suspended in physiological saline solution (10^6 cells/ml) and mixed with 20 ml of molten optimal KM agar. After 48 h of incubation at 25°C, killing activity was determined. Tetracycline (30 mcg) and amkin (30 mcg), as standard antibacterial agents, were used against such pathogens to evaluate the killing activity of killer yeasts.

RESULTS AND DISCUSSION

Screening for killer and sensitive phenotypes

The 38 yeasts tested in the present study for their sensitivity and ability to kill other yeasts showed that they varied greatly not only in the killing spectrum, but also showed different patterns for killing ability which indicated that they may secrete different killing factors. *Candida utilis* 22 was the most potent killer yeast. It revealed killing activity against 25 out of 38 tested yeasts (65.8%). *K. marxianus* showed strong killing activity against 3 out of the 38 tested yeasts (8%), while the others killed one to two yeasts out of the 38 (2.6-5%), respectively. Accordingly, *C. utilis* 22 and *K. marxianus* were selected as the most potent killer yeasts for further investigations. *K. marxianus* was killed by *C. utilis* 22 (Fig 1 C), indicating that they produced different types of toxins. It is well known that the producing yeasts remain immune to their toxins and to these produced by strains of the same killer group (Magliani *et al.,* 1997). Therefore, the present killer yeasts are belonging to different killer groups. On the other hand, 2 yeast isolates No. 16 and 19 (Fig. 2), taken from cheese and smoked fish, respectively were chosen in this study for their high sensitivity to both *C. utilis* 22 and *K. marxianus* (Figs. 1A & B) and were identified as *C. famata* var. *famata* of ascomycetes group and *Cryptococcus albidus* of basidiomycetes group. *C. albidus* has been implicated in human infection, and it is ubiquitous found on skin of humans and animals (Labreque *et al.*, 2005). Although infections with *C. albidus* are rare, it should be considered as a potential cause of ocular and systemic diseases in immune incompetent patients (Garelick *et al.*, 2004). *C. famata* var. *famata* in particular, has been poorly studied although it has been isolated from food and directly related to human infections (Abia-Bassey and Utsalo, 2006). In this connection, it is well documented that the present killer yeasts can be used as boicontrol agents in plants, humans, animals and fermentation medium as mentioned by Schmitt and Breinig (2002).

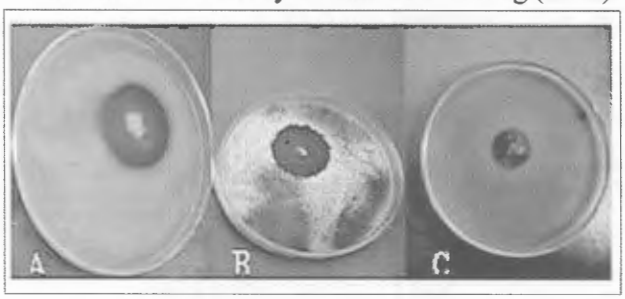

Fig. (1): Killer activity of *C. utilis* 22 against *C. famata var famata* (A), *K. marxianus* against *C. albidus* (B) and *C. utilis* 22 against *K. marxianus* (C).

