Yeasts Producing Killer Toxins: An Overview

El-Banna A. A.*, Malak A. El-Sahn & Shehata M.G.

Food Science and Technology Dept., Fac. of Agric., Alexandria Univ., 21545, El-Shatby, Alexandria, Egypt

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ABSTRACT

The production of exotoxins with antimicrobial activity on susceptible microorganisms by yeasts is a relatively common phenomenon. Exotoxins (generally proteins or glycoproteins) that are able to kill susceptible cells belonging to the same or congeneric species have been defined as killer toxins. Since first discovered in *Saccharomyces cerevisiae* , killer strains have been isolated from several yeast genera, including *Candida*, *Cryptococcus*, *Hanseniaspora* , *Kluyveromyces*, *Pichia* , *Torulopsis* , *Ustilago* , *Williopsis* and *Zygosaccharomyces*. Many types of killer toxins have been reported and their genomes were mapped on double-stranded RNA (*S. cerevisiae* K1, K2, K28,*Ustilage maydis* and *Hanseniaspora uvarum*) , a linear double-stranded DNA plasmid (*Kluyveromyces lactis*, *Pichia acaciae* and *Pichia inositovora*) or carried on a chromosome (*S. cerevisiae* KHS, KHR and *Williopsis mrakii*). During the last two decades, secreted killer toxins and toxin-producing killer yeasts have found several applications. For instance in the food and fermentation industries,killer yeasts have been used to combat contaminating wild-type yeasts which can occur during the production of wine, beer and bread . Killer yeasts have also been used as bio-control agents in the preservation of foods , in the bio-typing of medically important pathogenic yeasts and yeast-like fungi , in the development of novel antimycotics for the treatment of human and plant fungal infections, and finally in the field of recombinant DNA technology.

Key words: Killer yeasts, killer factors, yeast killer phenomenon, exotoxins, antimycotics, food industry, antimicrobial, bio-control, recombinant DNA

INTRODUCTION

The production of yeast-exotoxins with antimicrobial activity mediated by specific cell wall receptors on susceptible microorganisms is a relatively common phenomenon . The killer phenomenon of yeast cells was first reported by Bevan & Makower (1963). Killer yeasts are toxin-producing fungi that are immune to the activity of their own killer toxins. Killer yeasts can produce toxin proteins or glycoproteins (so-called killer toxins, killer factors or killer proteins) that can be lethal to susceptible yeast, fungi and bacteria strains . They have been isolated from environmental, clinical, industrial and agricultural sources. (Woods *et al.*, 1974, Rogers & Bevan,1978, Magliani *et al.*,1997, Waema *et al.,* 2009).Three phenotypes of yeasts were demonstrated ; namely, killer, neutral and sensitive , with respect to the killer character. Killer strains produce an extracellular toxin which kills sensitive strains, while neutral strains are neither produce toxin nor killed by it .The discovery of a fourth yeast phenotype termed 'killer/sensitive' was reported, this phenotype is sensitive to 'killer factor', but produces a new 'killer factor' which kills sensitive cells (Woods *et al.*,1974, Rogers & Bevan, 1978). The killer strain is immune to the effect of its own toxin. The effect of killer toxin is dependent both on its own potency and susceptibility of treated cells under selected conditions. The susceptibility of sensitive yeast to killer toxin is known to depend on various factors such as selected killer toxin, the exposed yeast strain, its growth phase and the state of culture under given experimental condition (Woods & Bevan, 1968, Mohamudha Parveen & Ayesha Begum, 2010).

After the initial discovery of the killer phenomenon in *S. cerevisiae*, it soon became evident that killer strains are not restricted to the genus *Saccharomyces* but can also be found among many other yeast genera; up to now, toxin-producing killer yeasts have been identified in *Candida*, *Cryptococcus, Debaryomyces, Hanseniaspora,Hansenula, Kluyveromyces, Metschnikowia, Pichia, Torulopsis, Ustilago, Williopsis and Zygosaccharomyces,* indicating that the killer phenomenon is indeed widespread among yeasts. There exists many classes of killer yeast strains differing particularly

in their spectrum of their activity against sensitive strains, in their cross reactivity, genetic determination of killer toxin, killer toxin immunity and the mechanisms of killer toxin action(Tredoux *et al.*,1986 , Magliani *et al.*,1997, Schmitt & Breinig, 2002, Mohamudha Parveen & Ayesha Begum, 2010).

The genetic basis for killer phenotype expression can be quite variable; in the few cases where killer determinants have clearly been identified, they are either cytoplasmically inherited encapsulated double-stranded RNA (dsRNA) viruses, linear dsDNA plasmids or chromosomal genes (Table 1). Structure of killer toxins , their specific receptors , and the recognized mechanisms of action of most investigated yeast killer systems are summarized in Table (2).

