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BIODIVERSITY AND KILLER TOXIN OF HALOTOLERANT YEASTS IN DIFFERENT ECOSYSTEMS

BY

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ABSTRACT

The biodiversity of halotolerant yeasts highly varied from one Egyptian ecosystem to another and depending on salt concentration in culture media. The yeasts were presented in 100%, 75%, 66.67%, 50% and 28.57% of the samples of the tested foodstuffs, plants, underground water, saline soil and salted water, respectively. Four hundred and thirty three yeast isolates were isolated from different ecosystems and classified according to their salt response into three categories namely: non or slight-halotolerant, moderate and extreme halotolerant in the percentage of 17.6%, 82.5% and 17.6% respectively. Only eight yeast isolates were selected according to variation in NaCl tolerance and fermentation capacity, then completely identified. The effect of osmotic potential of sodium chloride on the growth behavior and sugar consumption of these strains using shake flasks as a batch culture technique was evaluated. At high NaCl concentration, the lag phase of yeast growth was appeared and recorded the longest period (6 h) at osmotic potential ranged from 8.50 to 16.16 MPa, whereas the specific growth rate during the exponential phase decreased and the final yeast growth (O.D) was reduced. Also, there are direct relationships between NaCl osmotic potential and growth density or sugar utilization efficiency of all halotolerant yeast. The highest specific consumption rate of sugar was noticed during 4- 8h or 6- 12h of fermentation periods by the tested strains at different NaCl osmotic potential. In media containing 5% NaCl, no killer activity against halotolerant *Kl. bacillisporus* 3T was detected. Whereas *Pichia silvicola* 1T, *Pichia silvicola* 26T & *Kluyveromyces bacillisporus* 5T gave higher killer activities than on NaCl free medium but the vice versa was true for other strains. The maximum toxin production was noticed during the stationary phase growth of different strains. The activities of killer toxins against *Ps. lacremance*, *Staph. aureus*, *C. albicans* and fungal strains of *Fusarium* sp., *Tricoderma* sp. & *Aspergillu* sp were recorded.

Key words: Halotolerant yeasts, Egyptian ecosystems, Growth parameters, killer toxin, antimicrobial activity, *Rhodotorula minta*, *Pichia anomala*, *Pichia silvicola*, *Kluyveromyces bacillisporus*.

INTRODUCTION

The halotolerant yeast constitutes a heterogeneous group of yeasts belonging to very different genera and sharing the common capacity to resist low water activity in environments due to high osmotic pressure of salts (Tokuoka and Ishitani, 1991 and van-Eck *et al*, 1993). They are widely distributed in different salted ecosystems. They

are found on salted foodstuffs, salted water. The strongest evidence for this saline soil is the very low cell content: (on average from 10-100 cell g^{-1}). Exceptions can be seen in some forest soils where counts of 10^5- 10^6 g^{-1}were recorded (Martini, 1992). Khattab (2000) found that the count of halotolerant yeasts in marine waters at western parts of Alexandria decreased with increase in the concentration of NaCl. The highest numbers of yeasts recorded in summer. The yeasts that isolated from marine water were identified as *Aureobasidium sp.*, *Debaromyces hansenii*, and *Saccharomyces rosei*.

Gewaily *et al*, (2001) reported that the relatively high concentration of sodium chloride affects the growth parameters of yeasts i.e., growth rate, inoculum size, yield biomass, lag phase, ATPase and cell constituents. The ability of different NaCl concentrations varies greatly. They added that the two most important factors seem to affect this ability were the presence of mechanism for retaining a low concentration of NaCl within the yeast cells and the ability of enzymes to operate in the presence of high concentration of NaCl.

Killer yeasts secrete toxin lethal to sensitive yeasts but they are immune to their own toxins, since killer strains have been isolated from several yeast genera including *Kluyveromyces phaffi* (Ciani and Fatichenti, 2001). Salt may enlarge the activity spectra of killer yeast against the selected target strain (Llorente *et al*, 1997). Santos *et al* (2000) noticed that *P. membarnificiens* CYC1106 showed killer activity against *Candida boidnii* and *Sacch. cerevisiae* only in the presence of NaCl. Also, *C. nodaensis* gave the killer spectrum at 2M NaCl (Silva *et al*, 2003).

In view of these facts, the current work was designed to study the biodiversity of halotolerant yeasts in different Egyptian ecosystems such as salted foodstuffs, salted water, under ground water, plants and saline soil. The most tolerant isolates yeasts were identified in order to study their biological and killer activities at different NaCl osmotic potential. The antimicrobial activity of killer toxin produced by different yeast strains was also evaluated.

MATERIAL AND METHODS

Material:

1. Samples.

Different samples of five ecosystems such as salted foodstuffs, salted water, under ground water, plants and saline soil were collected from different sites in Egypt. Eight samples of salted foodstuffs including salted cheese, white cheese and old cheese as well as pickles of green olive, black olive, cucumber, lemon and union were collected from the local markets of Cairo. Saline soil samples were collected from 4 different sites of Egypt namely, Elwadi Elgadied, El-fayoum, Elsharkua and Elbosilly. Seven salted water samples were collected from Matruh, El Dakhla harbor & Ghaharby harbor of Mediterranean sea and Hurghada, Sharm Elsheikh & Ras as sidr sites of Red sea as well as Eltemsah lake water. Also, three salted water samples were collected in sterile bottles from Bani Suwayf, Elwadi Elgadyied and Siwah. Moreover, some fruits such as apple, figs, grapes and strawberries, as well as the

phyllosphere of fruit leaves were collected from different orchard in Egypt; such as Elfayoum, Elsharkua, Elbehaira and Qalyub.

2. Microorganisms used.

The yeast *Candida albicans* EMCC 105, was obtained from Egypt Microbiology Culture Collection, Cairo MIRCEN, Fac. of Agric. Ain shams univ., Cairo, Egypt, four fungal strains *Fusarium oxysporium, F. solani, Rhizoctonia solani* and *Penicillium expansium,* and two bacterial strains namely *Staphylococcus aureus & Salmonella typhimurium* were obtained from Dept. of Microbiology, Fac. of Agric. Ain shams univ., Cairo, Egypt. Whereas three bacterial strains and four fungal strains were obtained from Dept. of plant pathology, Fac. of Agric. Ain shams univ., Cairo, Egypt. These strains were *Erwinia amylovora, E. carotovora, Pseudomonas lacremance, Tricoderma reesi, T. viride, Aspergillus terrus* and *A. fumigatus.*

3. Media and solutions used.

- Malt extract agar medium (med. 1) [Lowes *et al*, 2000], was used for counting and isolation of the yeast obtained from salted foodstuffs and plants, malt yeast extract agar medium (med. 2) [Khattab, 2000] and Rose Bengal agar medium (med. 3) [Martin's, 1950] were used for counting and isolation of both non-halotolerant and halotolerant yeasts from salted foodstuffs or plants, salted water and saline soil, respectively.
- YM agar medium (med. 4), nutrient agar (med. 5) [Difco Manual, 1977] and Malt extract broth (med. 6) [Lodder and Kreger- van Rij, 1967] were used for the propagation, preservation and detection the killer toxin sensitivity of the tested yeast. bacterial and fungal strains, respectively.
- Basal broth medium (med. 7) [Andreishcheva *et al*, 1999], killer toxin agar medium (med.8) [Chen *et al*, 2000] and YEPD-MB agar medium (med. 9) [Soares and Sato, 1999] were used to study the growth behavior of tested yeast strains, detection the killer yeast phenotypes and determination of their killer activity, respectively. Whereas YEPD broth medium (med. 10) [Chen *et al*, 2000] was used for killer toxin production.
- Peptone physiological salt solution (PPS) (Nout *et al*, 1997) was used in preparation of standard inoculum of sensitive organisms.

