Determination of some optimum conditions for production of a killer toxin AY from the yeast *Saccharomyces cerevisiae*

Dr. Wala'a Shawkat Ali
Department of Biology/ College of Science
Al-Mustansiriya University.

ABSTRACT

Four samples of commercial brand of ready powdered *Saccharomyces cerevisiae* were collected from local market (Town and Angel/ China , Saf/ France and Pakmaya/ Turkey) for detection their ability to produce killer toxin against eleven isolates of microorganisms: *Lactobacillus acidophilus* , *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Staphylococcus aureus* , *Serratia marcescens* , *Cladosporium* spp., *Fusarium* spp., *Aspergillus* spp. and three isolates of *Candida albicans* , as well as *S. cerevisiae* isolates. Results showed that Angel yeast was the best one for production of killer toxin ,no production of killer toxin was observed by other isolates of *S. cerevisiae*. Also some optimum conditions for production of killer toxin was determined and the results showed that cultivation of yeast in glucose yeast extract peptone broth(GYEPB),adjusted at pH 5 and incubated at 25°C for 48 hours was the best to produce it .

INTRODUCTION

A wide range of antimicrobial substances has been successively characterized, including antibiotics, bacteriolytic enzymes, and bacteriocins. In 1963,Bevan and Makower [1] reported, for the first time in yeasts, that a few isolates of *Saccharomyces cerevisiae* secreted a substance that was lethal to other strains of the same species. Since the original discovery of the killer phenomenon in yeasts, several reports have addressed the question of the frequency of occurrence and range of specificity of yeast killer toxins [1].Yeast killer toxins (mycocins) are proteinaceous compounds which are active against members of the same species or closely related species, and the activities of these toxins are analogous to the activities of bacteriocins in bacterial species[2].

There is a high frequency of killer yeasts found in laboratory stocks and collections of commercial strains , but the frequency is higher still in natural habitats [3,4]. There are three basic phenotypes: killer, sensitive and neutral. The killer strains both produce a specific killer factor and resist to this toxin. The

resistant or neutral strains are insensitive to one or more killer factors and do not produce any toxin. The sensitive strains are totally susceptible to the killer toxin, which causes inhibition of macromolecule synthesis and cell disruption resulting in cell death. The wide occurrence of the killer phenomenon suggests that it plays an important role in yeast ecology, altering the distribution of the strains through competition by interference [5].The most thoroughly investigated yeast killer system is that of *S. cerevisiae*, which has been described in detail in many reviews. 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 [6,7].

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 animal fungal infections[8], as well as in the design and development of tools for recombinant DNA technology [9]. The yeast killer system was also applied for differentiation of etiological agents of nocardiosis and Gram-positive pathogenic bacteria [10].

The aim of this study was to detection of killer toxin production from *S. cerevisiae* and determination of some optimum conditions for its production.

MATERIALS AND METHODS

Yeast isolates

Four samples of commercial brand of ready powdered *Saccharomyces cerevisiae* were collected from local market:Town and Angel (China) , Saf (France) and Pakmaya (Turkey).

Indicator microbes

Lactobacillus acidophilus , *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Staphylococcus aureus* , *Serratia marcescens* , *Cladosporium* spp., *Fusarium* spp., *Aspergillus* spp. and 3 isolates of *Candida albicans* (Department of biology/College of Science/Al-Mustansiriya University) as well as *Saccharomyces cerevisiae* isolates ,were used as indicator microbes.

Culture media

Sabouraud agar (SA) (Hi-Media / India), Mueller Hinton agar (MHA) (Microgen /India), Malt extract broth (MEB) [11], pepton broth (PB), Yeast extract peptone broth(YEPB) and Potato dextrose broth(PDB) [12], Sabourouds dextrose broth(SDB) [13], Glucose yeast extract peptone broth(GYEPB) [7] and

wheat bran medium (WBM) which prepared by dissolving 5 gm wheat bran and 20 gm glucose in 1 liter of distilled water and autoclaved.

Activation of *Saccharomyces cerevisiae*

It was activated by dissolving 1 mg of powdered yeast in 5ml of GYEPB and incubated at 37°C for 24 hours, then streaked on SA and incubated at same conditions [14].