Classification of yeast killer phenomenon according to genetic basis

I- Cytoplasmically inherited encapsulated double-stranded RNA (dsRNA) viruses

Saccharomyces cerevisiae

The most thoroughly investigated yeast killer system is that of *S. cerevisiae*. Currently, the killer yeasts belonging to this species have been classified into three main groups (K1, K2, and K28) on the basis of the molecular characteristics of the secreted toxins, their killing profiles, the lack of cross-immunity, and the encoding genetic determinants. They are constituted by strains producing toxins encoded by double-stranded RNA (dsRNA), but other killer yeasts producing toxins named KHR and KHS, which are encoded on chromo-

Table 1: Genetic basis for killer phenomenon in most investigated yeasts

Genetic basis	Yeast produced toxin	Name of toxin gene	Toxin gene size (Kbp)	Name of killer toxin
Cytoplasmically inherited encapsulated double-stranded RNA (dsRNA) viruses	*Saccaromyces cerevisiae*	M1	1.8	K1
		M2	1.5	K2
		M28	1.9	K28
	Ustilago maydis	M1/M2	1.4	P1
		M2	0.98	P4
		M2	1.2	P6
	Haseniaspora uvarum	M	-	-
	Phaffia rhdozyma	M	-	-
	Zygosaccharomyces bailii	M	-	-
Linear double strand plasmids	*Kluyveromyces lactis*	pGkL 1	8.8	-
		pGkL2	13.4	-
	Pichia acaciae	pPac 1-1	13.6	-
		pPac 1-2	6.8	-
	Pichia inositovora	pPin 1-1	18	-
		pPin 1-3	10	-
Chromosomal genes	*Saccaromyces cerevisiae*	KHR	0.9	KHR
		KHS	2.1	KHS
	Pichia farinose	SMK1	0.6	KK1
	Pichia membranifaciens	-	-	PMKT
		-	-	PMKT2
	Willioposis mrakii	HMK	-	HM–1, K-500
	Willioposis saturnus	HSK	-	HSK
	Willioposis saturnus var mrakii	-	-	WmkT

Sources: Magliani, *et al*., 1997, Jijaki & Lepoivre, 1998, Guyard *et al.*,2002 , Schmitt &Preinig 2002 , Izgu & Altinbay, 2004, Santos *et al*., 2009 , Ochigava *et al*., 2011

Table 2: killer toxins , their structure , size , specific receptors and mechanism of action of most investigated yeasts

Yeast strain	Name of killer toxin	Structure of killer toxin	Size of killer toxin (KDa)	Receptor	Mechanism of action
Saccharomyces cerevisiae	K1	αβ dimer	19.0	β -1,6 D-glucan	Increase of membrane permeability to ions
	K2	αβ dimer	21.5		
	K28	αβ dimer	21.5	α-1,3- mannoprotein	Inhibition of DNA synthesis
Ustilago maydis	P1	αβ dimer	19.0	-	Increase of membrane permeability to ions
	P2	Monomer	11.1	-	
	P6	αβ dimer	17.7	-	
Kuyveromyces lactis	-	αβγ trimer	156.5	Chitin	Cell cycle arrest in G1 , chitinase activity
Pichia acaciae	-	Trimer	~ 190	Chitin	
Saccharomyces cerevisiae	KHR	Monomer	20	-	Increase of membrane permeability to ions
	KHS	Monomer	75	-	
Pichia anomala WC65	-	Monomer	83.3	β-1,6D- glucan	-
Pichia anomala NCYC434	K5	-	49	β-1,3D- glucan	Glucanase
Pichia farinose	SMK1	αβ dimer	14.2	-	Increase of membrane permeability to ions
Pichia kluyver		Monomer	19	-	Increase of membrane permeability to ions
Pichia membranifaciens	PMKT	-	-	B -1,6 D- glucan	-
	PMKT2	-	30	mannoprotein	-
Schwanniomyces occidentalis		Dimer	7.4,4.9	mannan	-
Williopsis mrakii	HM–1	Monomer	10.7	Cell wall β- glucan	Inhibition of β 1,3 glucan synthesis
	K-500	Monomer	1.8-5	Cell wall β- glucan	Increase of membrane permeability to ions
Williopsis saturnus	HSK	Monomer	-	-	-
Williopsis saturnus var mrakii	WmkT	Monomer	85	Cell wall β- glucan	Glucancase activity

Sources: Magliani, *et al*., 1997, Jijaki & Lepoivre, 1998, Guyard *et al.*, 2002, Schmitt &Preinig 2002, Izgu & Altinbay, 2004, Santos *et al*., 2009, Ochigava *et al*., 2011

somal DNA, have been defined (Goto *et al.,* 1990, Magliani *et al.*, 1997).

In *S. cerevisiae*, the killer phenomenon is based on the presence of cytoplasmically inherited double-stranded RNA (dsRNA) viruses . Persistent infection of yeast cells with these viruses is symptomless, yeast dsRNA viruses have no disadvantage for a single cell. In addition, yeast dsRNA viruses are considered non-infectious since no naturally occurring extracellular route of transmission has been identified. They were therefore designated 'virus-like particles' (VLPs). Based on the lack of cross-immunity, their molecular mode of action and their killing profiles, toxin-producing *S. cerevisiae* killer strains have been classified into three major groups (K1, K2 and K28), each of them secreting a unique killer toxin as well as a specific but as yet unidentified immunity component that renders killer cells immune to their own toxin (Schmitt & Breinig, 2002, Mohamudha Parveen & Ayesha Begum, 2010).

K1, K2, and K28 S. cerevisiae killer toxins are protein molecules secreted by killer strains. Killer strains are not susceptible (are immune) to their own toxin but remain susceptible to other killer toxins. Even though the toxins have different amino acid compositions and modes of molecular action, they show some general characteristics in their mechanisms of synthesis, processing, and secretion. Each toxin is synthesized as a single polypeptide pre-protoxin comprising larger hydrophobic amino termini than are usually found on secreted proteins; the pre-protoxins have similar overall structures. The pre-protoxins, once synthesized, undergo post-translational modifications via the endoplasmic reticulum, Golgi apparatus, and secretory vesicles, resulting in the secretion of the mature, active toxin(Magliani *et al.*,1997).