Methods:

1. Enumeration and isolation of halotolerant yeast.

Ten gram of salted foodstuff, fruits or saline soil, 10 ml of salted water and 10 cm2 of phyllosphere samples were mixed with 90 ml sterile saline solution (0.85 or 5% NaCl) and shaken for 15 min on orbital shaker. Plate techniques were used for yeast counting in salted food, fruits and the phyllosphere of fruit leaves using med. 1. Whereas med. 2 and med. 3 were used for counting yeast in seawater or underground water and soil samples. These media were supplemented with different levels of NaCl concentrations being 0, 5 and 10 % NaCl to determine the most halotolerant yeast in different samples. Then, the plates were incubated at 30°C for 2-4 days; the developing colonies on the plate were counted as colony forming units (CFU) per gram or ml samples. Colonies from counting plates were picked and purified by streak plate method under aseptic conditions (Nout *et al*, 1997) and stock cultures were maintained at 5°C on NaCl free med. 1 after incubation at 30°C for 48 hours.

2. Standard inoculum.

Standard inoculum of yeast isolates strains was prepared by inoculation of conical flask (250 ml volume) containing 100 ml of med. 7 with loop of the tested yeast. The inoculated flasks were incubated on orbital shaker 150 rpm for 24 hours at 30°C. The content of the flask was used as a standard inoculum (Gamal *et al*, 1998). One ml of this culture contained yeast cells ranged from 1.5 – 2.0 gL-1. Standard inoculums of sensitive yeast, bacteria and fungal strains were prepared by adding 10 ml of sterile PPS solution to each slant cultures (1- 5 days old) and shaken for 15 minutes. The suspension for each culture was collected and used as a standard inoculum (107- 108 cell ml-1 for yeast and bacterial cells whereas 104- 106 spores. ml-1 for fungal cells).

3. The salt tolerance of yeast isolates.

Eight trials of NaCl concentrations, ranging from 5 up to 20 % were used to study the salt response of different yeast isolates and detected their salt tolerant degree. The propagation was carried out in test tubes (25ml in volume) containing 10ml of med. 7. These tubes were inoculated with 0.1ml standard inoculum and incubated at 30°C for 3 days. At the end of incubation period, the developing growth was compared to control tubes to detect the positive results.

4. Yeast identification.

The tested yeast isolates were identified using their morphological and physiological characteristics according to Barnett *et al* (2000).

5. Biological activity of halotolerant yeast.

This experiment was constructed to study the effect of different NaCl osmotic potential ranged from 4.25 to 16.16 MPa on the growth behavior and sugar consumption of the tested yeast strains varied in their salt tolerance. The propagation was carried out in Erlenmeyer flasks (250 ml in volume) containing 100 ml of med. 7 supplemented with different NaCl concentrations ranged from 0 to 19%. These flasks were inoculated with 1ml of standard inoculum of the tested strains and shaken on rotary shaker (150 rpm) for 5 days at 30°C. Samples (5 ml) were taken from the growing culture periodically every 4 or 6 h under aseptic conditions to determine the optical density of growth spectrophotometrically at 620 nm using SPEKTROMOM 402. The samples were centrifuged at 5000 rpm for 15 min, and residual sugar was determined in the supernatant. The relation between incubation time and optical density of yeast was plotted using Excel programme. All parameters of yeast growth and sugar consumption were calculated.

6. Killer activity of halotolerant yeast strains

A- Detection of killer yeast.

The ability of the tested yeast strains to kill the sensitive yeasts was detected using cross-reaction assay (Soares and Sato, 1999). 150 µL standard inoculum of sensitive yeast strains were spread on the surface agar plates containing med. 8 with or without 5% NaCl and left to dry 1-2 h at 10°C. Then, these plates were inoculated with thick spot of tested yeast strain and incubated at 24± 1°C for 3-4 days. A clear inhibition zone lined with blue ring surrounded with the tested strain (killer yeast) was appeared is positive results.

B- Effect of incubation period on killing activity.

This experiment was carried out to detect the proper time for maximum toxin production by the selected halotolerant killer yeast strains. The propagation of these cultures was done as mentioned before for killer toxin production on med.10 during 4 days at 24± 1°C using shake flasks (125 rpm) as a batch culture. During fermentation period, 10 ml sample of culture fluid were centrifuged (8000 rpm/ 30 min) and tested for killing activity against sensitive yeast strains using agar diffusion method on med.9. At the end of incubation period, diameter of inhibition zone (mm) was measured (Barandica *et al*, 1999).

C- Anti-microbial activity of killer toxins.

This experiment was designed to study the anti-microbial activity of killer toxins produced by the selected halotolerant yeast strains on med. 10, at the optimum production time, against the growth of some pathogenic yeast, bacterial and fungal strains on their propagation media respectively, using agar diffusion method. The propagation was carried out as mentioned before at the end of the incubation period (4 days), and diameter of inhibition zone (mm) was thereafter measured.

Other experiment was carried out to detect the antifungal activity of high killer toxin concentration (5%) in liquid culture. The production of killer toxins was done as mentioned before. Then add to Erlenmeyer flasks containing 95 ml med. 6 to give final concentration of 5%. These flasks were inoculated with 1 ml of fungal standard inoculum then incubated at 24± 1°C for 4 days. At the end of incubation, dry weight of cells was determined and the percentage of killing activity was measured express as the dry weight loss %.

5. Calculation.

- The osmotic potential of NaCl was calculated according to the formula of http://en. Wikipedia. org/ wiki/ osmotic_pressure using the following equations:-

$\pi = MRT$

Where:

π= Osmotic potential

M= Osmolarity of NaCl conc.

R= Gas constant (22.41/273°K)

T= Thermodynamic temperature (absolute temp.)

of incubation = 273+°C

The specific growth rate (μ) and doubling time (t_d) were calculated from the exponential phase according to Painter and Marr (1963) and Excel programme. Number of generation (N) and multiplication rate (MR) were calculated according to Stanier *et al* (1970). The percentage of consumed sugar and specific consumption rate of sugar were also calculated.