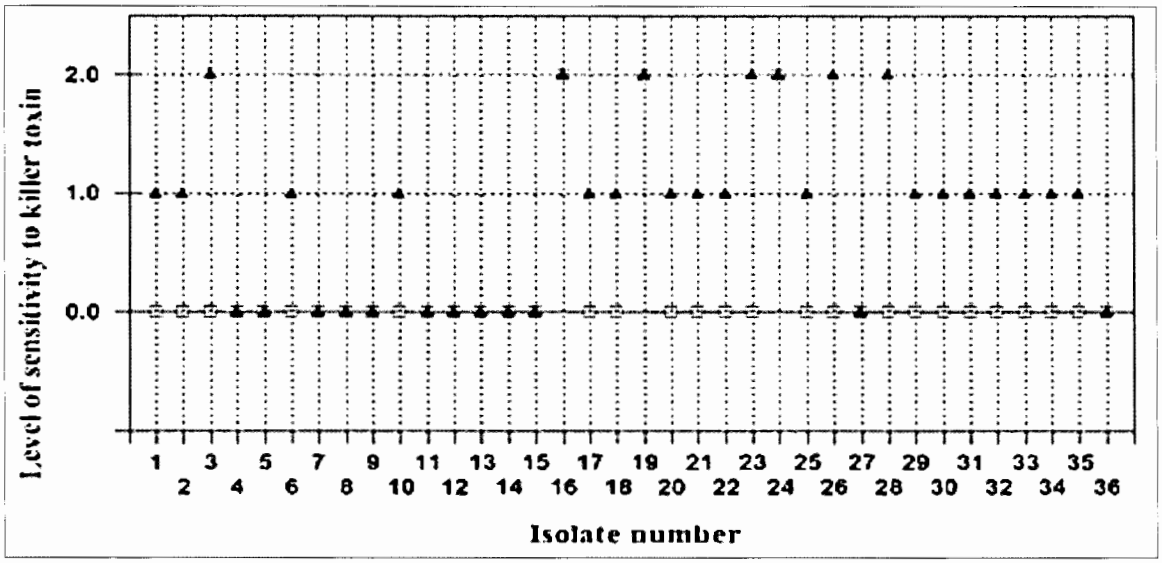

Fig. (2): Occurrence of resistance (0), weak sensitivity (1) and sensitivity (2) for the killer toxins of *C. utilis* 22 (▲) and *K. marxianus* (□).

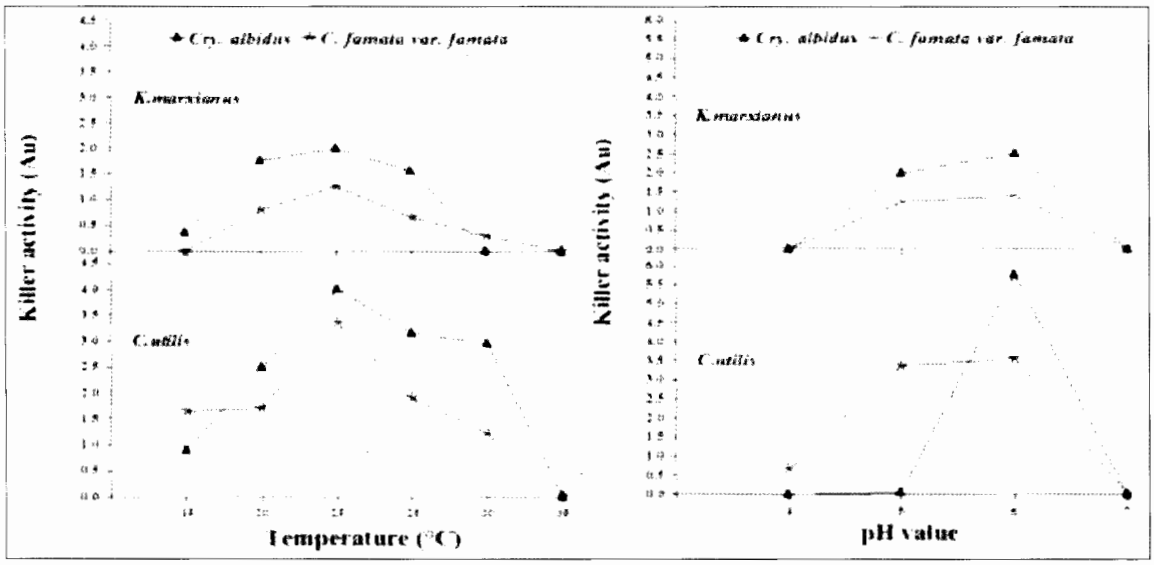

Fig. (3): Effect of different temperatures and pH values on killer toxin production by *C. utilis* 22 and *K. Marxianus.*