The best-studied and best-known killer toxin, K1 (19 kDa), is secreted as a molecule consisting of two distinct disulfide bonded unglycosylated subunits, termed α (9.5 kDa) and β (9.0 kDa), derived from a 42-kDa glycosylated precursor molecule (pro-toxin). The K2 and K28 toxins have been characterized more recently and less extensively than K1, but they seem to show a similar overall organization, mainly at the precursor level. K2 is synthesized as a 362-amino-acid precursor of 38.7 kDa. During the maturation process, the signal peptide is removed by peptidase cleavage, yielding the two subunits (α and β) that constitute the mature secreted toxin. The final α and β subunits are larger than those of K1 (172 and 140 amino acids, respectively), and α is N glycosylated at two positions.The recently published complete sequence of the M28 dsRNA has emphasized the similarity of pre-protoxin synthesis to that of K1 (Magliani *et al.*, 1997). Among the virally encoded killer toxins of S. cerevisiae, K1 and K28 are the best-studied proteins. Although both toxins differ significantly in their amino acid composition and their molecular mode of action, they share striking homologies with respect to their synthesis, processing and secretion. Each toxin is translated as pre-protoxin (pptox) showing similar structures, subsequently undergoing post-translational modifications via the endoplasmic reticulum, Golgi apparatus and secretory vesicles, finally resulting in the secretion of a mature α/β heterodimeric protein toxin. All three killer toxins of the best known killer strains (K1, K2, and K28) are encoded by different dsRNAs (M1, M2, and M28), differing in size (1.8, 1.5, and 1.9 kb, respectively) and showing similar organization (Magliani *et al.*, 1997, Schmitt&Breinig, 2002, Mohamudha Parveen & Ayesha Begum, 2010).

The killer toxins have different modes of action even though, they do have one thing in common: All viral toxins (K1, K2, K28) kill a sensitive yeast cell in a receptor -mediated two-step process (Fig. 1) . The first step involves a fast and energy-independent binding to a toxin receptor within the cell wall of a sensitive target cell. In case of K1 and K2, this primary receptor has been identified as

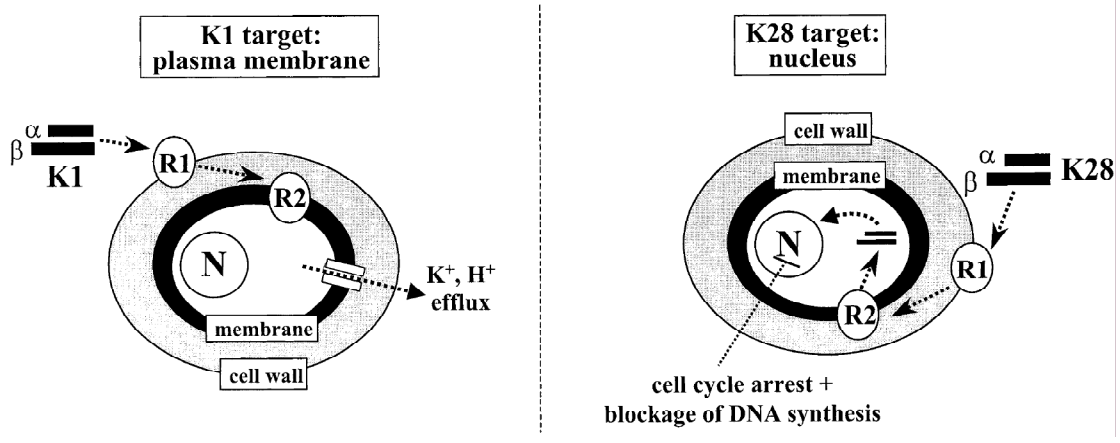

Fig. 1: Receptor-mediated mode of action of the yeast K1 and K28 viral toxins. Killing of a sensitive yeast cell is envisaged in a two-step process involving initial toxin binding to receptors at the level of the cell wall (R1) and the cytoplasmic membrane (R2). After interaction with the plasma membrane, K1 is acting from outside the cell and disrupts cytoplasmic membrane function, while K28 enters the cell by endocytosis in order to reach its final target, the yeast cell nucleus
(Source : Schmitt&Breinig, 2002)

β-1, 6-D-glucan, whereas the cell wall receptor for K28 is a high molecular mass α-1, 3-mannoprotein. The second, energy-dependent step involves toxin translocation to the cytoplasmic membrane and interaction with a secondary membrane receptor. After having reached the cytoplasmic membrane, the toxin (K1) exerts its lethal effect by ion channel formation and disruption of cytoplasmic membrane function. The lethal effect of K1 toxin involves the disruption of electrochemical ion gradient across the plasma membrane, which results from the increased permeability for H+ and an uncontrolled leakage of K+ ions and which is followed by a leakage of small molecules from the cell (amino acids and glucose). In contrast to the ionophoric mode of action in which K1 acts from outside the cell, K28 represents the first viral killer toxin for which it was demonstrated that it enters a sensitive target (yeast) cell by endocytosis. After receptor- mediated entry into the cell, the toxin traverses the secretion pathway in reverse (via Golgi and ER), subsequently enters the cytosol, and finally transduces its toxic signal into the yeast cell nucleus where the lethal events occur. Killer toxin K28 causes blockage of both DNA synthesis and budding cycle, thus causing a loss of cell viability (Bussey *et al.*, 1979, de la Pena *et al.*, 1981, Schmitt & Radler, 1988, Schmitt *et al.*, 1989, Santos *et al.,* 2000, Schmitt & Breinig, 2002 Mohamudha Parveen & Ayesha Begum, 2010).