RESULTS AND DISCUSSION

1. The biodiversity of halotolerant yeasts in different ecosystems.

Data presented in Table (1) clearly show that the log number of total viable yeast count (CFU/ unit) was varied from one ecosystem to another as

affected by salt concentration in cultivation media, where it decreased with the increase in the concentration of NaCl. These results are in agreement with those obtained by Blomberg (1997) who noticed that the yeast cells were losing their viability with increasing osmotic pressure, and cells fail to form colonies. The yeasts were presented in 100% of the foodstuffs samples at different NaCl concentrations and recorded the widest range of log CFU/g being 3.11 to 7.1 in cucumber (med. 1). Whereas the yeasts were presented in 33.3%, 44.44%, 25% and 14.3% of the tested plants, underground water, saline soil and salted water samples, respectively. The priority of foodstuffs may be due to their contents of simple carbon source as sugars, organic acids, polyols, and alcohols supporting yeast growth. The incidence of yeasts were detected in there ecosystem samples among 21 salted water, they were located in El Dakhla harbor (NaCl free & 5% NaCl) and Hurghada (NaCl free medium). Also it could be noticed that the total number of yeast in sea water were higher than that present in underground salted water.

The yeasts were detected in only two sites of saline soil samples Elsharkua site and El-fayoum site. In this respect, Martini (1992) reported that yeast population in soils affected by many factors such as chemical and physical composition, pH, organic matter, water availability, mineral fertilizers, herbicides, toxic heavy metals and soil salinity. With respect to yeast occurrence on plant samples, results also revealed that the yeast counts in fruits were higher than in their phyllosphere. The highest figure in plants was recorded in fig followed by grape either in med.2 (NaCl free or 5%).

Four hundred and thirty three yeast isolates were isolated from different ecosystem and distributed with the percentage of 36.95%, 21.7%, 27.02%, 8.77% and 5.57% in salted foodstuffs, plants, salted water, underground water and saline soil, respectively as shown in Table (2). The isolates were purified by streaking several time on proper media and checked by Gram staining. The highest percentage incidence of yeast isolates was obtained on media containing 10% NaCl followed on NaCl free medium being 61% and 27%, respectively.

Moreover, it could be arranged the sources of the most tolerant yeast isolates (at 10% NaCl) in decreasing order according to their percentage incidence as follows salted foodstuffs> salted water> plants> underground water> saline soil.

From the foregoing results, it could be concluded that the halotolerant and non halotolerant yeasts were existed in different ecosystems. The highest number of yeasts recorded in salted foodstuffs followed by plants, while the lowest counts in saline soil. Fifty and 266 out of 433 isolates were isolated on media containing 5 and 10% NaCl, respectively, so it called halotolerant yeast whereas 117 isolates were grown on NaCl free medium i.e. non halotolerant yeast. These isolates were used in further study to elucidate their salt response at different NaCl concentrations in cultivation media.

Table (1): The log number of total viable count of yeasts in different ecosystems at different NaCl concentrations %.

Ecosystem samples		Log of total yeast count CFU/ unit* at different NaCl concentrations (%)			Ecosystem samples		Log of total yeast count CFU/ unit* at different NaCl concentrations (%)		
		0	5	10			0	5	10
Salted foodstuffs	Salted cheese	6.42	6.07	5.09	Plants	Grape	4.1	3.08	-
	white cheese	6.71	6.15	4.15		Grape phyllosphere	2.34	-	-
	Green olive	5.215	5.04	3.81		Fig	4.15	3.34	-
	Union	6.21	6.17	5.04		Fig phyllosphere	3.58	-	-
	Lemon	6.23	6.04	3.41		Apple	2.11	-	-
	Black olive	6.24	4.83	4.73		Apple phyllosphere	-	-	-
	Cucumber	7.1	5.88	3.11		Strawberry	2.3	-	-
	Old cheese	6.76	6.16	4.15		Strawberry phyllosphere	-	-	-
Salted water	El Dakhla harbor	4.88	2.85	-	Salted undergro-und water	Siwah	2.04	1.477	-
	Ghaharby harbor	-	-	-		Bani Suwayf	2.18	1.95	-
	Matruh	-	-	-		Elwadi Elgadyied	-	-	-
	Ras as sidr	-	-	-	Saline soil	Elfayoum	3.477	-	-
	Sharm Elshekh	-	-	-		Elsharkua	2	1.69	-
	Eltemsah lake	-	-	-		Elbosilly	-	-	-
	Hurghada	2.6	-	-		Elwadi Elgadyied	-	-	-

- = total yeast count less than 30 or zero CFU/ unit Unit* = ml, gm or cm^2

2. The salt tolerance of yeast isolates.

According to NaCl tolerance of yeast isolates, it could be observed from data given in Table (3) that 71.8% of total yeast isolates, which grown in media containing NaCl concentrations ranged from 5 to 10%, were moderate halotolerant yeast. Whereas 17.6% of total isolates, which grown at 15 % NaCl concentrations, were extreme halotolerant yeast. The percentage of grown isolates was decreased with increasing the salt concentration to reach the minimum value being 1.85% at 19% NaCl. No yeast growth was detected by increasing NaCl more than 19%. Also, it could be noticed that only 41 isolates out of 117 non halotolerant isolates were

moderate halotolerant, whereas 65% of these isolates were classified as slight or non halotolerant. Moreover, 9 isolates out of 41 moderate halotolerant were classified as extreme halotolerant yeast which capable of growth on media up to 17% NaCl. On the other hand, one and seven isolates out of 50 and 266 in the percentage of 2% and 2.6% of total halotolerant isolates which isolated at 5 and 10% NaCl respectively, were grown at a wide range of NaCl concentration up to 19%.

Table (2): The percentage incidence of yeast isolates in different ecosystem samples at different NaCl concentrations.

NaCl concentrations in isolation medium	Total number of yeast isolates	Salted foodstuff		Plants		Salted water		Underground water		Saline soil	
		TN	%	TN	%	TN	%	TN	%	TN	%
0	117	38	32.5	30	25.6	31	26.5	11	9.4	7	6
5	50	35	70	8	16	0	0	0	0	7	14
10	266	87	32.7	55	20.7	86	32.3	27	10.15	11	4.13
Total	433	160	36.95	93	21.7	117	27.02	38	8.77	25	5.77

TN= Total number of yeast isolates %= The percentage of isolates

Table (3): The numbers of tolerance yeast isolates to different NaCl concentrations (%).

NaCl % in isolationmedium	Number of isolates	Number of yeast isolates grown on different NaCl concentrations %															
		5		10		15		16		17		18		19		20	
		TN	%	TN	%	TN	%	TN	%	TN	%	TN	%	TN	%	TN	%
0	117	41	35	21	18	9	7.7	2	1.7	1	0.85	0	0	0	0	0	0
5	50	50	100	24	48	11	22	11	22	10	20	5	10	1	2	0	0
10	266	266	100	266	100	56	21	55	20.7	38	14.3	31	11.7	7	2.6	0	0
Total	433	320	82.5	311	71.8	76	17.6	68	15.7	49	11.3	36	8.3	8	1.85	0	0

TN = Total number of yeast isolates

Only five out of 76 extreme isolates varied in their morphological characteristics and degree of glucose fermentation, in addition to non-halotolerant yeast isolates 28S and moderate halotolerant yeast isolates 17S and 1T were selected to complete their identification according to Barentt *et al* (2000). It was found that these isolates had characteristic similar to Rhodotorula minta 28S, Pichia anomala 17S & P. silvicola 1T, Kluyveromyces bacillisporus 1S, P. silvicola 26T, P. anomala 5H, K. bacillisporus 3T and K. bacillisporus 5T.

