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A N I N T E R N A T I O N A L J O U R N A L

Production and Optimization of Killer Toxin in *Debaryomyces hansenii* Strains

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ABSTRACT

Postharvest diseases of fruits and vegetables result in critical losses of production in worldwide. The losses often are caused by fungi and nowadays, most fungal pathogens are controlled by several strategies such as the use of fungicides. However, most of the fungicides are chemical-based compounds and are dangerous to human health and the nature. Therefore, the discovery of healthy and reliable strategies is crucial to control of fungal pathogens. In the paper, it was aimed to evaluate and characterize yeast isolates previously isolated from dairy products for the production of killer toxin. A total of 18 yeasts have been found to produce antagonistic behavior against susceptible fungal species. All of the yeasts expressing killer character were characterized by using several molecular techniques, and isolates TEM8 and 17 identified as D. hansenii have showed the strongest antifungal activities. Improvement of killer toxin production by the yeasts also has been studied, and the highest production was found in YMB medium containing NaCl (6%) and DMSO (1000 ppm) at pH 4.0 and 20°C. The killer characters of these yeasts have indicated the potential use of the yeasts as antagonists for the control of postharvest diseases in agricultural industries.

Key words: characterization, killer toxin, optimization, postharvest disease, yeast

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INTRODUCTION

Fungi are one of the major factors encountered in postharvest diseases of numerous vegetables and fruits and are considered to be a cause of high economic losses in all over the world[1-3]. Nowadays, synthetic fungicides are used generally in postharvest applications and are the most important agents for reducing fungal-based losses. Nevertheless, the use of synthetic compounds has been progressively restrained because of consumers' no longer want to see toxic or chemical residues in their foods[3]. Furthermore, the increasing occurrence of phytopathogens that are resistant to chemical agents encourages the necessitate to seek for different alternatives for disease control[4-6].

It is well known that members of certain groups of microorganisms have the ability to produce and secrete extracellular compounds that display antagonistic behaviors against others[7]. Biological control using antagonists seems to be a particularly promising approach to protect different fruits and vegetables from fungal infections[3,8]. Biological agents help reduce the use of chemical-based compounds and this is one of the most important reasons to consider their use. In most cases, due to their low or no toxicity to higher organisms they are safer to use. Moreover, they are more stable and less phytotoxic than chemical agents[3,9,10].

Recently, numerous researchers have reported the use of mechanisms of yeast antagonism for the control of fungal-based postharvest losses[11-13]. Probably the most significant antagonistic behavior by yeasts is the production of killer proteins (toxins) firstly discovered in *Saccharomyces cerevisiae* by Makower and Bevan[14]. These proteins are known to be active against organisms taxonomically related to their producers[10]. These toxins target the components of the fungal cell walls, and as mammalian cells lack the components, the agents do not damage the host cells. Killer character has been detected in above 90 yeast species[15].

Most of the killer yeasts have many characteristics, and these features make them efficient bio-agents in the preservation of fruits and vegetables. These yeasts have simple nutritional requirements, and are therefore easily produced in large-scales. Also, they do not harbor pathogenic or toxic potentials, in contrast to filamentous fungi[3,9]. Hence, in the present paper, we aimed to evaluate and characterize yeasts expressing killer phenotype as potential biocontrol agents for fungal diseases.

MATERIALS AND METHODS

Fungal strains and cultivation conditions

Yeast strains were provided from a previous research related to Turkish style homemade dairy products[16]. The yeast and mold strains were maintained at 4°C respectively on Yeast Malt (YM) agar [g/L, yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; and agar, 15] and Potato Dextrose Agar (PDA) [g/L, potatoes, 200; dextrose, 20; and agar, 20]. Fungi used as susceptible-test organisms for killer proteins are listed Table 1.