Ustilago maydis

Killer strains of *Ustilago maydis* can secrete one of the three different toxins that have been identified so far. These toxins, designated KP1, KP4, and KP6, have killer activity against susceptible cells of the same and closely related species (Koltin &Day, 1975). Each killer strain contains three to seven dsRNA segments, ranging in size from 0.36 to 6.2 kb, categorized into three groups: heavy (H), medium (M), and light (L). Six segments of dsRNA in P1 (H1 and H2, M1 to M3, and L), seven in P4 (H1 to H4, M2 and 3, and L), and five in P6 (H1, H2, M2, M3, and L) (Bozarth *et al.*,1981). On the basis of genetic and in vitro translation experiments, one of the M segments in each killer strain appears to code for the killer toxin: M2 in P6, M1 and/or M2 in P1, and M2 in P4 (Koltin *et al.*,1980). KP toxins have different specificities, so that cells resistant to one can be susceptible to another. Like the K toxins of *S. cerevisiae*, they seem to be synthesized as pre-protoxins that are successively processed intracellularly to yield the mature secreted bipartite (KP1 and KP6) and single subunit (KP4) toxins. Of these toxins, KP6 is the best characterized, since it has been highly purified. This toxin consists of two nonglycosylated polypeptides: α (78 amino acids; 8.6 kDa) and β (81 amino acids; 9.1 kDa) (Magliani *et al.*,1997). They are cleaved from a pre-protoxin of 219 amino acids (24.1kDa). In the mature toxin, α and β are not covalently linked. They interact as monomers with the susceptible cells independently and in a sequential manner, with the α subunit initiating the interaction and the β subunit exerting its effect only on cells previously exposed to α subunit. Receptors on the cell wall have not yet been identified, but the toxin binds to the cell wall. The spheroplasts are not susceptible to the toxin (Steinlauf *et al.*, 1988). Like KP6, the KP1 toxin is bipartite and the α and β subunits are not covalently linked but act independently as monomers. (Steinlauf *et al.*, 1988). The KP4 toxin has been recently purified and characterized (Koltin *et al.*, 1980). It is synthesized as a pre-protoxin of 127 amino acids (13.6 kDa). The mature toxin is secreted as a single polypeptide of 105 amino acids (11.1 kDa). This toxin does not appear to be glycosylated . Its mode of action is still unknown (Koltin *et al.*, 1980).

Hanseniaspora uvarum, Phaffia rhodozyma and Zygosaccharomyces bailii

The killer phenotype associated with double strand RNA mycoviruses, similar to those in *S. cerevisiae*, have been detected in the yeasts *Hanseniaspora uvarum* and *Zygosaccharomyces bailii*. Four dsRNA molecules associated with virus-like particles, encoding a killer system, have been isolated from *Phaffia rhodozyma* .Their molecular sizes were approximately 4.3, 3.1, 0.9 and 0.75 kilobase pairs (kbp) as determined by agarose-gel electrophoresis and they were designated as L, M, S1 and S2, respectively (Radler *et al.,*1990, Radler *et al.,*1993, Castillo & Cifuentes,1994).

II-Linear double strand DNA plasmid

Kluyveromyces lactis

Killer strains always contain 50 to 100 copies per cell of each of two cytoplasmically inherited linear plasmids designated pGKL1 (k1) and pGKL2 (k2), which are 8.8 and 13.4 kbp in size, respectively (Table 1). *K. lactis* killer strains secrete a heterotrimeric toxin that inhibits the growth of a wide range of susceptible yeasts in the genera *Candida*, *Kluyveromyces*, *Saccharomyces*, *Torulopsis*, and

Zygosaccharomyces, as well as non-killer strains of *K. lactis*. The toxin consists of three subunits: α polypeptide with a single asparagine-linked oligosaccharide unit, designated α (99kDa), and two smaller unglycosylated components, β (30 kDa) and γ (27.5 kDa). The precursor is targeted to the endoplasmic reticulum, where it is glycosylated, transported to the Golgi apparatus, and processed by a protease to form mature subunits. The mature toxin leads to the permanent arrest of susceptible cells in the unbudded (G1) phase of the cell cycle, in such a manner that they can never resume mitotic division. Despite previous reports, the toxin does not inhibit adenylate cyclase, but it causes a rapid and progressive loss of viability that is sufficient to explain the blockage of cell division (Sugisaki *et al.*, 1984, Magliani *et al.*, 1997).

The mode of action of the toxin is still poorly understood. However, two effects, arrest in G1 and loss of viability, are apparently mediated by different mechanisms. Only the γ subunit appears to be required to arrest proliferation, acting against an unidentified intracellular target. The α subunit shows sequence homology to both plant and bacterial chitinases and has the essential chitinase activity that is required for the toxin to act on susceptible yeast cells. As this activity cannot mediate the cell cycle arrest induced by the toxin, it is most probably required by the γ subunit to gain entry to the susceptible cell. It is probably involved in the interaction with a carbohydrate receptor in the cell wall or, alternatively, in the degradation of cell wall carbohydrate structures, thus providing the toxin with a route of entry into the cell (Butler *et al.*, 1991). Chitin may represent the cell wall receptor for the killer toxin. After the toxin binds to the chitin in the cell wall, it is likely that part or all of the molecule is translocated across the membrane into the cytoplasm. The function of the β subunit is still unknown. However, since its molecule shows four hydrophobic regions, it has been hypothesized that this subunit may play a role, together with α subunit, in the binding and membrane translocation of the toxin, allowing the γ subunit to enter the cell or resulting in the loss of viability of the treated cells, as the γ subunit alone can account only for the G1 arrest(Butler *et al.*,1991, Magliani *et al.*,1997).