Biological activity of halotolerant yeast strains.

Growth parameters.

The growth behavior of halotolerant yeasts at different NaCl osmotic potential was studied and comparing with that obtained by non halotolerant yeast strain. Data. illustrated by Fig. (1) show that the non halotolerant yeast strain (*Rhodotorula minuta* 28S) and moderate halotolerant yeast *Pichia anomala* 17S grew exponentially during the first 8h of incubation period on med. 7 at 0.36 and 4.25 MPa NaCl osmotic potential. The perioc' of exponential phase increased to range from 12 to 28 h for the growth of extreme halotolerant yeasts at different

NaCl osmotic potential. Increasing the osmotic potential higher than 4.25 (by increasing NaCl more than 5%) resulted in delaying the exponential phase to start after 6 h for the growth of all extreme halotolerant yeast strains at osmotic potential higher than 8.50 MPa (NaCl higher than 10%) except the treatment of *P. silvicola* 1T, *P. silvicola* 26T & *K. bacillisporus* 3T at 8.50 MPa, which recorded lag phase of 4 h. The highest figures of growth of all tested yeast strains (expressed as optical density) at the end of the exponential phase was observed on NaCl free medium (0.36 MPa osmotic potential) except extreme halotolerant yeast strains *Pichia anomala* 5H, *Kluyveromyces bacillisporus* 3T and *Kluyveromyces bacillisporus* 5T which gave the highest growth at 4.25, 4.25 and 8.50 MPa osmotic potential, respectively. Also, it could be noticed that there are direct relationship between the growth of all tested halotolerant yeast strains and NaCl osmotic potential of med. 7, where the lowest growth was recorded at lowest osmotic potential as shown in Fig. (2).

Regarding, the growth parameters during exponential phase of all tested strains, it could be reported that specific growth rate, number of generation and multiplication rate were decreased with increasing the NaCl concentration to give the lowest figures at osmotic potential ranged from 13.61 to 16.16 MPa. The lowest doubling time at 0.36 MPa osmotic potential was obtained by *P. anomala* 17S (1.28 h) followed by *K. bacillisporus* 5T (2.1) (not shown). Generally, it could be concluded that the growth of halotolerant strains varied from one strain to another and affected by their ability to grow in the presence of high NaCl concentration. At high NaCl concentration, the lag phase of yeast growth was appeared and recorded the longest period (6 h) at osmotic potential ranged from 8.50 to 16.16, whereas the specific growth rate during the exponential phase decreased and the final yeast growth (O.D) was reduced. In similar studies Gamal *et al* (1998) noticed that a long lag period (3 days) of *Rhodotorula mucilaginosa* T38 growth was recorded at 15% NaCl and resulted scant growth after 6 days being 1.3 (as optical density). Also, the growth of *Zygosaccharomyces rouxii* was delayed in the start of exponential growth in the presence of 15% NaCl (Hosono, 2002). Andreishcheva *et al* (1999) stated that only at high NaCl concentrations NaCl significantly affected the final biomass yield, extended the lag phase and reduced growth rate.

Sugar consumption.

The amount of consumed sugar increased gradually during the growth of all tested strains to reach the maximum value after 24 or 28 h as seen in Fig. (3). The amount of consumed sugar by the extreme halotolerantyeast strains and moderate halotolerant*Pichia silvicola* 1T was decreased with increasing the concentration of NaCl to record the minimum value at highest osmotic potential for each strain. The highest figures of consumed sugar by moderate and extreme halotolerant yeasts ranged from 18.84 to 19.94 gl^{-1} and from 4.4 to 19.5 gl^{-1}, respectively at different NaCl osmotic potential. Whereas the lowest range of consumed sugar was between 4.4 to 5.6 gl^{-1} and obtained at lower NaCl osmotic potential of med. 7 ranged from 12.76 to 16.16 MPa after 24h of fermentation period at 30°C.

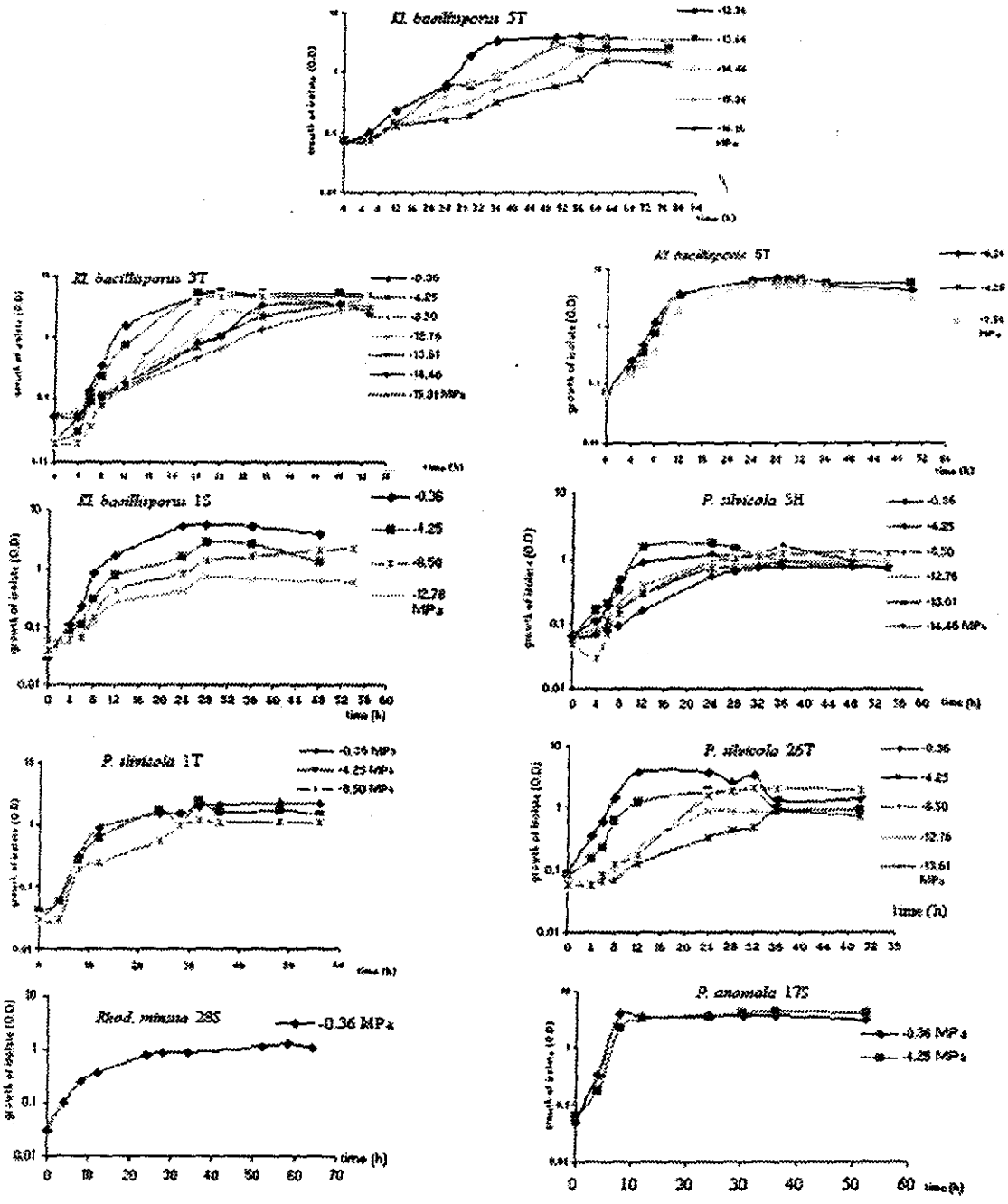

Fig. (1): Growth curve of yeast strains on med. 7at different NaCl osmotic potential during 77 h incubation period at 30°C using shake flasks as a batch culture.