Table 1 - Fungi used as susceptible-test organisms

Aspergillus niger NRRL 326	*Kluyveromyces lactis* NRRL Y-8279
Candida albicans NRRL Y-7875	*Kluyveromyces marxianus* NRRL Y-1109
Candida rugosa NRRL Y-95	*Saccharomyces cerevisiae* NRRL Y-12632
Candida tropicalis NRLL YB-366	*Wickerhamomyces anomalus* NRRL Y-366
Candida zeylanoides NRRL Y-1774	*Yarrowia lipolytica* CBS 6124
Debaryomyces hansenii NRRL Y-7426	

Screening of killer phenotype

Killer phenotype was determined in triplicate on Killer Medium (KM) agar [g/L, yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10; agar, 15; and methylene blue, 0.03; buffered at pH 4.0 with 100 mM Na_2HPO_4-citrate buffer]. Media pHs were checked before and after autoclaving and the differences were never exceeded 0.1-0.2 units.

Screening for yeasts against several fungi was conducted according to the method by Hashem and Alamri[13] with minor modifications. The overnight yeast cultures and four-day-old mycelium of *Aspergillus niger* NRRL 326 were suspended in sterile distilled water (dw) and appropriate amounts taken from the cell suspensions were inoculated into KM agar (kept at 45°C) to get 2 x 10^6 cells/ml after pouring. After the plates incubated at 20°C for 72h, isolates were considered killer protein producers if they formed an inhibition zone against the susceptible organisms.

DNA sequencing and restriction fragment length polymorphism (RFLP) analysis of ribosomal RNA regions

Genomic DNAs were isolated according to Liu et al[17]. The amplification reactions of ribosomal RNA regions and oligonucleotide primers used were given in a previous paper[18].

The sequences from D1/D2 domain of 26S rRNA regions produced using primers NL1 and NL4 were matched using a BLASTN search and aligned with the sequences in GenBank database using CLUSTAL W2.0 software.

For RFLP analyses, PCR amplicons of ITS1-5.8SrRNA-ITS2 and 18S rRNA regions obtained with the primer pairs ITS1 - ITS4 and P108 - M3976, respectively, were used. Amplification products were digested separately by fast digest restriction endonucleases (Fermentas) *Hae*III, *Hinf*I, *Sau*3AI, *Sau*96I and *Taq*I, and reaction mixtures were incubated according to the manufacturer's instruction. To predict and verify the size of each restriction fragment, Nebcutter V2.0 programme, available at http://tools.neb.com/NEBcutter2, was used.

Production and improvement of killer toxin

The most efficient killer toxin producing yeasts were grown in 1 liter Erlenmeyer flasks with 500 ml of Yeast malt broth (YM) medium buffered with 100 mM Na_2HPO_4-citric acid (pH 4.0). The optical densities of the cultures were adjusted at 600 nm in a spectrophotometer. Aliquots of 50 ml were transferred in sterile falcon tubes to determine the toxin production.

Table 2 - The components of culture media used in the improvement of the killer toxin production

Media	**Components (g/L)**
Yeast carbon base broth (YCB)	Bacto yeast carbon base, 11.7; peptone, 5
Yeast extract peptone glucose broth (YEPD)	Yeast extract, 10; peptone, 20; glucose, 20
Yeast nitrogen base broth (YNB)	Bacto yeast nitrogen base, 6.7; glucose, 5
Yeast malt broth (YMB)	Yeast extract, 3; malt extract, 3; peptone, 5; glucose, 10
Potato dextrose broth (PDB)	Potato infusion, 4; glucose, 20

The overnight yeast cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of buffered media. To improve killer toxin production, different media [Yeast carbon base broth (YCB), Yeast extract peptone glucose broth (YEPD), Yeast nitrogen base broth (YNB), Yeast malt broth (YMB) and Potato dextrose broth (PDB)] were investigated as a first step for the toxin production. The components of the media used are listed Table 2. All media buffered with 100 mM Na_2HPO_4-citric acid (pH 4.0) were incubated at 20°C in a rotary bed shaker at 150 rpm. Having selected the best producing medium, the medium was then supplemented with

different additives [Ammonium sulfate, Dimethyl sulfoxide (DMSO), Glycerol, Phenylmethylsulfonyl fluoride (PMSF), Sodium dodecyl sulfate (SDS), Sorbitol, Triton-X 100 and Tween 80] using two concentrations (100 and 1000 ppm), different NaCl concentrations (0-10%, w/v) and evaluated with different growth temperatures (10 to 30°C) and pHs (3.0 to 6.0). The killer activities of the samples were determined and compared with the preliminary production rates.

