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RESEARCH ARTICLE

STUDY OF YEAST KILLER TOXINS AND THEIR INTERACTIONS.

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Abstract

Yeast killer toxins were identified as proteins which were named killer factors or killer toxins and the producing strains were termed killer yeast. Killer yeasts secrete proteinaceous killer toxins lethal to susceptible yeast strains. The killer strains are insensitive to their own toxins.We aimed at this work to Screen for the killer and sensitive strains among different yeast strains and to study the effect of the environment on this killer character and the different curing treatments for detecting the genetic determinants responsible for the killer toxins. Thirty two yeast strains were used for cross-reaction assay to select some killer strains against 8 yeast strains. Five of these strains were used to study the effect of the selected killer yeasts on their growth. The killer strains obtained were characterized in relation to the optimal temperature and pH for activity. Most killing yeast strain was *Candida glabrata* . The killed yeasts represented all different genera under study except for S.Cervisciae ATCC 6037. Curing experiments showed that dsDNA plasmid are responsible for the killer character in these killer strains except for the killer strain S.Cervisciae. While all these strains except for C. krusie CMGB94 lost their killing activity with elevated temperature curing. All strains showed complete curing and lost their killing activity at both PH3 and PH8, while showed optimum killing activity at PH4 and 5, while partial curing occurred at PH 6 and 7.Also all strains except for C. krusie CMGB94 showed dsrRNA as genetic element responsible for the killer character. Yeast killer toxins are thought to serve the purpose of competitor killing and thereby provide a selective advantage to the producing species. Killer yeasts and their toxins have many potential applications in environmental, medical and industrial biotechnology.

Introduction:-

The killer system in yeasts has been extensively investigated since it was first described in *Saccharomyces cerevisiae* by Bevan and Makower (Bevan, and Makower 1963). Killer strains secrete a protein toxin which is lethal

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to sensitive strains of the same genus and, less frequently, strains of different genera (Petering et al 1991). Among the yeasts, killer, sensitive, and neutral strains have been described. Eleven distinct patterns of the range of killer activity against killer yeast have been found (K1-K11) according to the interaction between the killer yeasts (Soares and Sato 1999).

Yeast strains belonging to diverse species produce and secrete proteins or glycoproteins, known as killer toxins (KTs), that are lethal to susceptible strains (Bostian , et al 1980a). This property, which offers a competitive advantage to self-immune killer yeasts in their ecological niches, has found several applications in the biological control of plant pathogens and spoiling yeasts in the food and fermentation industries (Bostian , et al 1980b). In the medical field, KTs have been used for the biotyping of pathogenic microorganisms, in epidemiological studies, and for the identification of novel cellular targets in microbial cells and the development of new antimicrobials (Goto, et al 1990). Some KTs, such as K1 and K28 from Saccharomyces cerevisiae, have a narrow spectrum of activity, limited to susceptible strains of the same species, while other KTs show a wide killing spectrum (Bostian , et al 1980b). In particular, some Wickerhamomyces anomalus (formerly Pichia anomala) KTs proved to be active against a wide range of microorganisms, including other yeast species, filamentous fungi, bacteria, and protozoan parasites (Kitano , et al 1984).

A killer phenomenon in yeast, namely, the ability to secrete a toxic agent by some strains of a defined species that is toxic to sensitive individuals of the same species and related species, was initially reported in Saccharomyces cerevisiae by Bevan and Makower (1963). A similar phenomenon was earlier reported in bacteria and the secreted agents were referred to as colicins (Fredericq, 1956).

Following the initial observations, which limited the activity of killer toxins to restricted species of yeasts, the finding that, the killer activity could be displayed against a great variety of unrelated eukaryotic and prokaryotic microorganisms. This finding led to the reevaluation of the yeast killer phenomenon, with special emphasis on the surprising susceptibility of microorganisms of clinical interest such as Candida albicans, Pneumocystis carnii and Mycobacterium tuberculosis (Baeza et al., 2008).

Genetic studies have shown that the killer phenotype of S. cerevisiae is inherited cytoplasmically and has been linked to the presence of a double stranded RNA (dsRNA) associated with virus-like particles within the cytoplasm of the killer cells. However, dsRNA need not always be the determinant. In other genera, like Kluyveromyces lactis, the information for the killer phenotype is carried by linear dsDNA . The killer character of Candida species is encoded by chromosomal genes, not by extrachromosomal ones (Maqueda et al, 2011).