Preparation of killer toxin

The method of Mohamudha and Ayesha [15] was used, with minor modifications. The culture of *S. cerevisiae* was grown in GYEPB for 48 h at room temperature. After centrifugation at 6000 rpm for 15 min at 4°C, the supernatant was incubated for 4 h at 4°C and the precipitate was collected by centrifugation at 6000 rpm for 20 min and re-suspended in equal volume of 0.1M citrate phosphate buffer pH 4.2. This toxin supernatant was stored at 4°C until use.

killer toxin activity assay

Pour plates were prepared from SA seeded with indicator fungi while pour plates were prepared from MHA seeded with indicator bacteria. Wells (6 mm diam.) cut into the pour plates and filled with 100 µl of the toxin supernatant. The plates of fungi were incubated at 25°C for 2-3 days and plates of bacteria were incubated at 37°C for 24 h.The presence of killer toxin was indicated by inhibition zones around the wells. The killing activity of each sample was measured and defined as the mean zone of inhibition of replicate wells [3]. The antimicrobial activity of killer toxin was determined in all experiments of this study.

Production of killer toxin in different media : To determine the best medium for production of killer toxin, an activated yeast was inoculated into different culture media: MEB, PB , YEPB , SDB , PDB , GYEPB and WBM. Cultures were incubated at 25°C for 48 h.

Effect of medium pH : GYEPB was adjusted to pH 3.5, 4, 4.5, 5, 5.5 and 6 respectively, and inoculated with an activated yeast then incubated at 25°C for 48 h.

Effect of incubation temperature : GYEPB was inoculated with an activated yeast and incubated at 25, 37 and 40°C for 48 h.

Effect of incubation periods : GYEPB was inoculated with an activated yeast and incubated at 25°C for 24 , 48 and 72 h.

RESULTS AND DISCUSSION

The assay method currently employed to distinguish killer strains was first described by Makower and Bevan in 1963. Woods and Bevan refined this method and established the optimal culture conditions for toxin production and activity. The killing ability of these compounds may be underestimated or may even remain unnoticed depending on the selection of the appropriate sensitive

strain and other experimental conditions [4]. As shown in table 1,only one isolate of *S. cerevisiae* (Angel yeast) referred as AY was able to produce killer toxin against some isolates of microorganisms. The highest activity was observed against *C. albicans* isolates and *C. albicans* OR was the most sensitive one to killer toxin AY, therefore *C. albicans* OR was selected as indicator isolate in all subsequent experiments in this study . Lowest activity was observed against *P. aeruginosa* , *P. fluorescens*, *S. marcescens* and *S. aureus* . No activity was observed against other microorganisms used in this study. The patterns of sensitivity observed were likely to be as a result of a number of factors including: site of isolation (which undoubtedly influenced species and strain type found) ; micro-environmental conditions within the niche (which may have induced morphological changes in the isolates) ; the status of the individual (which may have influenced isolate type) ; and cell wall characteristics. A combination of these factors could affect the interaction between the toxin molecules and their cell surface receptors and the type and number of receptors available may differ between isolates [3].

Previous finding of positive results for inhibitory action of killer toxins produced by *Saccharomyces* against yeasts and bacterial isolates is consistent with the present observation [1,14,16].It was apparent that not all strains of the same species produced the same patterns of activity and such diversity of action suggests that the toxins produced by each may be biochemically distinct from one another [3].However, The nature of the interaction(s) between the killer yeasts and strains of other microbial groups still remains to be elucidated [10].

Table -1: Antimicrobial activity of killer toxin of *Saccharomyces cerevisiae* isolates against indicator microbes .

Indicator microbes	Isolates of *Saccharomyces cerevisiae*			
	PY	SY	TY	AY
Lactobacillus acidophilus	-	-	-	-
Pseudomonas aeruginosa	-	-	-	+
Pseudomonas fluorescens	-	-	-	+
Staphylococcus aureus	-	-	-	+
Serratia marcescens	-	-	-	+
***Cladosporium* spp.**	-	-	-	-
***Fusarium* spp.**	-	-	-	-
***Aspergillus* spp.**	-	-	-	-
***Candida albicans* V1**	-	-	-	+
***Candida albicans*V2**	-	-	-	+
***Candida albicans* OR**	-	-	-	+
***Saccharomyces cerevisiae* PY**	-	-	-	-
***Saccharomyces cerevisiae* SY**	-	-	-	-
***Saccharomyces cerevisiae* TY**	-	-	-	-
***Saccharomyces cerevisiae* AY**	-	-	-	-

+ : inhibition zone , - : no inhibition zone.

Optimal medium for killer toxin production

As shown in table 2, much higher production was observed in GYEPB against *C. albicans* OR than in PDB , WBM and SDB. Low killer toxin production was observed in YEPB and MEB. No production of killer toxin was observed in PB . The production of killer toxins may be strongly affected by the culture conditions and optimal conditions may need to be found empirically [17].Previous studies have shown that killer toxin production is enhanced by the presence of yeast extract and organic nitrogen compounds in the growth medium [17]. The yeasts secret glycoprotein toxins that kill the sensitive strains in grape must , YEPD and in many other culture media [18].However, the variance of production of killer toxin in different culture media used in this study might be due to the differences of type and quantity of carbon and nitrogen sources as well as the other components.