Determination of protein content and killer toxin assay

Protein concentrations of the samples were estimated according to the Bradford method using bovine serum albumin as standard[19].

Killer toxin assay was performed using paper discs (6 mm) on KM agar inoculated with *Candida tropicalis* NRLL YB-366 strain. The supernatants obtained from 50 ml culture samples centrifuged at 5000 rpm and 4°C for 10 min were filtered using a 0.45 μm syringe membranes and the protein contents in the supernatants were precipitated with ice-cold ethanol to a final concentration of 70% (v/v). The pellets were recovered by centrifugation at 7000 rpm and 4°C for 10 min and resuspended in 100 mM Na_2HPO_4-citric acid (pH 4.0) buffer[20]. The killer activity was measured as the diameter of the inhibition zone, and the activity was expressed in arbitrary units (AU). Under the conditions studied, a linear relationship was observed between the diameter of the inhibition zone and the logarithm of the protein concentration in the solution tested. The toxin resulting in an inhibition zone with a 1 mm diameter is defined as 1 AU.

RESULTS AND DISCUSSION

To control the targets and reduce the usage of different inappropriate environmental methods, prioritizing the use of agents isolated from their intrinsic environments reveals a giant potential during postharvest points[5,8]. Isolating novel strains harboring antifungal properties from different habitats is of great importance for postharvest disease control[4,6]. Some yeasts exhibit antagonistic behaviors toward other microorganisms such as yeasts and molds by producing killer toxins. This phenomenon is quite important and it has potential for the use of postharvest practices.

In present paper, it was aimed to evaluate and characterize yeasts expressing killer character, and all of the isolates investigated were obtained from domestic sources (cheese and yoghurt). These isolates and susceptible-test organisms were maintained at 4°C on appropriate media and killer toxin producing isolates were determined on KM agar supplemented with methylene blue at 20°C for 72h. From the initial yeast collection, 18 isolates (32%) produced an inhibition zone against at least one susceptible strain and were classified as killer yeasts (Table 3). The blue zone of cellular death was not considered to be an evident and these yeast isolates were regarded as antagonists. As seen in Table 3, two isolates (named as TEM8 and 17) have showed strong antifungal activity to most (9 of 11) of the tested organisms, followed by TEM31 (7 of 11) and TEM9 and 21 (6 of 11), respectively. The efficacy of killer character seems to different from strain to strain among the targets compatible with previous reports[21,22]. For this reason, it is very important to investigate the potential use of yeast strains isolated from different sources for the control of fungal pathogens. Killer yeasts are often isolated from foods and can inhibit growth of other fungal pathogens. Bleve et al.[23] tested yeasts to select isolates showing a killer phenotype against *Aspergillus carbonarius* and *A. niger*, and authors identified 28 isolates exhibiting the antagonistic behaviors from an initial group of 144 yeast isolates. The results from inhibition experiments indicated that

some of the yeasts significantly reduced the colonization of the pathogenic fungi on wounded berries.