We aimed at this work to Screen for the killer and sensitive strains among different yeast strains and to study the effect of the environment on this killer character and the different curing treatments for detecting the genetic determinants responsible for the killer toxins. The killer strains obtained will be characterized in relation to the optimal temperature and pH for activity, and the kinetics of the production of killer activity

Materials and Methods

1. Strains

This study was conducted on 32 yeast strains that were provided from Faculty of science , King Abdul-Aziz University- Jeddah, Saudia Arabia. These thirty two yeast strains were used for cross-reaction assay to select some killer strains against 8 yeast strains. Five of these strains were used to study the effect of the selected killer yeasts on their growth.

Yeast strains:

Saccharomyces cerevisiae UQM 49 (14), NRRLY-17007 (43), NRRLY-17008 (44), NRRLY-17009 (45), NRRLY-1208 (49), LBC-1269 (53), LBC-254 (57), Kluyveromyces lactis NRRLY-1140 (9), Kluyveromyces fragilis DSM 70292 (29), Candida pelliculosa (C1), (C2), Candida utilis (C3), Candida tropicalis (C4), Saccharomycopsis fibuligera NRRLY-1062 (D) and Saccharomycopsis lipolytica DSM 70562 (27). All were used in this study.

2. Media

Yeast extract peptone dextrose medium (YEPD) was routinely used for maintenance and preparation of different yeast cultures. It has the following composition: 1% yeast extract, 2% peptone, 2% glucose and 2% agar. Cross-reaction and assay of killer character during this study were carried out on the killer medium (KM) (Somers and

Bevan, 1969) which contains 10 g yeast extract, 10 g peptone, 20 g glucose, 20 g agar, 110 ml of 1M K2PO4-citric acid buffer (pH 4.5), 30 mg methylene blue, and distilled water up to 1000 ml.

3. Assay of killer character

The Killer and resistance characters were assayed as described by Somers and Bevan (1969), using killer medium. For examination of the killer phenotype, cells of the strain to be tested for producing killer toxin (s) were streaked on cells of the strain to be tested for sensitivity which was uniformly suspended in 20 ml of KM agar maintained at 45^{o}C, since methylene blue is a specific stain for dead yeast cells. After two days of incubation at 25^{o}C, results were categorized as three types: Negative (confluent growth of indicator strain and streak without color effects); Intermediate killing (streak surrounded by clear inhibition zone without blue edge or indicator turned blue without inhibition zone); Complete killing (spot surrounded by clear inhibition zone lined with blue ring of dead indicator cells). Resistant colonies showed no reaction on either killer or sensitive agar plates.

4. Curing experiments

Yeast strains were cured of their killer phenotype by cultivation in liquid YEPD medium in the presence of 20 μg/ml ethidium bromide at 30^{o}C for 72 hours (Gunge and Sakaguchi, 1981) or 150 ng/ml of cycloheximide for 72 hours (Fink and Styles, 1972) or at elevated temperature of 37^{o}C for 72 hours (Wickner, 1974) or at different PH range (Tredoux et al., 1986). Samples were removed once a day from each culture of elevated temperature treatment or different PH and once at the end of ethidium bromide and cycloheximide treatment. Several dilutions were plated onto YEPD. A total of 100 single colonies were randomly isolated and tested for the killer phenotype.

Results:-

1. screening for killer and sensitive yeast strains

Thirty two yeast strains belonging to Twelve species and four genera were used in this study. They were tested for differences in both killing and immunity cross-reactions as shown in table 1.

Table 1:-Killing and sensitivity cross-reaction assay of different yeast strains

Strains tested for sensitivity		**Strains tested for killing ability**							
		S. cerevisiae LBC 2504	C.tropicalis CMGB165	Saccharomycopsis lipolytica NRRLY-1062	S. lipolytica DSM 70562	s.Crvisciae ATCC201583	C. krusie CMGB 94,	S.Cervisciae ATCC 4126	S.Cervisciae ATCC 6037
Saccharomyces strains									
S. cerevisiae UQM 49	1	-	-	-	-	-	-	-	-
S. cerevisiae NRRLY-17007	2	-	-	-	-	-	-	-	-
S. cerevisiae NRRLY-17008	3	-	-	-	-	-	-	-	-
S. cerevisiae NRRLY-17009	4	-	-	-	-	-	-	-	++
S. cerevisiae NRRLY-1208	5	-	++	++	-	-	-	-	-
S. cerevisiae LBC 1269	6	-	+	+	-	-	-	-	-
S. cerevisiae LBC 2504	7	-	-	-	-	-	-	-	+
Candida strains									