On the other hand, the highest figure of specific consumption rate of sugar (μ_S) was noticed during 4- 8 h fermentation period by all moderate and extreme halotolerant yeast strains at NaCl osmotic potential ranged from 0.36 and 8.50 MPa and during 6- 12 h fermentation period by extreme halotolerant yeasts *Kluyveromyces bacillisporus* 3T and *Kluyveromyces bacillisporus* 5T at lower NaCl osmotic potential ranged from 12.76 to 16.16 MPa, then a sharp decrease in specific consumption rate of sugar was obtained by increasing the fermentation period.

Regarding to the relation between consumed sugar and growth density, the previous data clearly show that all halotolerant yeast strains recorded higher growth density and sugar utilization % than non halotolerant *Rhodotorula minta* 28S at the end of the exponential phase (24 h) on NaCl free med. 7 except extreme halotolerant *Kluyveromyces bacillisporus* 5T which gave only low sugar utilization (41.75 %). Both the highest values of growth and sugar utilization (94%) were attained by moderate halotolerant strains and extreme halotolerant *K. bacillisporus* 3T at NaCl osmotic potential ranged from 0.36 to 8.50 MPa. Decreasing the osmotic potential lower than 8.50 MPa led to decrease in the growth density and sugar utilization for both *Kluyveromyces bacillisporus* 3T and *Kluyveromyces bacillisporus* 5T whereas the former strain gave the higher figures than the latter strain. From the aforementioned data, it could be stated that there are indirect relationship between NaCl concentrations and growth density or sugar utilization efficiency of all halotolerant yeast which varied in their behavior according to fermentation capacity.

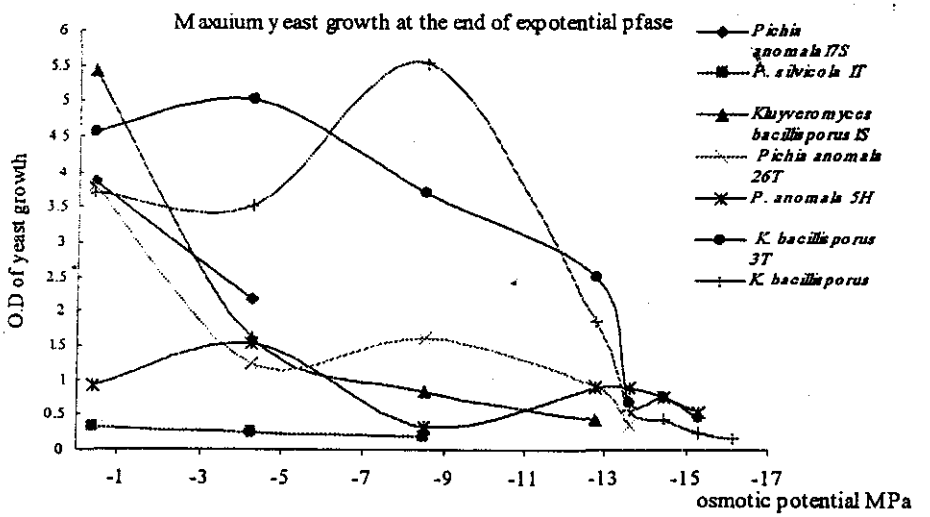

Fig. (2): Maxuium growth of haloterant yeast strains at the end of expotential phase on med. 7 at different NaCl osmotic potentialat 30°C using shake flasks as a batch culture.