Table 3 - Antagonistic activities of yeast isolates against susceptible-fungal species

Strain No.	Inhibition Zones (Diameter of inhibition zone – Diameter of organism, mm)										
	Susceptible Fungi										
	A.n	***C.a***	***C.r***	***C.t***	***C.z***	***D.h***	***K.l***	***K.m***	***S.c***	***W.a***	***Y.l***
TEM1	*w*	*w*	—	—	—	9	*w*	*w*	—	3	—
TEM5	3	*w*	—	—	8	5	*w*	—	—	*w*	*w*
TEM6	—	*w*	3	3	—	*w*	*w*	—	6	9	15
TEM7	—	*w*	3	2	—	*w*	—	—	8	9	16
TEM8	8	3	12	11	—	*w*	11	3	11	7	21
TEM9	2	*w*	5	5	—	*w*	*w*	—	6	9	18
TEM10	*w*	*w*	3	3	—	*w*	—	—	6	8	15
TEM14	—	*w*	5	6	—	*w*	*w*	—	7	9	15
TEM15	*w*	*w*	5	6	—	*w*	*w*	—	5	9	16
TEM17	7	3	12	11	—	—	8	4	5	11	20
TEM18	4	*w*	—	—	—	*w*	*w*	—	4	—	6
TEM21	*w*	5	6	7	—	*w*	*w*	—	6	9	16
TEM22	5	*w*	—	—	—	*w*	*w*	—	4	*w*	*w*
TEM29	4	*w*	—	—	—	*w*	5	*w*	—	3	—
TEM31	*w*	8	2	2	10	—	—	—	5	14	19
TEM35	2	*w*	—	—	—	—	7	—	5	*w*	*w*
TEM52	2	6	—	—	—	6	7	—	5	—	*w*
TEM60	4	*w*	—	—	5	5	*w*	—	5	*w*	*w*

A.n, *A. niger* NRRL 326; *C.a*, *C. albicans* NRRL Y-7875; *C.r*, *C. rugosa* NRRL Y-95; *C.t*, *C. tropicalis* NRLL YB-366; *C.z*, *C. zeylanoides* NRRL Y-1774; *D.h*, *D. hansenii* NRRL Y-7426; *K.l*, *K. lactis* NRRL Y-8279; *K.m*, *K. marxianus* NRRL Y-1109; *S.c*, *S. cerevisiae* NRRL Y-12632; *W.a*, *W. anomalus* NRRL Y-366; *Y.l*, *Y. lipolytica* CBS 6124; *w*, weak

In the present paper, killer toxin producing isolates were characterized by using sequencing of D1/D2 domain of 26S rRNA gene and RFLP analysis of ITS1-5.8SrRNA-ITS2 and 18S rRNA gene regions. According to the BLAST results of D1/D2 domain, 10 of 18 isolates including the most efficient killer yeasts TEM8 and 17 were successfully identified as *Debaryomyces hansenii*, and the others were *Candida zeylanoides*, *Meyerozyma guilliermondii* and *Pichia kudriavzevii* (Table 4).

PCR amplification of ITS1-5.8SrRNA-ITS2 gene region produced amplicon sizes in 530 and 640 bp (Table 4). Digestion of these fragments by restriction endonucleases (*Hae*III, *Hinf*I, *Sau*3AI and *Sau*96I) revealed that isolates belonging to the closely related species represented similar RFLP profiles at least for these endonucleases. In other words, RFLP analysis did not differentiate the yeasts from each other accurately. Only the isolate TEM5 identified as *P. kudriavzevii* by sequencing showed completely different RFLP patterns for the all tested endonucleases. In addition to these results, RFLP analysis led to possible false readings and experimental mistakes as such in isolates TEM5 and 8. In cases of TEM5 and 8, RFLP patterns obtained from the isolates were not matched to that of any yeast species and type species used. Therefore, ITS-RFLP analysis was performed again for these isolates, and it was found that the previous results were incorrect. Ultimately, the finally obtained RFLP patterns were compatible with that of belonging to the species and type species by sequencing.

PCR amplification of 18S rRNA gene region rendered amplicon size in 1800 bp, and such in the other gene region, RFLP patterns of this region produced similar results for the closely related species (Table 4). Besides, two isolates TEM1 and 5 identified respectively as *C. zeylanoides* and *P. kudriavzevii* by sequencing showed partly different RFLP patterns for the all tested endonucleases.