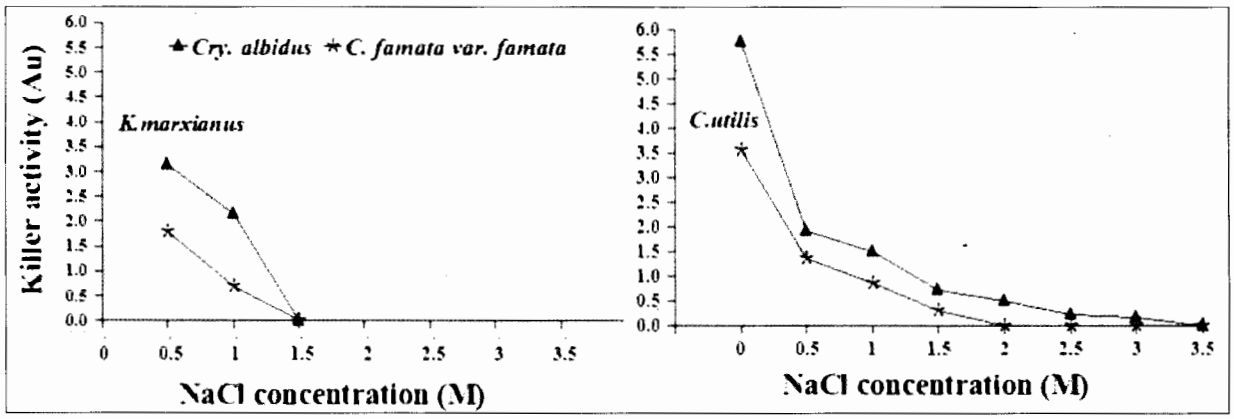

Fig. (4): Effect of different NaCl concentrations on killer toxin production by *C. utilis* 22 and *K. Marxianus.*

The interaction between killer gene and environment was investigated. The cell free supernatants obtained from the killer yeasts *C. utilis* 22 and *K. marxianus* reached their maximum activity (inhibition zone 1.5 Au and 1.0 Au, respectively) after 24 h of their growth, which corresponds to the end of the rapid growth phase and at the beginning of the stationery growth phase (data not shown). The same behaviour was found for other killer toxins which are produced and secreted during exponential growth phase of the producing yeast and increased up to the beginning of stationary phase (Santos and Marquina, 2004 and da Silva *et al.*, 2008).

Improvement of killing activity

It is well known that the phenotype of a living organism is a direct result of the interaction between genotype and the whole environmental conditions surrounding such genotype. Changing the environment may lead to a different phenotype due to changing the expression level of some genes. The killer toxin production is strongly affected by the culture conditions and the optimal conditions have to be found empirically (Marquina *et al.*, 2002). The present results of the effect of culture conditions, temperature, pH, NaCl, carbon and nitrogen sources in assay medium indicated that different killer yeasts had differential responses to the surrounding environmental conditions. The killer yeasts and their toxins were pH- and temperature-dependent, which cause membrane permeability change in sensitive cells (Robledo-Leal *et al.*, 2014). Optimal conditions for the killer toxin of *C. utilis* 22 and *K. marxianus* showed greatest activities between 20-28°C and pH 5-6 and the maximum activity displayed at 25°C and pH 6. Regarding the sensitive yeasts, *C. albidus* exhibited increasing sensitivity than *C. famata* var. *famata* to the killer toxins under the mentioned conditions (Fig. 3), similar observations were reported previously (Hernández *et al.*, 2008). Several yeasts were described as killer when assayed for killer phenotype in the presence of NaCl (da Silva *et al.*, 2008). In contrast, the toxins produced under any given NaCl concentration exhibited lower activity than without any NaCl concentration (Fig. 4). Similar observation was reported by Aguiar and lucas (2000) who reported that killer phenotype does not require salt induction and doesn't need salt to display activity. Moreover, the salt influence was associated with sensitive strain sensitivity which is consistent with the pore-formation mode of action that was described (Llorente *et al.*, 1997) or inferred for some yeast killer system (Magliani *et al.*, 1997). The results (data not shown) revealed that glucose was the best carbon source that enhanced the production of *C. utilis* 22 and *K. marxianus* killer toxins. It was reported that production of exo - β-1, 3-glucanase by *C. oleophia* was also glucose-dependent (Bar-Shimon *et al.*, 2004). The killer toxin production depends on the nitrogen source supplied to the growth medium and in particular, yeast extract and ammonium sulphate may be stimulatory as additives which stimulate protein synthesis and increase the stability of the produced killer toxin (Marquina *et al.*, 2001). Accordingly, much higher production by *C. utilis* 22 and *K. marxianus* strains in the presence of yeast extract plus peptone and ammonium sulphate respectively was found (data not shown).