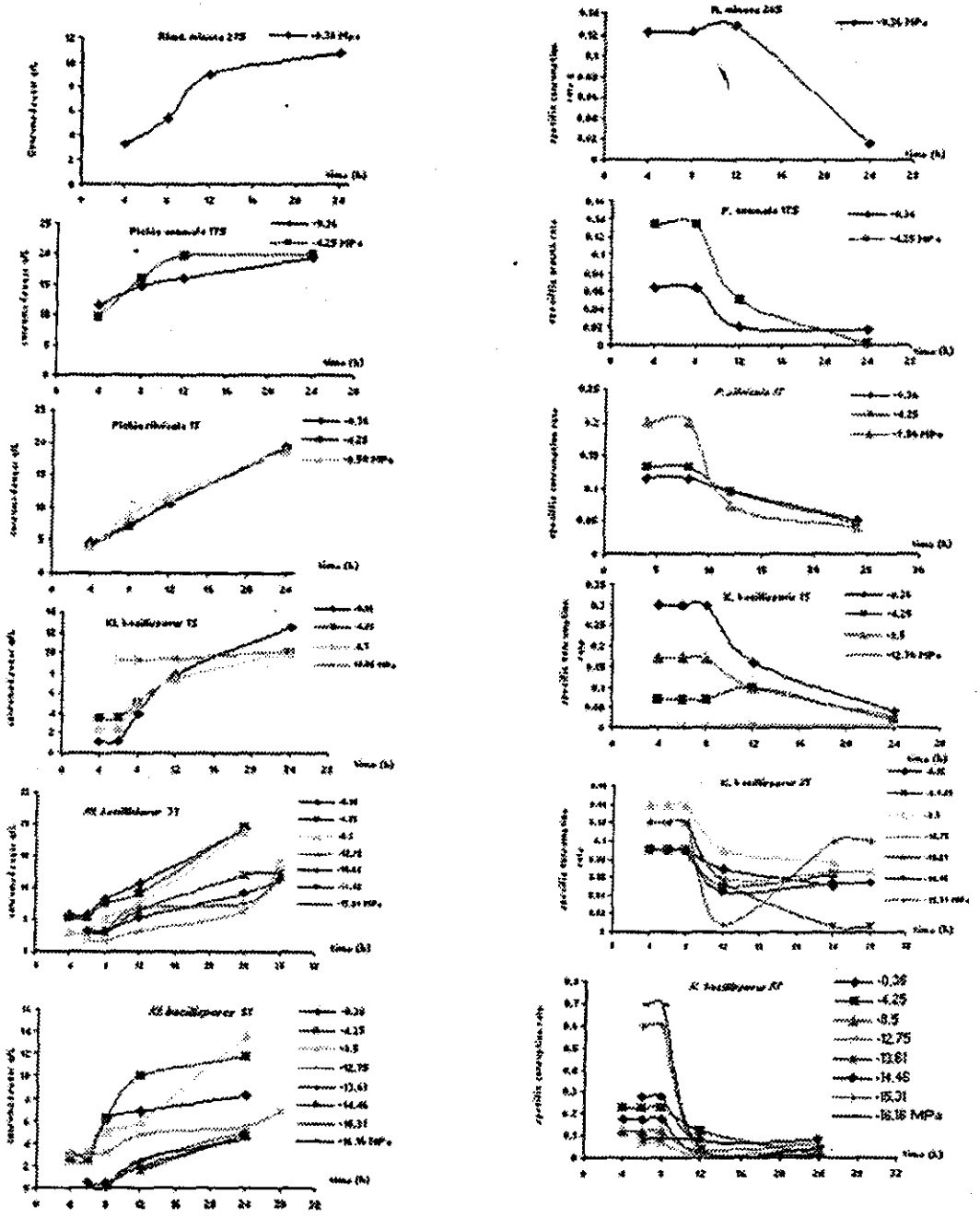

Fig. (3): Consumed sugar (g/L) and specific consumption rate (h^{-1}) of yeast strains on med. 7during exponential phase at different NaCl osmotic potential using shake flasks as a batch culture (150 rpm).

5. Killer toxin production.

A. Detection of killer yeast.

Data given in Table (4) reveal that all tested strains have the ability to produce killer toxin with different killer spectra. In free NaCl med.8 non-halotolerant *Rhodotorula minta* 28S was inhibited by all halotolerant yeast strains and affected on 5 out of 7 halotolerant strains resulted killing activity 71.43% of tested strains. On the contrary, *Kluyveromyces bacillisporus* 3T which inhibit the growth of non-halotolerant *Rhodotorula minta* 28S failed to inhibit the growth of all halotolerant yeast strains. The widespread killer activity was obtained by *Pichia anomala* 17S followed by *Pichia anomala* 5H against 7 and 6 yeast strains representing 100% and 85.71% of tested strains, respectively. Both *Pichia anomala* 1S and *Pichia silvicola* 26T gave the same percentage of killer activity being 57.14% for their different sensitive strains, whereas *Kluyveromyces bacillisporus* 5T and *Pichia silvicola* 1T inhibited the same strains (*Rhodotorula minta* 28S and *Pichia anomala* 5H) and gave the same killer activity (28.57%). Also, it could be observed that both *Kluyveromyces bacillisporus* 3T and *Kluyveromyces bacillisporus* 5T were sensitive for killer toxin produced by *Rhodotorula minta* 28S, *Pichia anomala* 17S, *Pichia silvicola* 26T and *Pichia anomala* 5H strains.

In med.8 supplemented with 5% NaCl, only three killer yeast out of 7 strains recorded higher killer activity than on NaCl free medium (*Pichia silvicola* 1T, *Pichia silvicola* 26T and *Kluyveromyces bacillisporus* 5T), whereas the vice versa was true by other three killer yeasts namely, *Pichia anomala* 17S, *Kluyveromyces bacillisporus* 1S and *Pichia anomala* 5H. Only one killer yeast *Kluyveromyces bacillisporus* 3T failed to inhibit the growth of all tested halotolerant yeast strains in both medium treatments.

In similar studies, Aguiar and Lucas (2000), noticed that *Pichia jadinii* and *P. membranaefaciens* decreased their toxic spectra in the presence of salt, whereas *C. tropicalis* was more sensitive. The latter strain was killed by 3.4% of total strains in the absence of salt, and by 5.2, 8.8 and 10.4% in the presence of 0.5, 1.0 and 1.5 M NaCl, respectively. The possibility that the toxin work against some mechanisms for resisting pressure has been suggested by Suzuki and Nikkuni (1989), and the observed lack of binding ability of this toxin to β- 1, 6- glucan may have functional implications (Suzuki and Nikkuni, 1994). Also, Llarente *et al* (1997) noticed that the presence of salt in the assay medium may be necessary to reveal the killer phenotype of some yeasts. They added that salt may enlarge the activity spectra of a killer yeast against the selected target strains. Silva *et al* (2003) found that the maximum killer specteum of *C. nodaensis* was recorded at2M NaCl.

B. Effect of incubation period on killer toxin production.

Results illustrated by Fig. (4) show that the killer activity (expressed as inhibition zone diameter) of each killer toxin was varied from one sensitive yeast strain to another. The inhibition zone diameter of killer toxins tested was increased by increasing the incubation period till reaching the maximum during the first 24- 72h period produced by *Pichia silvicola* 26T, *Pichia anomala* 5H, *Pichia silvicola* 1T and *Rhodotorula minta* 28S and during 48- 96h period of *Kluyveromyces bacillisporus* 1S and *Pichia anomala* 17S against all sensitive yeast strains i.e during the stationary

growth phase of producer strains. Then, the killer activity of the tested toxin produced was stopped or decreased with incubation period increased. In this respect, Barandica *et al* (1999) stated the relationship between toxin production rate and the growth rate was non-liner. Pommier *et al* (2005) added that the lag phase before the beginning of the killer effect, a period of toxin accumulation in the medium, to give high enough concentration for sensitive population. Actually, it has been already reported that a minimum quantity of toxin molecules has to be fixed on the cell wall of a sensitive yeast before it dies.

Table (4): Anti-yeast activity of non and halotolerant yeast strains as affected by both the absence and presence of 5% NaCl on med. 8 incubated at 23- 25°C after 3 days in solid culture.

Killer yeast strain	Without NaCl								
	Target yeast								
	28S	17S	1T	1S	26T	5H	3T	5T	%
28S	□	□	■	■	□	■	■	■	71.43
17S	■	□	■	■	■	■	■	■	100
1T	■	□	□	□	□	■	□	□	28.57
1S	■	□	■	□	■	■	□	□	57.14
26T	■	□	□	□	□	■	■	■	57.14
5H	■	■	□	■	■	□	■	■	85.71
3T	■	□	□	□	□	□	□	□	14.29
5T	■	□	□	□	□	■	□	□	28.57
	With 5% NaCl								
	Target yeast strains								
	28S	17S	1T	1S	26T	5H	3T	5T	%
28S	-	-	-	-	-	-	-	-	0
17S	-	□	■	■	■	■	■	■	100
1T	-	■	□	■	■	□	■	□	66.67
1S	-	□	■	□	■	□	□	□	33.33
26T	-	□	■	□	□	■	■	■	66.67
5H	-	□	□	□	□	□	■	■	33.33
3T	-	□	□	□	□	□	□	□	0
5T	-	■	□	□	■	□	□	□	33.33

■ Sensitive strains (clear inhibition zone) □ Resistance strains (no inhibition zone)
- No growth of *Rhodotorula minuta* 28S % Percentage of killer activity
17S *Pichia anomala*, 1T *Pichia silvicola*, 1S *Kluyveromyces bacillisporus*,
26T *Pichia silvicola*, 5H *Pichia anomala*, 28S *Rhodotorula minuta* 28S
3T *Kluyveromyces bacillisporus*, 5T *Kluyveromyces bacillisporus*

C. Antimicrobial activity of killer toxin.

The antimicrobial activity of killer toxin produced from selected killer yeast strains on submerged culture was investigated against 14 tested pathogenic organisms including G^+ & G^- bacteria, fungi and yeast. The inhibitory effect of killer toxins was detected using agar diffusion method.