Pichia acaciae

P. acaciae killer strains have been shown to possess two linear plasmids, designated p*Pac*1-1 (13.6 kbp) and p*Pac*1-2 (6.8 kbp). These plasmids are quite similar in both function and structural organization to those found in *K. lactis* . Despite important similarities to *K. lactis* killer toxin, significant functional differences exist. *P. acaciae* toxin seems to be composed of three subunits (110, 39, and 38 kDa) with an associated chitinase activity.Chitin binding is essential to the activity of the toxin, which causes G1 cell cycle arrest. This toxin shows a wide range of activity, differing from but overlapping with that of *K. lactis*. All the linear plasmids so far identified, besides those associated with killer phenotypes, show similar promoter-like elements in their sequences, suggesting the existence of a unique but highly conserved expression system for these extrachromosomal elements (Bolen *et al.*, 1994).

Pichia inositovora

The presence of three linear dsDNA plasmids, of approximately 18, 13, and 10 kbp, has been reported in a killer toxin-producing strain of *P. inositovora*. Only two of them (p*Pin*1-1 and p*Pin*1-3) seem to be associated with the killer phenotype, while the loss of p*Pin*1-2 has no effect on toxin production or susceptibility. The killer toxin apparently is an acidic heat-labile glycoprotein whose characterization and range of actions have not yet been determined (Ligon *et al.*, 1989, Magliani *et al.*, 1997).

III- Killer phenotype associated with chromosomal genes

Saccharomyces cerevisiae

As mentioned above, other killer toxins have been described in *S. cerevisiae* (Tables 1&2), and two of them, which are chromosomally encoded, have been designated KHR (killer of heat resistant) and KHS (killer of heat susceptible), because they differ in their thermostability and optimum pH. The mature KHR and KHS toxins are single proteins, with molecular masses of about 20 and 75 kDa .The mature toxins have molecular masses lower than those of their precursors, some protein processing is thought to occur during maturation. The *KHR* and *KHS* genes consist of 888 and 2,124 bp, respectively, with no homology to other killer genes (Goto *et al.,* 1990).

KHR encodes a pre-protoxin of 33 kDa, which has a possible hydrophobic signal sequence in the N-terminal site region, four competent sites for glycosylation, and five cleavage sites that might be cut by a protease. The mature KHR toxin does not

have clearly hydrophobic regions, and so its mode of action is probably different from that of K1.The purified toxin had an isoelectric point of pH 5.3. The toxin had novel killer activity against Candida glabrata and *S. cerevisiae*, but did not affect bacteria, fungi, or other yeasts (Goto *et al*., 1990, Magliani *et al*.,1997). KHS encodes for a 79-kDa precursor with a hydrophobic N-terminal sequence that is probably spliced to produce the mature toxin. The mature KHS toxin shows three clusters of hydrophobic amino acid sequences that might have an ionophore function similar to that of K1 or K2 toxins. (Goto *et al*., 1990, Magliani *et al*., 1997).

Pichia anomala

The killer system of *P. anomala* has an activity against a wide range of unrelated microorganisms, such as yeasts, hyphomycetes, and bacteria, including important opportunistic pathogens such as *C. albicans* and the mycelial and yeast forms of the dimorphic fungi (Polonelli&Morace,1986). A killer toxin, purified from a strain (WC 65) of *Pichia anomala*, has been characterized and demonstrated to be an acidic glycoprotein of 83.3 kDa (Table 2). , stable between pH 2.0 and 5.0. Studies on the growth rates of a susceptible *C. albicans* strain in the presence of various toxin concentrations suggest the presence of two non-mutually exclusive binding sites for the toxin. One of them (probably a surface β-1,6-D-glucan-related binding site) binds noncompetitively, and the other binds competitively.

A second toxin produced by the killer strain *P. anomala* ATCC 96603, previously known as UP 25F, has been extensively investigated for its antimicrobial activity. This toxin is supposed to be a glycoprotein encoded by nuclear genes. It acts on a wide spectrum of susceptible microorganisms that are characterized by the presence of specific cell wall receptors and lack of immunity systems. The toxin cell wall receptors in *C.albicans* seem to be distributed mainly in budding cells and germination tubes, supporting previous findings that the *P.anomala* killer toxin is more active against young yeast cells. Ultrastructural studies on the secretion of the toxin, carried out by immunoelectron microscopy with a specific monoclonal antibody, suggest that it undergoes heterogeneous secretion by the killer cells and concentration in the cell wall layers before being exported outside the cells . The most important characteristic of this toxin is its wide range of activity, which suggests the existence of a ubiquitous form of yeast toxin receptor, probably constituted by β-glucan(Polonelli & Morace, 1986, Magliani *et al.*, 1997).