Killer toxins stability

Stability of the killer toxins is strongly dependent on pH, temperature and NaCl concentration of the solution. These common properties are consistent with the proteinaceous nature of the killer toxin which is also obvious from the susceptibility of most toxins to proteolytic enzymes. As happens with activity, the majority of killer toxins were irreversible inactivated above pH 5 and were stable only in a narrow pH range, usually between 2 and 5 (da Silva *et al.*, 2008). In contrast, killer toxins of *C. utilis* 22 and

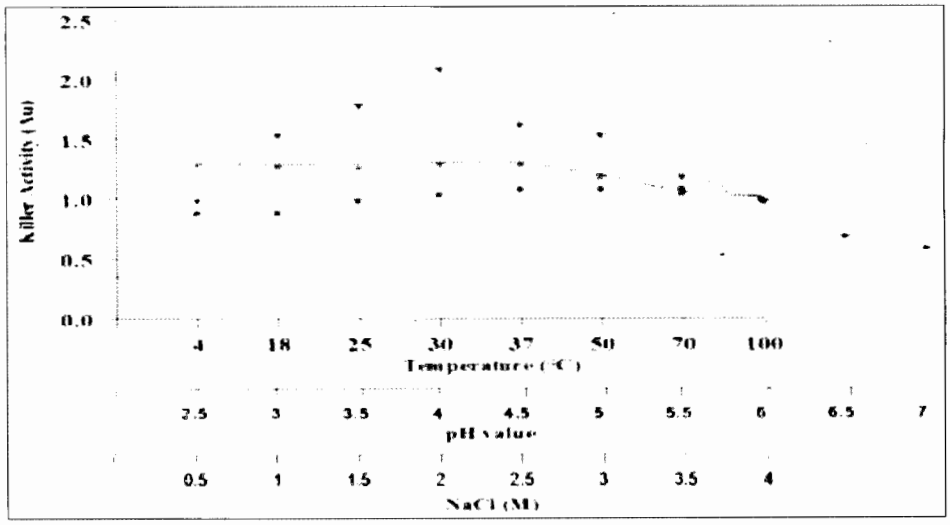

Fig. (5): Thermal (✳), pH (◆) stability for 1h and NaCl (▼) stability for 12 h of *C. utilis* 22 killer toxin.

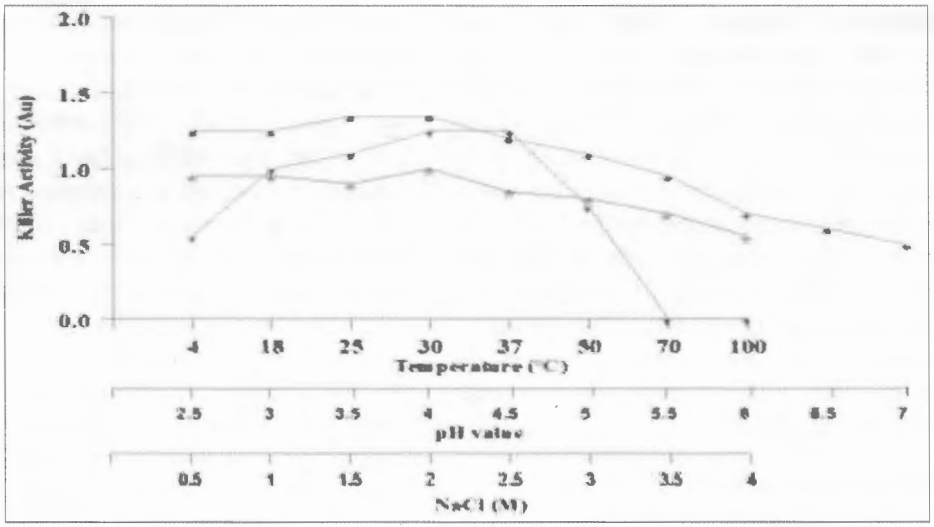

Fig. (6): Thermal (✳), pH (◆) stability for 1h and NaCl (▼) stability for 12 h of *K. marxianus* killer toxin.

K. marxianus had a broad pH stability range (2.5-7) (Figs. 5 & 6), in accordance with Komiyama *et al.* (1995) who reported that the killer toxin of *Hansenula saturnus* had a broad pH stability range (2-11).