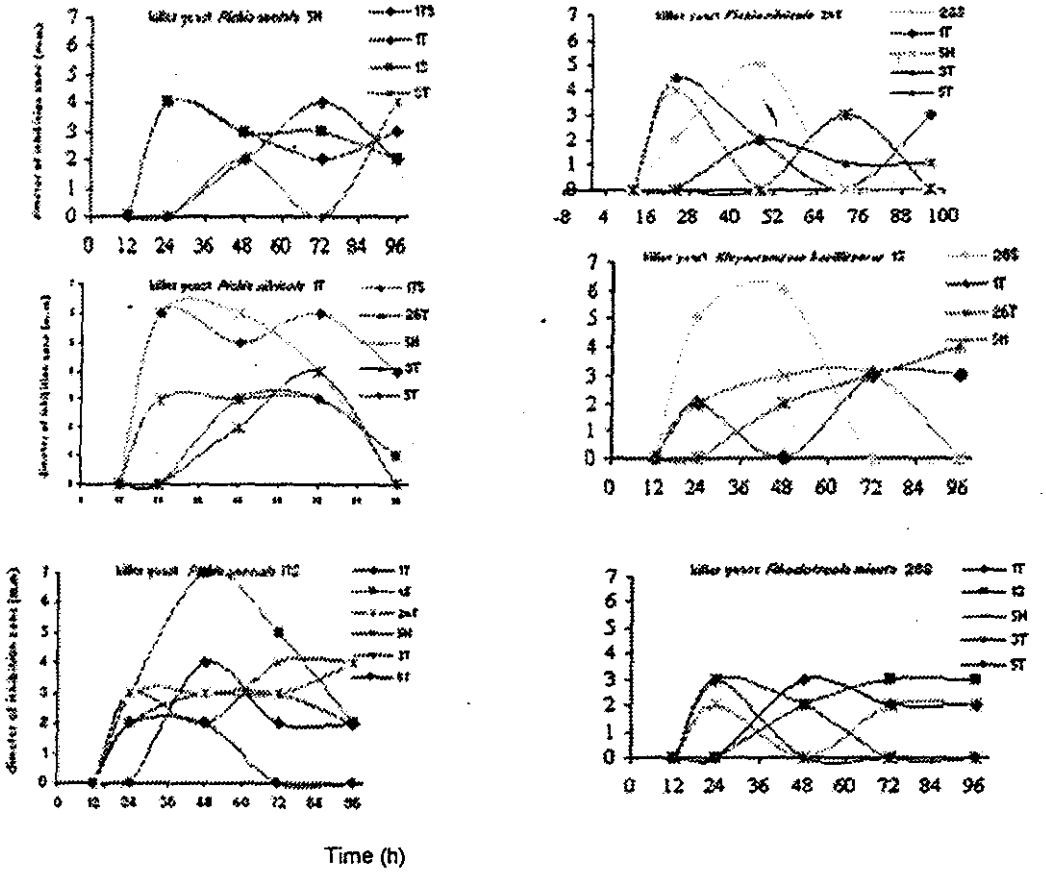

Fig. (4):Anti-yeast activity of killer toxin produced by most efficient halotolerant killer yeast strains during 96h incubation period using cross killing reaction on med..8

Data presented in Table (5) indicated that the bacterial strains were more resistance for killer toxin than fungal strains. Only two bacterial strains namely *Pseudomonas lacremance* and *Staphylococcus aureus* were inhibited by both killer toxins of *Pichia anomala* 5H and *Pichia silvicola* 26T giving inhibition zone of diameters 5 mm for the first strain and 2.5 & 5 mm for the second strains. Other tested killer toxins had no anti-bacterial activity against all tested strains (5 strains). These results are in line with those obtained by İzgü and Altinbay (1997). They reported that certain mycocens had the inhibitory effect on some pathogenic G^{+ve} bacteria, including *Staph. aureus*.

The anti-yeast activity was only recorded by *Kluyveromyces bacillisporus* 1S, *Pichia anomala* 17S and *Rhodotorula minta* 28S toxins against *C. albicans* showing 5, 3 and 2 mm inhibition zone diameter, respectively. Also, the inhibitory effect of *Pichia anomala* NCYC 422 was detected by İzgü *et al* (2006) against a variety of yeasts including pathogenic species of *Candida*.

Also, it could be noticed that all tested killer toxins failed to inhibit the growth of *Penicillium expansium*, *Rhizoctonia solani* and *A. famigatus*. Whereas *A. terrus* was sensitive only to killer toxin of *Kluyveromyces bacillisporus* 1S showing 3 mm inhibition zone. The contrary was noticed on *Fusarium oxysporium*, as it inhibited by all tested killer toxins. The largest inhibition zone diameter was recorded by *Pichia anomala* 17S toxin against *Tricoderma viride* (15 mm) followed by *Fusarium solani* (7.5 mm). Moreover, it could be arranged the killer toxins in decreasing order according to efficacy as follows *Pichia anomala* 17S> *Pichia silvicola* 26T> *Pichia silvicola* 1T> *Pichia anomala* 5H > *Kluyveromyces bacillisporus* 1S > *Rhodotorula minta* 28S. There are some reports in the literature of wide range intergeneric killing spectrum of *Pichia* toxin and their relative high stability in comparison to toxin of other killer yeasts (İzgü *et al*, 2006; Druvrefors *et al*, 2005 and Barandica, 1999).

Table (5): Anti-microbial activity of killer toxin production by some tested yeast strains using diffusion method.

Pathogenic organisms	Inhibition zone diameter (mm) of killer toxin					
	1T	5H	1S	17S	26T	28S
Bacterial strains						
Erwinia amylovora	0	0	0	0	0	0
Erwinia carotovora	0	0	0	0	0	0
Pseudomonas lacremance	0	5	0	0	5	0
Staphylococcus aureus	0	2.5	0	0	5	0
Salmonella typhimurium	0	0	0	0	0	0
Yeast strain						
Candida albicans	0	0	5	3	0	2
Fungal strains						
Fusarium oxysporium	2	3.5	3	4	4	4
Fusarium solani	0	0	0	7.5	4	0
Penicillium expansium	0	0	0	0	0	0
Rhizoctonia solani	0	0	0	0	0	0
Aspergillus terrus	0	0	0	3	0	0
Aspergillus fumigatus	0	0	0	0	0	0
Tricoderma reesi	1.4	4	3	0	4	0
Tricoderma viride	4.5	0	0	15	3.3	0

28S= *Rhodotorula minuta*, 17S= *Pichia anomala* 17S,
1T= *Pichia silvicola* 1T, 1S= *Kluyveromyces bacillisporus* 1S,
26T= *Pichia silvicola* 26T, 5H= *Pichia anomala* 5H,
3T= *Kluyveromyces bacillisporus* 3T,
5T= *Kluyveromyces bacillisporus* 5T