K5-type yeast killer protein produced by *Pichia anomala* NCYC 434 (Table 2) was concentrated and purified ,it had a molecular mass of 49000 Da. The pI value of the K5-type yeast killer protein was measured at 3.7 . It is a glycosylated protein. Its internal amino acid sequence is 100% identical to exo-β-1,3-glucanase of *P. anomala* strain K which is a glycoprotein of 45.7kDa with pI of 4.7. The purified protein was highly stable at pH values between 3 and 5.5 and temperature up to 37ºC. *Pichia anomala* NCYC 434(k5) has a broad killing spectrum among fungi with relatively growth inhibitory effect towards fungal cell walls predominantly composed of β-1,3-glucan such as *Candida albicans , Torulaspora delbrueckii* and *Kluveromyces maxianus(*Izgu & Altinbay, 2004).

Pichia farinosa

A novel type of killer toxin produced by the halotolerant yeast *P. farinosa* (Tables 1&2) has been recently described. This toxin, termed SMK (salt-mediated killer toxin), is a heterodimer (14.214 kDa), whose subunits (α, 6.6 kDa; β, 7.9 kDa) are tightly linked under acidic conditions. It shows its maximum killer activity in the presence of 2 M NaCl. Although there is no sequence similarity to other toxins, the 222-amino acid *P. farinosa* pre-protoxin resembles the *S. cerevisiae* K1 toxin in overall structure, hydrophobicity profile, and processing, suggesting that the target of the toxin is the membrane (Suzuki & Nikkuni, 1994).

Pichia kluyver

P. kluyveri killer toxin (Table 2) a 19-kDa acidic glycoprotein, induces the formation of ion-permeable channels, as does *S. cerevisiae* K1, which causes leakage of potassium ions and ATP, decrease of the cellular pH, and inhibition of amino acid uptake (Middelbeek *et al.*, 1979).

Pichia membranifaciens

Pichia membranifaciens CYC 1086 secretes a killer toxin (PMKT2) that is inhibitory to a variety of spoilage yeasts and fungi of agronomical interest. The killer toxin in the culture supernatant was concentrated by ultrafiltration and purified to homogeneity by two successive steps. Biochemical characterization of the toxin (Table 2) showed it to be a protein with an apparent molecular mass of 30

kDa and an isoelectric point of 3.7. At pH 4.5, optimal killer activity was observed at temperatures up to 20 ºC. Above approximately this pH, activity decreased sharply and was barely noticeable at pH 6. Strains of *P. membranifaciens* produced at least two different types of toxins, named PMKT and PMKT2. PMKT2 had physico-chemical properties and molecular mass similar to PMKT, but their spectra of biological activity against a variety of fungal and yeast strains were different, indicating that they were different toxins . PMKT inhibits strains of *Saccharomyces* but PMKT2 does not, and PMKT was not able to inhibit several *B. bruxellensis* isolates, whereas PMKT2 did so. These differences in killer spectra could be accounted for by the preliminary differences observed between the two toxins in the nature of the primary receptors present in the sensitive strains. PMKT was found to have affinity for (1,6)-β-D-glucans whereas PMKT2 was found to be adsorbed by cell-wall mannoproteins, suggesting the presence of two different primary receptors for these toxins (Santos *et al*., 2009).

Schwanniomyces occidentalis

The yeast *Schwanniomyces occidentalis* produces a killer toxin lethal to sensitive strains of *Saccharomyces cerevisiae*. Killer activity is lost after pepsin and papain treatment, suggesting that the toxin is a protein. The killer protein was composed of two subunits with molecular masses of approximately 7.4 and 4.9 kDa, respectively. Maximum killer activity was between pH 4.2 and 4.8. The protein was stable between pH 2.0 and 5.0 and inactivated at temperatures above 40°C. The killer protein was chromosomally encoded. Mannan, but not β-glucan or laminarin, prevented sensitive yeast cells from being killed by the killer protein, suggesting that mannan may bind to the killer protein (Table 2). Identification and characterization of a killer strain of *S. occidentalis* may help reduce the risk of contamination by undesirable yeast strains during commercial fermentations (Chen *et al.*, 2000).

Williopsis(Hansenula) mrakii , Williopsis saturnus and Williopsis saturnus var mrakii

At least two different killer toxins have been described in *Williopsis mrakii*: a toxin designated HM-1, first described in 1983; and a second one, described more recently and designated K-500 (Table 1) (Hodgson *et al.*,1995). Unlike most of the reported toxins, HM-1 is a basic unglycosylated polypeptide (pI 9.1); is composed of 88 amino acids (10.721 kDa), 10 of which are cysteines; and shows high thermostability (100°C for 10 min) and pH stability between pH 2 and 11. The exceptional stability of this cysteine-rich molecule appears to be due to the existence of many disulfide bonds, which also seem to be essential for its biological activity, as demonstrated by the inactivation of the toxin by reducing agents such as 2-mercaptoethanol and dithiothreitol. HM-1 kills susceptible strains by a unique mechanism, presumably involving interference with the synthesis of β-1,3-glucan, thus rendering the wall osmotically fragile or defective and ultimately resulting in lytic cell death. HM-1 inhibits the in vitro activity of b-1,3-D-glucan synthase (Table 2), suggesting that the toxin can perturb the synthesis of the yeast cell wall by inhibiting the glucan synthesis at the budding sites or the conjugating tubes, which results in cell lysis (Hodgson *et al.*,1995, Magliani *et al.*,1997) .