In the literature, there is scarce data on the effect of incubations on killer toxins stability, regarding their conservation possibilities. This information is though determinant if some biotechnological applications are to be considered, like the exploitation of killer toxins as biological control agents in agriculture (Marquina *et al.*, 2002) and as natural food preservatives (da Silva *et al.,* 2008). Killer toxins of *C. utilis* 22 and *K. marxianus* exhibited high thermal stability up to 37 and 30°C, respectively and only about 23.7% activity loss was observed at 100°C after 1h treatment (Figs. 5 & 6), compared with the killer activity of *D. hansenii* when kept at 30°C for 24 h where more than half of the activity was lost (Marquina *et al.*, 2002). Moreover, high salt stability at 3 and 4 M NaCl was observed after 12 h for toxins of *K. marxianus* and *C. utilis* 22, respectively (Figs. 5 & 6). Similar observations were reported by da Silva *et al.* (2008) that killer toxin of the halotolerant yeast *C. nodaensis* still active at very diverse and aggressive environmental conditions (4 M NaCl). These results suggest that salt stimulation underlying phenomenon might be of chemical rather than biological nature, hypothesising an interference of salt of the protein structure/stability. The positive influence of salt on protein temperature sensitivity could be compatible with the role of Na^+ on protein structure (da Silva *et al.*, 2008).

Estimation of killer toxin molecular weight

Molecular weight of the killer toxin of *C. utilis* 22 was expected to be around 47.07, 50.4, 52.2 or 99.8 KDa, whereas it was estimated to be around 46.9, 50, 52 or 99.7 KDa for *K. marxianus* toxin (black arrow heads) (Fig. 7). Supporting this finding,

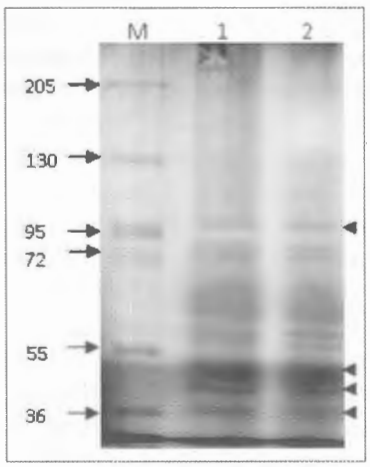

Fig. (7): Toxin protein banding patterns of *C. utilis* 22 (lane 1), *K. marxianus* (lane 2) and M refers to standard protein marker.

Marquina *et al.* (2002) reported that the molecular weight of *K. fragilis* killer toxin was 42 KDa and *Candida* SW-55 had two toxins, each purified toxin giving a marked band with molecular mass of 36 KDa (Yokomori *et al.*, 1988). In addition, there is a possibility of the present killer toxins as a trimetric protein toxin (α, β, γ) as reported by Marquina *et al.* (2002) that *K. lactis* killer toxin was described as protein dissociated into three subunits with molecular mass of γ (28 KDa), β (31 KDa) and α (97 KDa). Such finding needs further investigations.

Curing treatment for detecting the genetic determinant responsible for the killer toxins

Responsible genes for the killer toxins may be carried on a chromosome, on dsRNA or on linear dsDNA plasmids. To identify the position of the genetic determinants of the killer toxins in *C. utilis* 22 and *K. marxianus,* several plasmid curing treatments were performed; ethidium bromide and the growth at elevated temperature. After the treatment of the presented two killers with ethidium bromide and/or

after elevated temperature (37°C) of incubation, a total of 50 single colonies was randomly chosen isolated and tested for their killer activity against the sensitive yeasts. All of the 50 colonies retained their killer activity. Ethidium bromide curing experiment which was reported to cure dsDNA plasmids (Magliani *et al.*,1997) and growth at elevated temperatures (37°C or above) curing experiments which can cure infected strains of their M1 dsRNA (Weinstein *et al.*,1993) gave negative results. These results provide evidences that neither dsDNA plasmids nor dsRNA virus-like particles are responsible for the killer character in the present killer strains (data not shown), indicating that the nuclear genes may be responsible for the killer characters, similar observation was reported by da Silva *et al.* (2008).

Antimicrobial activity of *C. utilis* 22 and *K. marxianus* killer yeasts

Several potential applications for the killer yeasts have been studied using some plant and human pathogenic fungi, in addition to some pathogenic bacteria. The killer toxins were demonstrated to have antifungal activity against pathogenic fungi; *C. utilis* 22 was very effective against *Fusarium* sp., *A. alternata* and *A. niger* as plant pathogens. It was found to reduce their radial growth by 56, 41.4 and 33%, respectively (Table 1), while it produced 0.1 Au inhibition zone against *C. albicans* (data not shown). On the other hand, *K. marxianus* toxin reduced the radial growth of *Fusarium* sp. and *A. alternata* by 59, 32.8% (Table 1) but had no inhibitory effect neither on *A. niger* nor *C. albicans.*