Table 4 - Molecular characterization of yeasts expressing killer character

Strain No.	ITS1-5.8S rDNA-ITS2					18S rDNA				D1/D2 domain of 26S rDNA		
	PCR (bp)	Restriction Fragments (bp)				PCR (bp)	Restriction Fragments (bp)			PCR (bp)	Homology (%)	Identification Result (GenBank Accession Number)
		*Hae*III	*Hinf*I	*Sau*3AI	Sau96I		*Hae*III	*Hinf*I	*Taq*I			
TEM1	640	420+140+80	320+320	400+220	500+140	1800	510+290+270+180+160+150+120+80	830+600+240+110	410+400+380+240+210	549	99	*Candida zeylanoides* (JQ277230)
TEM5	530	380+90	220+150+140	370+130	530	1800	500+230+210+180+160+150+120+70	800+540+230+130	590+500+290+240	559	99	*Pichia kudriavzevii* (JQ277231)
TEM6	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	571	99	*Debaryomyces hansenii* (JQ277232)
TEM7	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	572	99	*Debaryomyces hansenii* (JQ277233)
TEM8	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	570	98	*Debaryomyces hansenii* (JQ277234)
TEM9	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	561	99	*Debaryomyces hansenii* (JQ277235)
TEM10	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	557	99	*Debaryomyces hansenii* (JQ277236)
TEM14	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	559	99	*Debaryomyces hansenii* (JQ277237)
TEM15	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	561	99	*Debaryomyces hansenii* (JQ277238)
TEM17	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	563	99	*Debaryomyces hansenii* (JQ277239)
TEM18	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	542	99	*Meyerozyma guilliermondii* (JQ277240)
TEM21	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	565	99	*Debaryomyces hansenii* (JQ277241)
TEM22	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	549	99	*Meyerozyma guilliermondii* (JQ277242)
TEM29	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	539	99	*Meyerozyma guilliermondii* (JQ277243)
TEM31	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	560	98	*Debaryomyces hansenii* (JQ277244)
TEM35	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	565	99	*Meyerozyma guilliermondii* (JQ277245)
TEM52	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	566	99	*Meyerozyma guilliermondii* (JQ277246)
TEM60	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80	567	99	*Meyerozyma guilliermondii* (JQ277247)
C.z	640	420+140+80	320+320	400+220	500+140	1800	510+290+270+180+160+150+120+80	830+600+240+110	410+400+380+240+210			
D.h	640	420+140+80	320+320	400+220	500+140	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80			
M.g	640	390+140+80	320+320	400+220	400+140+80	1800	500+270+180+170+160+150+120+110+80	810+600+240+110	410+400+390+260+200+80			
P.k	530	380+90	220+150+140	370+130	530	1800	500+230+210+180+160+150+120+70	800+540+230+130	590+500+290+240			

C.z, C. zeylanoides NRRL Y-1774; *D.h, D. hansenii* NRRL Y-7426; *M.g, M. guilliermondii* ATCC 6260; *P.k, P. kudriavzevii* ATCC 24210

In the last decades, several identification techniques have been recommended as alternatives instead of time-consuming classical methods. However, as in conventional methods, there are several reports presenting a single molecular technique lead to misidentification of an organism in some cases. For instance, Arias et al.[24] compared several approaches for characterization, and in terms of accurate identification, partial sequencing of the 26S rRNA was found to yield the best results, followed by classical techniques and ITS analysis. On the other hand, in several studies, it was stated that D1/D2 domain of 26S rRNA region could not differentiate closely related taxa. In a study performed by Daniel and Meyer[25], several gene sequences were investigated for the effectiveness of species differentiation. As a result of their experiments, researchers concluded that D1/D2 domain of 26S rRNA region is not specific for closely related taxa and recommended the use of more than one gene region for identification purposes. In our study, considering the aforementioned cases and Table 4, it was seen that the use of one gene region or method did not provide the accurate identification of the closely related species. Above mentioned possible false readings and experimental mistakes, and lack of full database for each enzyme can cause false identification, and this situation reduces the reliability of RFLP analysis. This study has demonstrated once again that using of a combination consisting of several different methods is a crucial stage for the accurate identification of an organism.