In contrast, the K-500 killer toxin is an acidic polypeptide with a relative molecular mass between 1.8 and 5.0 kDa. It is readily inactivated by high temperature and pH values above 4.0. It possesses extensive anti-*Candida* activity. The toxin has not yet been completely characterized, and its killing mechanism remains unclear. It is significantly smaller than any of the other known yeast killer toxin polypeptides. There is some evidence that the toxin can act in a manner similar to *S. cerevisiae* K1 and *P. kluyveri* toxins by producing channels in the susceptible cell and thus promoting the loss of ions that leads to cell lysis. (Hodgson *et al.*, 1995, Magliani *et al.*, 1997).

W. saturnus produces HSK toxin which is very similar to HMK produced by *Williopsis mrakii* (Tables 1& 2). The genes of these toxins encode precursors of 125 and 124 amino acids, respectively, showing an N-terminal signal sequence of 37 amino acids, which may be removed by a signal peptidase, followed by mature proteins of 88 and 87 amino acids, respectively. The molecular mechanisms of killing are probably identical (Kimura *et al.*, 1993).

Williopsis saturnus var *mrakii* MUCL 41968 (Table 2) secretes a 85-KDa glycoprotein killer toxin (WmKT) that displays a cytocidal activity against a wide range of microorganisms, making WmKT a promising candidate for the development of new antimicrobial molecules. The toxin was proposed to bind to the surface of sensitive micro-

organisms through the recognition of β-glucans. WmKT exhibits amino acid sequence similar to glucanase. WmKT acts on yeast cells through a hydrolytic activity directed against cell wall β-glucans that disrupts the yeast cell wall integrity leading to death (Guyard *et al.*, 2002).

Promising applications

I- Food and fermentation industries

The food and beverage industries were among the first to explore the application of killer – toxin producing yeasts to kill spoilage microorganisms (Lowes *et al.*, 2000). Yeast strains often achieve competitive advantage by producing killer toxins, which kill off competing species sensitive cells belonging to either the same or a different species (Santos *et . al*., 2009). The use of killer toxins to control yeast population during fermentation has been postulated for beer , wine and bread (Tredoux *et.al*.,1986 , Schmitt & Breinig , 2002, Antonini *et.al*.,2005 , Lopes&Sangorrin, 2010).In food industry , the use of killer yeasts as starter culture could protect against spoilage yeasts (Waema *et.al*.,2009).

Genetically engineered specific killer strain of *S.cerevisiae* can be used as commercial starter cultures in wine fermentation to prevent the growth of wild strains of *S.cerevisiae* and other closely related *Saccharomyces* during and after alcoholic fermentation, thereby protecting the final product from fermentation and production of a biofilm (Heard&Fleet, 1987). Also, of potential industrial interest is the finding of osmophilic killer yeasts, whose toxic activity was demonstrated only in the presence of high concentration of salts such as NaCl or KCl. For example, osmophilic *Kluyveromyces* strains with killer activity against *Zygosaccharomyces rouxii* is useful in developing natural preservatives to prevent refermentation of salted fermented foods (Magliani *et.al*., 1997, Hernández *et al.*, 2008). Santos *et al.* (2009) reported that P MKT2,a new killer toxin from *Pichia membranifaciiens* can be used in wine fermentation to avoid the development of spoilage yeasts . PMKT2 was able to inhibit *Brettanomyces bruxellensis* while *S.cerevisiae*, the fermentation strain, was full resistant.

On the other hand, food spoilage caused by microorganisms is a serious problem for the food industry. The exploration of killer yeasts as producers of mycocins active against these undesired microorganisms seems to be promising. Hence, the use of selected killer yeasts as a bio-control method may be related to the improvement of the food industry by reducing the use of chemical preservatives.

II- Killer yeast as potential antimicrobial agents

The finding that the killer activity could be displayed against a great variety of eukaryotic and prokaryotic microorganisms led to a re-evaluation of the yeast killer phenomena, with special emphasis on the surprising susceptibility of microorganisms of clinical interest such as *Candida albicans*, *Pneumocystis carinii* and *Mycobacterium tuberculosis* (Magliani *et.al.,* 1997).

As antifungal agent

Antifungal research is currently focusing on the possible use of yeast killer toxins as novel antifungal agent (Schmitt & Breinig, 2002). Killer toxins in future might find application in the treatment of fungal infection (Magliani *et.al*., 1997). Within this group, secreted killer toxins mainly produced by non-*Saccharomyces* yeasts show a broad spectrum of killing activity against a great number of human and plant pathogens (Schmitt & Radler, 1988).

The killer toxin –producing yeasts has a clinical significance due to the search for new antimycotic agents medically important strains that cause human and animal fungal infections (Rogers & Bevan, 1978; Schmitt & Radler, 1988) for example, killer toxin activity of *Pichia anomala* was reported to be fungistatic for *Candida albicans* (Vadkertiova&Slavikova, 2007).The use of selected toxins against pathogenic yeasts that cause systemic mycoses has also been suggested by Walker *et.al*. (1995). However, most yeast killer proteins exhibit their cytotoxic activity only within a narrow pH range and temperature between 20ºC and 30ºC and, therefore yeast toxins are probably not suitable for oral and /or intravenous administration, but applications in the treatment of superficial lesions might well be possible (Schmitt & Breinig, 2002).

Some yeasts are potential as biological control agents against plant pathogenic fungi. *Pichia membranifaciens* might have the potential to control *Botrytis cinerae* , which causes the gray mold disease (Santos *et.al.,* 2004).Also , yeast killer toxins have been shown to have inhibitory effects on some wood decay and plant pathogenic fungi (Waema *et.al*., 2009) .