The control of phytopathogenic molds by yeasts was studied with great potential usage, mainly inhibiting molds that cause fruit rotting in post-harvesting period, because the yeast is a good competitor for nutrient and space. Positive results are found in literature for the biocontrol of plant diseases by yeasts in field situations (Rosa-Magri *et al.*, 2011). Killer toxins produced by *Hansenula, Pichia and Kluyveromyces* strains were demonstrated to have antifungal activity against pathogenic fungi that threat human and plant hosts (Magliani *et al.*, 2002). Secreted killer toxins mainly produced by non-*Saccharomyces* yeasts showed a broad spectrum of killing activity against a great number of plant and human pathogens, including the anti-*Pneumocystis carinii* activity in a described killer strain of the yeast *Pichia anomala* (Seguy *et al.*, 1998). Whilst killer strains of *Saccharomyces cerevisiae* and *Pichia anomala* markedly inhibited the growth of certain wood decay basidiomycetes and plant pathogenic fungi, suggesting that such yeasts might have a potential as novel antimycotic biocontrol agents (Waema *et al.*, 2009). Beside, Cabral *et al.* (2009) tested killer yeasts against the phytopathogen *Moniliophthora preniciosa* and found that *Dipodascus capitatus* and *Candida* sp. strains inhibited its growth. Schmitt and Breinig, (2002) suggested that the genetic information on toxin production could be integrated into and expressed from the genome of maize plants (*Zea mays*) to render such transgenic plants resistant to the attack of yeast phytopathogens such as *Ustilago maydis*. Regardingly, the role of killer yeasts in agricultural biotechnology as biocontrol agents is now beginning to be exploited. This change is in part being driven by a desire for more sustainable alternative to chemical pesticides. Bio-prospection for yeasts as biocontrol agents has recently resulted in their employment in post-harvest protection of fruit from molds (Rosa-Magri *et al.*, 2011). Such findings need further investigations.

Besides, killer yeasts were able to kill some members of both Gram positive and Gram negative bacteria (Table 2). *C. utilis* 22 produced 3.1 and 2.4 Au inhibition zone against *P. aeruginosa* and *S. Aureus,* respectively, while *E. coli* showed resistance. On the other hand, *K.marxianus* produced 2.3 Au inhibition zone against *P. aeruginosa* comparing with tetracycline, while *S. Aureus* and *E. Coli* showed resistance. These findings are in agreement with Izgü and Altinbay (1997) and in conflicting with those of Ozhovan *et al.* (2002), who reported that the yeast killer toxins are not active against bacteria exhibiting only fungicidal or fungistatic action.

Table (1): *In vitro* antifungal activity of *C. utilis* 22 and *K. marxianus* killer yeasts against fungal pathogens

Strains	Radial growth reduction (%)					
	C. utilis 22			*K. marxianus*		
	G1	G2	X	G1	G2	X
A. niger	7.85	5.25	33.12	7.85	8.00	0.00
A. alternate	3.50	2.05	41.42	3.50	2.35	32.85
Fusarium sp.	5.00	2.20	56.00	5.00	2.05	59.00

G1 = growth of pathogenic fungus in control plates.
G2 = growth of pathogenic fungus in treated plates.
X = % of radial growth reduction.

Table (2): *In vitro* antibacterial activity of *C. utilis* 22 and *K. marxianus* killer yeasts against bacterial pathogens

Strains	*C. utilis* 22	*K. marxianus*	Tetracycline (30 mcg)	Amkin (30 mcg)
	Inhibition zone (Au)		Inhibition zone (cm)	
S. aureus	2.4	-ve	2.4	-ve
P. aeruginosa	3.1	2.3	3.9	-ve
E. coli	-ve	-ve	-ve	-ve

In conclusion, *C. utilis* 22 and *K. marxianus* killer yeasts produce different killer toxins (proteinic nature). These killer yeasts have a wide range of killing activity against plant and human pathogens. Moreover, each one has its own spectrum and exhibits high thermal, pH and salt stability. These characteristics make killer toxins a promising candidate for several biotechnological applications as biocontrol agents in agriculture and medical purposes and in the high salt food products preservation from spoilage other yeasts, especially chromosomal gene(s) may be responsible for both killer toxins.

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