In most studies, it is indicated that the culture conditions influence the production of killer toxin and the optimal conditions should be found empirically[20,26]. Therefore, in the present paper, the production of killer toxins by *D. hansenii* TEM8 and 17 in different media was compared (Fig. 1). Although all media supported the growth of the killer yeasts, the production of killer toxin in YNB medium was not observed. Accumulation of the killer toxins in extracellular medium at pH 4, 20°C and 150 rpm was empirically found to be high in YM medium. Also, the medium yielded killer toxin with a high specific activity than the other media used, followed by YCB, YEPD and PDB medium, respectively. Because of YM medium is a more minimized medium and provide higher specific activity than the others, the medium was chosen for the further studies.

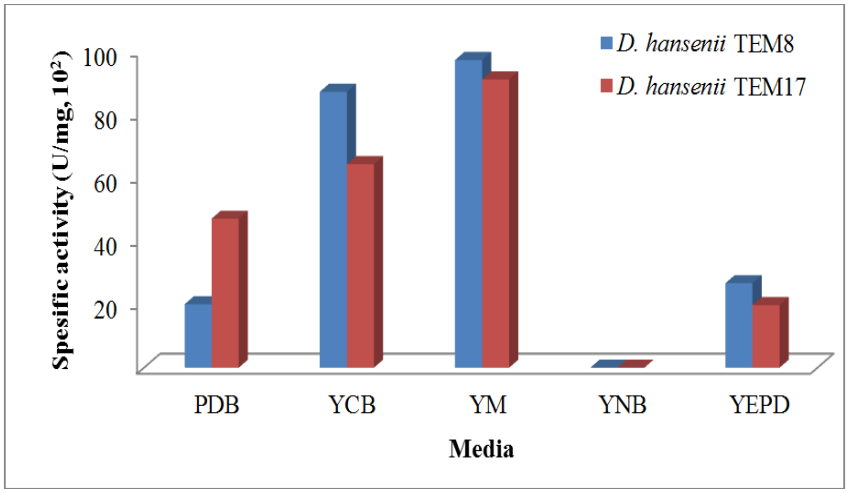

Figure 1 - Media used in killer toxin production at pH 4.0 and 20ºC

To improve the production of killer toxin, YM medium was supplied with different agents and evaluated at varied growth temperatures and pHs. Marquina et al.[20] reported that the optimal temperature of toxin production by *D. hansenii* strain used was at 20°C and occurred between 5 and 35°C. Researchers also indicated that above

pH 5.0 irreversibly inactivate most toxins and killer toxins are active particularly in a narrow pH range. In our experiments, different temperatures (10 to 30°C, Fig. 2) and pHs (3.0 to 6.0, Fig. 3) have supported the killer toxin production by *D. hansenii* strains in different amounts. The toxin production reached the highest levels at pH 4.0 and 20°C. These findings were compatible with the literatures[20,26]. However, interestingly, the toxin production has still continued above pH 5.0, and it stopped completely at pH 6.5. These findings indicated that our strains have the ability to produce the killer toxins in a wide range of temperature and pH.