As antibacterial agent

Killer activity of yeast might operate over bacteria and could be used for the bio- control of contaminating bacteria for alcoholic fermentation. (Polonelli & Morace, 1986, Meneghin *et al.*, 2010). It was reported that toxins from *C. glabrata*, *P. anomala* and *T. figueirae* were found to be active against *Lactobacillus plantarum* and *Bacillus subtilis* (Polonelli & Morace 1986). Also, killer toxin of *Candida kruseii*, isolated from fermented vegetables, exhibited growth inhibition against *E.coli*, *S.aureus, S .typhimurum* and *B. cereus* (Waema *et al.*, 2009).The killer activity of *Saccaromyces cerevisiae* against bacterial strains was reported by Meneghin *et.al.* (2010). Also, Polonelli & Morace (1986) mentioned that *S.cerevisiae* was only capable to inhibit Gram negative bacteria. However, the inability of K9 killer toxin from *Williopsis saturnus* var *marki* NCYC500 to kill *Streptococcus penumoniae* was recently demonstrated by Ochigava *et al.* (2011).

III- Yeast killer system in bio-typing

Killer system may be effective and inexpensive tool for yeast finger printing and could be used for intraspecific characterization of industrially and clinically interesting yeast cultures (Ochigava *et al.* 2011). The first application of the yeast killer system for intraspecific differentiation of pathogenic fungi was reported for *Candida albicans* isolates. The killer system has proven to be fruitful not only in differentiation of important slowly growing pathogenic, such as the mycobacteria, but also in the differentiation of faster-growing Gram positive (Izgu *et al.*, 1997) and Gram negative bacteria (Polonelli *et al.*, 1987).

Evidence that selected killer yeasts may display their inhibitory effect on different species of molds other than yeasts prompted evaluation of the potential of the yeast killer system to differentiate strains of *Pseudallescheria boydii* , *Aspergillus niger* and *Sporothrix schenckii*. The opportunity to differentiate members of the genus *Aspergillus* was of great value for studying the impact of fungal pathogen *A. fumigatus* in hospitalized patients during outbreak of aspergillosis (Magliani *et al.*,1997) .

IV- killer yeasts in recombinant DNA technology and transgenic plants

Killer plasmids of *Saccharomyces cerevisiae*, which code for killer toxins, have been used as cloning vectors in recombinant DNA technology for the expression of foreign protein. Killer toxins which are naturally produced and secreted by virus-infected strains of the fungal pathogen *U.maydis* have been shown to be attractive and unique model for the introduction of fungal resistance into tobacco plants (Kinal *et.al*., 1995, Soares & Sato, 2000).

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الخمائر المنتجة للسموم : نظرة شاملة

عمرو عبد الرحمن البنا- ملاك أحمد الصحن- محمد جمال شحاته

قسم علوم وتكنولوجيا الأغذية ، كلية الزراعة – جامعة الإسكندرية – الرقم البريدى ٢١٥٤٥- الشاطبى – الإسكندرية مصر

يعتبر إنتاج الخمائر لسموم خارجية لها فعل مضاد للميكروبات على الميكروبات الحساسة لها بمثابة ظاهرة معروفه نسبيا.هذه السموم الخارجية عاده ما تكون بروتيناً أو جليكوبروتين ويكون في مقدورها قتل الخلايا الحساسة من ميكروبات تنتمى لنفس نوع أو أنواع قريبة من الميكروب المنتج لها وقد سميت هذه السموم بالسموم القاتلة وكذا السلالة المنتجة لها باسم السلالة القاتلة . أول اكتشاف لسلالة قاتله كان من الخميرة *Saccharomyces cerevisiae* تلي ذلك اكتشاف سلالات قاتلة من أجناس خمائر أخرى تشمل :

Candida, Cryptococcus, Hanseniaspora, Kluyveromyces, Pichia,

Torulopsis, Ustilago, Williopsis and Zygosaccharomyces

تم التعرف على العديد من السموم القاتلة وتمييز ثلاثة أنواع منها طبقا للجينوم المنتج لها وهى على النحو التالي : سموم منتجة بواسطة RNA مزدوج السلسلة موجود في السيتوبلازم (مثل سموم K28, K2, K1 المنتجة بواسطة *S. cerevisiae* ، سموم منتجه بواسطة *Ustilage maydis, Hanseniaspora uvarum*)، سموم منتجة بواسطة سلاسل مزدوجة خطية من DNA موجود في بلازميد (مثل سموم *Kluyveromyces lactis, Pichia inositovora, Pichia acaciae*) أو سموم منتجة بواسطة الكر وموسومات نفسها (مثل KHR, KSH المنتج بواسطة *S. Cerevisiae* وسموم *Williopsis mrakii*) وتجدر الإشارة إلى أن للسموم القاتلة والخمائر القاتلة العديد من التطبيقات وذلك خلال العقدين الأخيرين ، فقد استخدمت الخمائر القاتلة فى مجال صناعات التخمير والأغذية لقتل سلالات الخميرة الملوثة أثناء إنتاج النبيذ والبيرة والخبز. كما استخدمت الخمائر القاتلة كعوامل تحكم حيوية في حفظ الأغذية ، فى تصنيف الخمائر والفطريات أشباه الخمائر الممرضة ، كمضاد جديد للفطريات فى معالجه العدوى الفطرية فى الإنسان والنبات وأخيرا في مجال تكنولوجيا DNA معاد التوليف.