C. pelliculosa	8	-	++	++	++	-	++	-	++
C. albicans	9	++	+	++	++	-	++	-	++
C.albicans	10	-	-	-	-	-	-	-	-
C. tropicalis	11	-	-	-	-	-	-	-	-
C.albicans	14	-	-	-	-	-	-	-	-
C.albicans	15	-	-	-	-	-	-	-	++
C.albicans	16	-	-	-	-	-	-	-	-
C.famata	17	-	-	-	-	-	-	-	+
C.famata	18	-	-	-	-	-	-	-	++
C.famata	19	-	-	-	-	-	-	-	-
C.famata	20	-	-	-	-	-	-	-	-
C.lusitania	21	-	-	-	-	-	-	-	-
C.glabrata	22	-	-	-	-	-	-	-	-
C.glabrata	23	+	++	++	++	++	++	+	++
C.parapsilosis ATCC 22019	24	-	++	+	+	+	+	-	-
C.parapsilosis	25	-	+	++	+	+	-	-	-
C. tropicalis	26	+	+	+	-	++	-	+	-
C. tropicalis	27	-	++	-	-	-	-	-	-
C. tropicalis	28	-	-	-	-	-	-	-	-
C. tropicalis	29	-	++	-	++	+	-	-	-
C. tropicalis	30	-	++	++	++	+	+	+	++
C.krusei ATCC14243	32	+	++	++	++	++	+	+	++
Kluyveromyces strains									
K. lactis NRRLY-1140	12	-	-	-	-	++	-	-	-
K. fragilis DSM 70292	13	-	-	-	-	-	-	-	-
Cryptococcus strain									
Creptococcus	31	-	++	-	-	+	-	-	-

neoformans									

S.: Saccharomyces. , C.: Candida , K.: kluyveromyces. ++ = complete killing; + = intermediate killing; - = nonkilling.

Different curing treatments for detecting the genetic determinants responsible for the killer toxins as shown in table 2.

Table 2:-Curing of killer yeast using sensitive strain No 23:

	Method of curing											
Killing strain	growth at 20°C	growth at 25°C	growth at 37°C	growth at 40°C	PH 3	PH 4	PH 5	PH 6	PH 7	PH 8	Cycloheximide	Ethidium bromide
C.tropicalis CMGB165	++	++	+	-	-	++	++	+	+	-	-	-
S. lipolytica DSM 70562	++	++	++	-	-	++	++	+	+	-	+	-
S.Cerviscia e ATCC 6037	++	++	+	-	-	++	++	+	+	-	-	++
S.Cerviscia e ATCC 4126	++	++	+	-	-	++	++	+	+	-	-	-
C. krusie CMGB94,	++	++	++	+	-	++	++	+	+	-	++	-

++ Complete killing , + Partial killing , - non killing (curing).

Discussion:-

Following the initial observations, which limited the activity of killer toxins to restricted species of yeasts, the finding that the killer activity could be displayed against a great variety of unrelated eukaryotic and prokaryotic microorganisms led to a reevaluation of the yeast killer phenomenon, with special emphasis on the surprising susceptibility of microorganisms of clinical interest such as Candida albicans and Mycobacterium tuberculosis (Polonelli et al., 1986).

Results obviously showed that yeast strains under investigation not only varied greatly in killing spectrum, but they also showed different patterns of killing ability, indicating that they may secrete different killer factors. The killing spectrum of the killer strains was previously used to identify the killer phenotype of the screened killer yeasts (Rogers and Bevan, 1978).

Results in table (1) show that the most killing yeast strain is Candida glabrata strain 23, which revealed killer activity against seven out of the eight tested strains (87.5%). The killed yeasts represented all different genera under study except for S.Cervisciae ATCC 6037. It is important to mention that Candida glabrata was described by Helguera, et al (2012**)** as a killer against a Saccharomyces cerevisiae W303 sensitive strain. Also Candida albicans was the 2nd most killing strain following Candida glabrata as it killed 6 out of 8 sensitive strains (75%). The killed yeasts represented all different genera under study except for S.Cervisciae ATCC 6037 and S.Cervisciae ATCC 4126. Worldwide, the prevalent cause of invasive candidiasis remains C. albicans, although the epidemiology of candidal infections has gradually shifted towards non-albicans species, such as C. glabrata and C. krusei (Enoch et al 2017). Also , C. tropicalis strain No 26 showed complete killing activity against 4 tested strains 50% , moderate killing activity against 2 out of 8 killing strains 25% and non killing activity against the remaining 2 strains. On the other hand only 2 strains of S.cerviaisae code no 5 and 6 showed both complete and moderate killing activity respectively against 2 (25%) only of the tested strains and the remaining isolates were non killing against all 8 tested strains. This was in agreement with the report of Young and Yagiu (1978) who suggested that killer strains of S. cerevisiae showed an antiyeast spectrum restricted to sensitive Saccharomyces strains (Table 1).