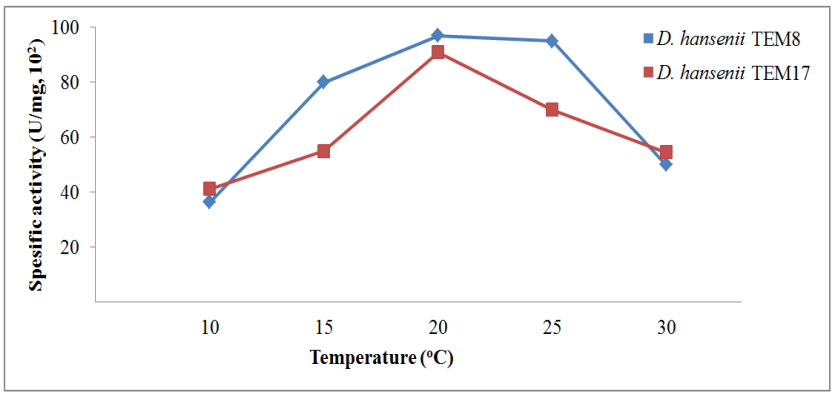

Figure 2 - Effect of temperature on killer toxin production at pH 4.0

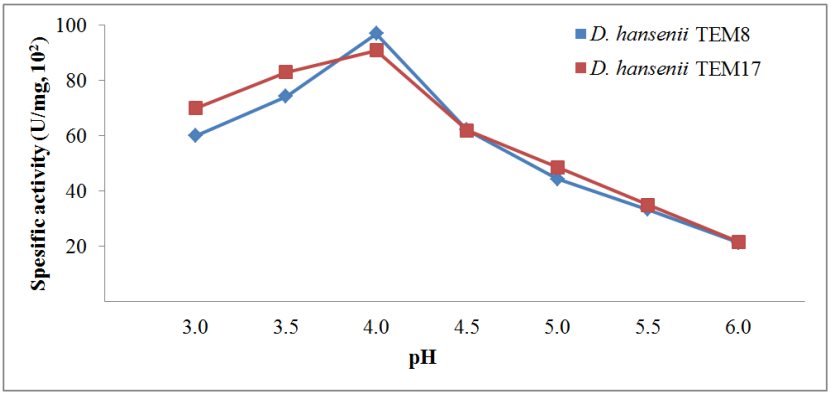

Figure 3 - Effect of pH on killer toxin production at 20ºC

The addition of reagents, including organic solvents, polyhydric alcohols, protease inhibitors, salts and non-ionic and ionic detergents, is known to stabilize some unstable proteins by preventing inactivation in solutions[27,28]. Marquina et al.[20] found the most efficient additive to be DMSO (100 ppm) on the toxin production by *D. hansenii* strain investigated. Besides, researchers reported that PEGs also were effective, although sorbitol, glycerol and PMSF did not improve the production. In our killer strains, *D. hansenii* TEM8 and 17, DMSO also increased the toxin production considerably at the concentration of 1000 ppm, and the addition of other additives did not improve the production at the concentration analyzed (Fig. 4). On the other hand, ammonium sulfate and non-ionic detergents significantly reduced the killer toxin production, and ionic detergent SDS inhibited the growth and toxin production completely compatible with previous observations[20,26].

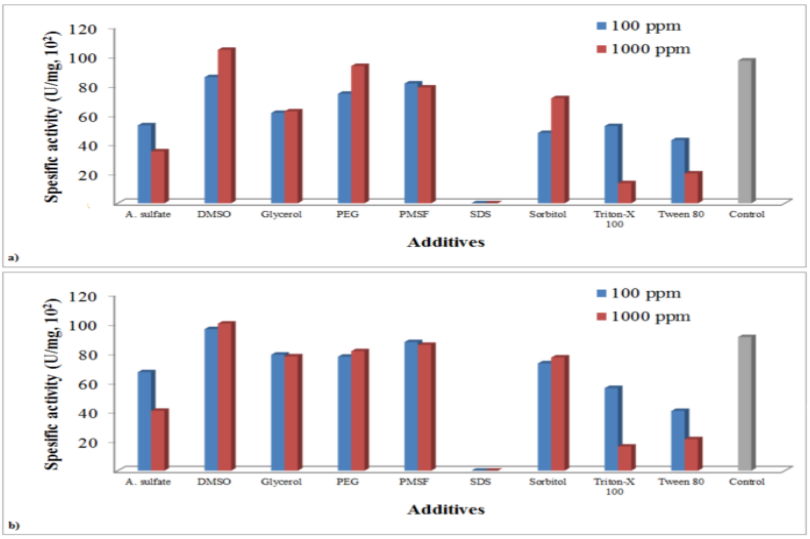

Figure 4 - Effect of different additives on killer toxin production at pH 4.0 and 20ºC; a) *D. hansenii* TEM8, b) *D. hansenii* TEM17