In liquid culture, all tested killer toxins were added (med. 6) to assay medium in concentration of 5% to study their effects on fungal dry weight. The highest figure of antifungal activity of killer toxin (expressed as the percentage of fungal dry weight loss) was obtained by *Pichia silvicola* 26T against the growth of *Tricoderma viride* (96%) followed by *Pichia anomala* 5H and *Pichia silvicola* 1T toxin (95 & 94%). The former fungal strain was also affected by *Pichia anomala* 17S and *Pichia silvicola* 1T toxins showing 84% and 74% of fungal

weight loss, respectively. Five out six tested killer toxins were active to inhibit the growth of *Fusarium oxysporium* and *T. reesi* in the range of killer activity ranged from 60 to 92% and from 66 to 95%, respectively. *A. terrus* was also affected by *Pichia anomala* 17S toxin in submerged culture with 86.5% of dry weight loss (Fig. 5). In this respect, *Pichia anomala* J121 inhibited the growth of a wide varity of molds, e.g. *Botrytis cinerea*, *Aspergillus candidus*, *Penicillium roqueforti* and other plant-pathogenic of wood decay fungi, stored apple, grapevine plants, grain in airtight storage and wood (Druvefors *et al*, 2005).

From the aforementioned results, it could be concluded that both tested non-halotolerant and halotolerant yeasts produced killer toxins lethal to sensitive yeasts but they were immune to their toxins. Only non halotolerant yeast strain failed to grow and produce killer toxin in the presence of 5% NaCl whereas some halotolerant yeasts recorded the maximum killer activity. The maximum production of killer toxins was attained during the stationary phase of yeasts growth. Killer toxin of *Pichia silvicola* 26T gave the highest antimicrobial activity on 42.86% followed by *Pichia anomala* 17S and *Pichia anomala* 5H on 35.71% and 28.57% of tested strains. Also, *Pichia silvicola* 26T, *Pichia anomala* 17S and *Pichia silvicola* 1T toxins recorded the widest spectrum as antifungal activity. The highest antifungal activity of tested killer toxins was recorded against *Tricoderma viride* and *T. reesi*. *Fusarium oxysporium* was affected by all tested killer toxins in both solid and liquid culture. So it could be recommended to test the role of *Pichia silvicola* 26T, *Pichia anomala* 17S and *Pichia anomala* 5H to control the plant disease.

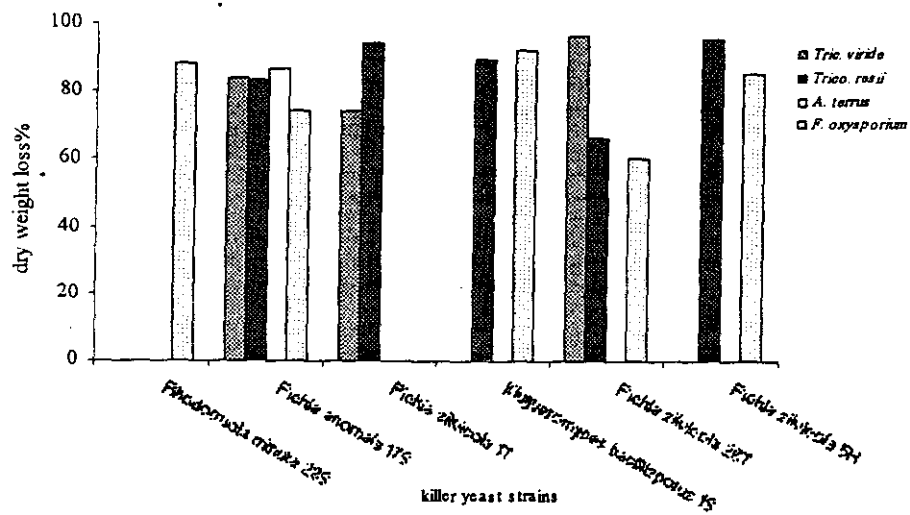

Fig.(5): Antifungal activity of killer toxin (5% v/v) prouduced by teasted yeast strains in med. 6 using shake flasks as a batch culture (125 rpm).

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التنوع الحيوى و التوكسينات القاتلة للخمائر المتحملة للملوحة فى النظم البيئية المختلفة

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يختلف التنوع الحيوى للخمائر المتحملة للملوحة فى الوساط البيئية المصرية بدرجة كبيرة من وسط بيئى الى اخر متاثرا بتركيزات الملح الموجودة فى بيئات الزرع،فينخفض بزيادة تركيزات كلوريد الصوديوم تواجد الخمائر بنسبة ١٠٠%، ٧٥%، ٦٦,٦٧%، ٥٠٠%و ٢٨,٥٧% فى عينات الاغذية المملحة و النباتات و مياه الابارو التربة الملحية على الترتيب.اربع مائةو ثلاثةو ثلاثون عزلة خميرة تم عزلهم من الاوساط البيئية المختلفة، و قسمت تبعا لتحملها للملوحة الى ثلاث فئات: غير او ضعيفة تحمل الملوحة، متوسطة التحمل وقوية التحمل بالنسب التالية ١٧,٦%و ٨٢,٥%و ١٧,٦% على الترتيب. اختير ثمان عزلات خميرة المتحملة فقط تبعا لاختلاف تحملها للملوحة و قدرتها التخميرية و عرفت تعريفا كاملا. يؤثر الضغط السموزى لكلوريد الصوديوم على نمو و استهلاك السكر لعزلات الخميرة، فالتركيزات المرتفعة من كلوريد الصوديوم اظهرت مرحلة النمو الاجى للعزلات المختبره فكان اقصى طور لاجى ٦ ساعات عند جهد اسموزى يتراوح ما بين ٨,٥٠و ١٦,١٦ ميجا بسكال، فى حين انخفض معدل النمو التخصصى و كمية النمو النهائية فى نهاية النمو الوغارتمى. كذلك توجد علاقة مباشرة بين الجهد الاسموزى لكلوريد الصوديوم و كمية انمو و معدل استهلاك السكر، اعلى معدل استهلاك تخصصى للسكر بعد ٤-٨س او ٦-١٢س من فترة التخميرفى وجود جهود اسموزية مختلفة. لم يظهر تاثير قاتل على سلالة *Kluyveromyces bacillisporus* 3T فى وجود ٥% كلوريد صوديوم. فى حين ان سلالات الخميرة *Pichia silvicola* 26T و *Pichia silvicola* 1T و *Kluyveromyces bacillisporus* 5T اظهرت نشاط قاتل اعلى من البيئة الخالية من الملح و العكس صحيح مع العزلات الاخرى. يصل انتاج التوكسين اعلاه فى مرحلة نمو الثبات للعزلات المختلفة. اظهرت توكسينات الخميرة نشاط مضار للميكروبات الاخرى منها *Pseudomonas lacremance* و *Staphylococcus aureus* و الفطريات *Tricoderma* و *Aspergillus* و *Fusarium* و *C. albicans*.