It was observed that Candida strains code 8,9,23,30 and 32 were the most sensitive strains. They were killed by strains code 5,6,8,7 and 8 out of the 8 tested killer strains respectively. On the other hand there were 10 strains out of the tested 32 strains proved to be resistant to killing they included 3 S.Cereviciae strains code 1,2,3 which included, S. cerevisiae UQM 49 , S. cerevisiae NRRLY-17007, S. cerevisiae NRRLY-17008. Also K. fragilis DSM 70292 code 13 was resistant, 6 candida strains code 14,16,19,20,21,28. Any of the killer strains could affect all these strains growth (Table 1).

Taking into consideration all these results, C.tropicalis CMGB165, Saccharomycopsis lipolytica NRRLY-1062, S.Cervisciae ATCC 6037 which showed the broadest killer spectrum were chosen for tests of curing against sensitive strains code 8,9,23,30 and 32 , which showed the largest inhibition zone.

The responsible genes for the killer toxins may be carried on a chromosome, on dsRNA or on linear dsDNA. To locate the position of the genetic determinants of the killer toxins in the three chosen killer yeasts, several plasmid curing treatments were performed. Ethidium bromide treatment has been reported to cure dsDNA killer plasmids (Gunge and Sakaguchi, 1981) , While, cycloheximide treatment (Carroll and Wickner 1995) and growth at an elevated temperature (Wickner, 1974) have been reported to eliminate dsRNA killer plasmids.

A total of 100 single colonies were randomly isolated and tested for the killer phenotype against sensitive strain 23 after the treatment of the five killers; with ethidium bromide. All these colonies except those of the killer strain S.Cervisciae ATCC 6037 lost their killer activity after 48 hours .These results provide evidence that dsDNA plasmid are responsible for the killer character in these killer strains except for the killer strain S.Cervisciae ATCC 6037 , since the treatment with ethidium bromide, which was reported to cure dsDNA plasmids gave positive results.

The replication of M1 dsRNA virus is naturally temperature sensitive, so that the growth at elevated temperatures (37^{o}C or above) can cure infected strains of their M1 dsRNA (Weinstein et al., 1993).

As shown in table (2) all single colonies isolated from the chosen five killer strains were subjected to elevated temperature treatment, all these strains except for C. krusie CMGB94 strain lost their killing activity against the sensitive strain 23. Only C. krusie CMGB94 49 showed remained partially cured . With increasing the temperature of the treatment. Partial curing of M dsRNA by the growth at elevated temperature was previously reported by Wickner (1974) and Weinstein et al. (1993) using S. cerevisiae killer strains. Cells of the original killer strain were streaked on cells of the nonkiller isolates to examine their sensitivity to the original killer strain. All the heat-cured colonies obtained after elevated temperature curing proved to be sensitive to the killer toxin produced by the original strain. This means that the cured isolates lost the genetic material responsible for the killing activity as well as the immunity to the toxin.

As shown in table (2) all single colonies isolated from the chosen five killer strains were subjected to different range of PH, with temperature 25 ^{o}C. All strains showed complete curing and lost their killing activity against the sensitive strain 23 at both PH3 and PH8, while showed optimum killing activity at PH4 and 5, while partial curing occurred at PH 6 and 7. Tredoux et al., 1986 studied Effect of temperature and pH on killer action of Killer yeast: they found that the optimum PH for killer action of S.cerviaciea strain against WE500 (a sensitive strain in the South African wine industry that were selected from the culture collection) was either at pH 4.8 or above (Tredoux et al., 1986). Also Yap 2000 found in his thesis that there is a variation in killer yeast activity with changes in PH levels with different types of killer yeasts (Yap, 2000).

Conclusion:-

Results obviously showed that yeast strains under investigation not only varied greatly in killing spectrum, but they also showed different patterns of killing ability, indicating that they may secrete different killer factors. Most killing yeast strain was Candida glabrata ,while Candida albicans was the 2nd most killing strain. The killed yeasts represented all different genera under study except for S.Cervisciae ATCC 6037. It was observed that the most sensitive strains were from Candida species , while the most resistant strains were from Saccharomyces species. Curing experiments showed that dsDNA plasmid are responsible for the killer character in these killer strains except for the killer strain S.Cervisciae. While all these strains except for C. krusie CMGB94 lost their killing activity with elevated temperature curing. All strains showed complete curing and lost their killing activity at both PH3 and PH8, while showed optimum killing activity at PH4 and 5, while partial curing occurred at PH 6 and 7. Also all strains

except except for C. krusie CMGB94 showed dsrRNA as genetic element responsible for the killer character. In challenging for limited resources, 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 growth arrest of other species or genera. Although of all potential applications of killer yeasts or their killer toxins, killer yeast strains can be a problem in commercial processing because they can kill desirable strains. Carful study and further molecular testing are needed to confirm their presence and subsequently their usefulness.

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