Because of *D. hansenii* strains are known to grow better in media containing sodium chloride, we also investigated the effect of NaCl concentrations on the production of killer toxin (Fig. 5). The toxin production was found to be best at a concentration of NaCl at 6% (w/v). Above and below this concentration, the production decreased gradually and these results were also compatible with previous findings[20,26].

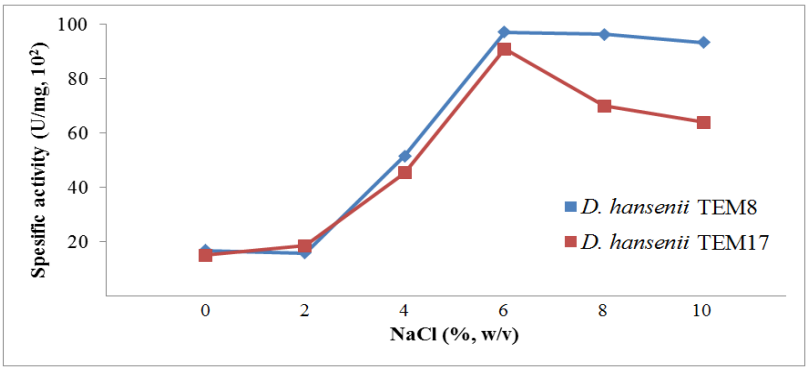

Figure 5 - Effect of NaCl concentration on killer toxin production at pH 4.0 and 20ºC

The use of bio-agents to control postharvest diseases of fruits and vegetables has been investigated as an alternative to the use of synthetic fungicides and a few commercial products are available nowadays. Discovery of novel microorganisms having antagonistic behaviors is important task because antagonistic microorganisms lived in domestic sources may be more effective against the pathogenic fungi present in that areas[2]

The characteristic properties of the genus *Debaryomyces* that endows it with such a potential has recently been reviewed by Breuer and Harms[29]. It is a yeast with numerous application areas in biotechnological processes such as dairy and meat fermentations, synthesis of fine chemicals and production of proteolytic and lypolytic enzymes. Besides, the antagonistic activity of *D. hansenii* of dairy and non-dairy origins is relatively well known. Payne and Bruce[30] investigated its activity on fungal spoilage of sawn *Pinus sylvestris* timber and reported that this yeast considerably reduced disfiguration of autoclaved sapwood blocks inoculated with mixtures of common wood-inhabiting molds or blue-stain fungi. Also, Hernández-

Montiel et al.[31] studied the biocontrol performances of *D. hansenii* strains isolated from marine environments and they suggested it can be used for biocontrol of postharvest diseases caused by *Penicillium italicum* on Mexican lime. On the other hand, several researchers evaluated killer character of *D. hansenii* strains isolated from dairy products and they also suggested it as an efficient biological agent to inhibit growth of several pathogenic yeast and moulds[22,32].

The outcomes defined in this paper revealed that *D. hansenii* strain TEM8 and 17, isolated from cheeses, had also great potentials to manage diseases caused by fungi. The toxin production in these strains was improved in YM medium, and the addition of DMSO contributes to the maximum levels of toxin production. No doubt, more studies are needed before the incorporation of these strains in biotechnological applications. Therefore, additional studies are planned to elucidate the structures and in vivo biocontrol efficiencies of killer proteins produced from these strains.

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