Table -2: Antimicrobial activity of killer toxin AY of *Saccharomyces cerevisiae* AY which cultivated in different media against *Candida albicans* OR

Cultural medium	Diameter of inhibition zone (mm)
Malt extract broth (MEB)	11.5
Pepton broth (PB)	-
Yeast extract peptone broth(YEPB)	12
Sabourouds dextrose broth(SDB)	14.5
Potato dextrose broth(PDB)	15
Glucose yeast extract peptone broth(GYEPB)	18
Wheat bran medium (WBM)	15

Optimal pH for killer toxin production

As shown in table 3 , pH 5 was the best value for toxin production. No production was observed at pH values 3.5 , 5.5 and 6 ,while significant reduction in toxin production was observed at pH values 4 and 4.5.

The optimum pH for production and stability of different killer toxins was varied ,for example, the optimum pH for production and stability of K1killer toxin ranges from 4.6 to 4.8, while the optimum pH for K2 toxin ranges from 2.9 to 4.9 [4].In general, *S. cerevisiae* is an acidophilic organism and, as such, grows better under acidic conditions. The optimal pH range for yeast growth can vary from pH 4 to 6, depending on temperature, the presence of oxygen, and the strain of yeast [19].

Optimal temperature for killer toxin production

Best production of killer toxin was occurred at incubation temperature 25°C,while at 37°C the production was reduced. No production was observed at 40°C (table 3). Similar results were recorded for other killer toxins [7,16].Most of the yeast colonies formed at 37 or 40 °C showed no evidence of killing and all strains showed increasing frequency of non killer colonies with increasing temperature [20], for example, *Candida nodaensis* killer toxin displays killer activity when assayed at temperatures ranging from 18 to 30 °C and loses killer

phenotype at 40 °C.This profile is similar to what has been described for other killer toxins [9]. Interestingly, the killer yeasts displayed their toxic activity under remarkably different conditions of pH, temperature, and oxygen concentration [6] and the pair of killer and sensitive strains tested [7].

Optimal incubation period for production

As shown in table 3,when *S. cerevisiae* AY was grown in GYEPB medium at 25°C, low production of killer toxin was observed at 24 h of incubation , maximum production of it was observed at 48 h, while at 72 h ,no production was observed. The measured killing activity increased with cell titer during the exponential phase of growth and leveled off as cells reached their stationary phase [3]. A behavior also found for other killer toxins, whose production and secretion occurs during exponential growth of the producer yeast increasing up to the beginning of stationary phase [3,9]. Hodgson *et al.* [3] reported that to facilitate the production of workable titers of active killer factor under static culture conditions it was necessary to grow the yeast over extended periods of time.

Depending on results above it can be concluded that when the yeast *S. cerevisiae* AY was grown in the GYEPB medium with pH 5 at 25 °C for 48 h , it could produce the highest amount of killer toxin.

Table -3:Effect of pH ,temperature and incubation period on the production of killer toxin AY by *Saccharomyces cerevisiae* AY against *Candida albicans* OR

Production conditions pH	Diameter of inhibition zone(mm)
3.5	-
4	13
4.5	16.5
5	25
5.5	-
6	-
Temperature(°C)	
25	25
37	15
40	-
Incubation period(hours)	
24	17
48	25
72	-

REFERENCES

1- Polonelli, L. and Morace, G.(1986) Reevaluation of the yeast killer phenomenon. J. Clin. Microbiol., 24(5): 866-869.

2- Lowes, K. F. ; Shearman, C. A. ; Payne, J. ; Mackenzie, D. ;Archer, D. B. ; Merry, R. J. and Gasson, M. J.(2000) Prevention of Yeast Spoilage in Feed and Food by the Yeast Mycocin HMK. Appl. Environ. Microbiol.,66(3): 1066–1076.

3- Hodgson,V.J.; Button,D. and Walker,G.M.(1995) Anti-Candida activity of a novel killer toxin from the yeast *Williopsis mrakii*. Microbiol., 141: 2003-2012.

4- Marquina , D. ; Santos, A.; Peinado, J.M.(2002) Biology of killer yeasts. Int. Microbiol., 5: 65–71.

5- Antonini, S.R.C.; Sanino, A.; Araújo, J.C. and Tosta,C.D.(2005) The Killer yeasts and the alcoholic fermentation. Braz. J. Food Technol., 5:40-46.

6- Magliani,W.; Conti,S.; Gerloni,M.; Bertolotti, D. and Polonelli, L.(1997) Yeast killer systems. Clin. Microbiol.Rev., 10(3): 369–400.

7- Rodríguez-Cousin˜o, N. ; Maqueda, M.; Ambrona,J. ; Zamora,E.; Esteban, R. and Ramírez,M.(2011) A new wine *Saccharomyces cerevisiae* killer toxin (Klus), encoded by a double-stranded RNA virus, with broad antifungal activity is evolutionarily related to a chromosomal host gene. Appl. Environ. Microbiol.,77(5): 1822–1832.

8- Schmitt, M. J. and Breinig,F.(2002) The viral killer system in yeast: from molecular biology to application. FEMS Microbiol. Rev., 26: 257-276.

9- Silva, S.; Calado,S.; Lucas,C. and Aguiar, C.(2008) Unusual properties of the halotolerant yeast *Candida nodaensis* Killer toxin, CnKT. Microbiol. Res.,163 : 243-251.

10- Meneghin,M.C.; Reis,V.R. and Ceccato-Antonini,S.R.(2010) Inhibition of bacteria contaminating alcoholic fermentations by killer yeasts. Braz. Arch. Biol. Technol.,53 (5): 1043-1050.

11- Ellis,D.H.(1994) Clinical Mycology: the human opportunistic mycosis.Gillingham,Australia.

12- Atlas, R.M.;Parks, L.C. and Brown, A.E.(1995) Laboratory Manual of Experimental Microbiology.Mosby-Year Book, Inc.USA.

13- Harley, J.P. and Prescott, L.M.(1996) Laboratory Exercises in Microbiology.3rd ed.WCB/McGraw-Hill company, Boston, USA.

14- Al-Gosha'ah,F.A.S.(2005) Studying the effect of inhibitory substances produced by *Saccharomyces boulardii* on virulence factors of some enteric bacteria. Ph.D thesis, Al- Mustansiriya University, College of Science.

15- Mohamudha, P. R. and Ayesha, B. J.(2010) Production and effect of killer by *Saccharomyces cerevisiae* on sensitive yeast and fungal pathogens. Int. J. Pharmaceutical Sci. Rev. Res.,3(1):127-129.

16- Al-Zubaidii,Z.M.E.(2001)Extraction and purification of antimicrobial agents from *Saccharomyces cerevisiae* against some isolates of bacteria, yeasts and molds when grown in different media. Ph.D thesis, Al-Mustansiriya University, College of Science.
17- Santos,A. and Marquina,D.(2004) Killer toxin of *Pichia membranifaciens* and its possible use as a biocontrol agent against grey mould disease of grapevine. Microbiol. ,150: 2527–2534.
18- Silva,G.A.; Poli,J.S.; Poletto, C.M.; Schaker,P.D.C. and Valente,P.(2011) Production of functional killer protein in batch cultures upon a shift from aerobic to anaerobic conditions. Braz. Arch. Biol. Technol.,54 (3): 601-612.
19- Narendranath,N.V. and Power,R.(2005) Relationship between pH and medium dissolved solids in terms of growth and metabolism of Lactobacilli and *Saccharomyces cerevisiae* during ethanol production. Appl. Environ. Microbiol.,71(5): 2239–2243.
20- Wickner, R.B.(1974) "Killer character" of *Saccharomyces cerevisiae*: curing by growth at elevated temperature. J. Bacteriol., 117(3): 1356-1357.

تحديد بعض الظروف المثلى لإنتاج السم القاتل AY من خميرة الخبز *Saccharomyces cerevisiae* AY

م .د. ولاء شوكت علي

قسم علوم الحياة/كلية العلوم/ الجامعة المستنصرية

الخلاصة

تم جمع اربع عينات من خميرة الخبز *Saccharomyces cerevisiae* التجارية الجاهزة من السوق المحلية (تاون و انجل / الصين و ساف/ فرنسا و باكمايا/ تركيا) للتحري عن قابليتها على إنتاج السم القاتل ضد إحدى عشرة عزلة من الأحياء المجهرية والتي شملت: *Pseudomonas aeruginosa* و *Pseudomonas fluorescens* و *Lactobacillus acidophilus* و *Aspergillus* spp. و *Fusarium* spp. و *Cladosporium* spp. و *Staphylococcus aureus* و *Serratia marcescens* وثلاث عزلات من *Candida albicans* فضلا عن عزلات خميرة الخبز نفسها. واظهرت النتائج إن خميرة انجل كانت الأفضل في إنتاج السم القاتل ولم تظهر بقية العزلات من خميرة الخبز أية إنتاجية للسم القاتل ، ايضا تم تحديد بعض الظروف المثلى في إنتاج السم القاتل وأظهرت النتائج إن تنمية الخميرة في وسط Glucose yeast extract peptone broth(GYEPB) ذو الأس الهيدروجيني 5 بدرجة 25 °م لمدة 48 ساعة هو الأفضل لإنتاجه.