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Optimum conditions of killer toxins produced by *Torulaspota delbrueckii* and *Wickerhamomyces anomalus* and their action as antifungal agents

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Abstract

Background: This study was conducted to estimate the antifungal activity and efficiency of purified killer toxins (TK and WK) produced by *Torulaspota delbrueckii* and *Wickerhamomyces anomalus* as alternative drugs of resistant strains against several pathogenic fungi (*Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*). A specific focus was on the antifungal activity of such purified proteins by using a simple and economical tools under controlled conditions of fermentations.

Results: The results showed that the optimum conditions of these experiments were detected at 30 °C, 150 rpm, pH 4.5, 3 days incubation period and by using one culture medium (yeast malt extract broth with 2% SDS). The extracted crude toxin had an excellent effect on *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*. The inhibition zone values were 35 and 30 mm for WK and TK, respectively, against *Candida albicans*. While the lowest values of inhibition zone were recorded at 25 mm for WK and 18 mm for TK against *Aspergillus flavus*. The WK killer toxins were in general more active against the three clinical strains compared to the TK that had the lowest inhibitory effect.

Conclusion: This study is conducted for the first time in Iraq to determine the optimum conditions for producing two types of killer toxins and testing their antifungal activity. The optimal killer activity was obtained at low pH and mesophilic temperature. The two yeast strains had a good role in the reduction of radial growth of tested pathogenic fungi

Keywords: Alternative drugs, Killer toxins, Primary screening, Optimum condition, Yeasts, *Torulaspota delbrueckii*, *Wickerhamomyces anomalus*

Background

More than 1500 species of yeast have been described so far, many of them have been used in various fermentation processes (Kręgiel et al. 2017). Numerous species of yeasts have been reported to produce toxic proteins called exotoxin or mycocins against several pathogenic yeasts (Abu-Mejdad et al. 2020; Ferraz et al. 2016). For example, *Torulaspota delbrueckii* (a species of yeasts belongs to the family of Saccharomycetaceae) and *Wickerhamomyces anomalus*

(belongs to the family of Wickerhamomyceteae) have been reported to produce strong and active toxic proteins or glycoproteins as killer toxins. These antimicrobial substances can play an important role in the inhibition growth of different genera of yeasts (Becker and Schmitt, 2017; Weiler and Schmitt, 2003). The production of killer toxins was firstly described in 1963 for *Saccharomyces cerevisiae*; after that, the activity of killer toxins has been reported in 100 yeast species belonging to more than 20 genera (Maturano et al. 2012). The mode of action of these mycocins may be related to several mechanisms that involve DNA damage or inhibition of DNA replication, attacking the cell membranes of targeted yeast cells and pore

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formation. The pore formation can lead to cell death due to changes in the permeability of cytoplasmic membrane, loss of potassium ions, and energy (Camilli and Quintin, 2018).

According to Ruyters et al. (2015), there are three basic phenotypes for killer toxin production. First, killer strains that produce both a specific killer factor and resist to this toxin. Second, neutral strains that resist to one or more killer toxins and do not produce any killer factor. Third, sensitive strains that are totally susceptible to killer toxin causing inhibition of macromolecule synthesis and cell disruption resulting in cell death indicated the interactions between yeasts and filamentous fungi (killer-sensitive K-S strains). The K-S strains form killer factors, which are also sensitive to other killer yeasts (Chen and Chou, 2017; Marquina et al. 2002)

Although yeasts are known in their biological roles in the environment, however, there are much more actions of yeast modes have not been discovered in particular environments as well as their behaviors as antifungals against other organisms (Brauer et al. 2019; Ferraz et al. 2016)

According to Ruyters et al. (2015), sensitive cells have been reported to be killed by the viral K1 toxin of *S. cerevisiae* due to the function disruption of cytoplasmic membranes and the formation of lethal ion channels. Other behaviors of toxins, such as the viral K28 toxin of *S. cerevisiae* and the plasmid-driven killer toxin of *K. lactis*, result in early S phase or cell cycle arrest in G1, respectively (Weiler and Schmitt, 2003). In this perspective, the experiments of sensitive fermentation and killers have come to be relevant. On the other hand, inconsistent consequences have been stated concerning the appropriate preliminary killer/sensitive proportion that allows killer yeasts to prevail fermentation environments. As stated by Maturano et al. (2012), when the temperature of incubation is ranged between 18 and 28 °C, the expression of *Saccharomyces cerevisiae* killer toxin would be allowed. A number of studies informed that toxins produced by *Saccharomyces* and other killer genera ordinarily become inactive at higher temperatures (35 or 40 °C). Many species of yeasts have been reported their incubation period affecting on the production of antifungal agents (Samoticha et al., 2019)

The majority of killer yeasts have various characteristics that make them effective bio-agents in the conservation of fruits and vegetables. These killer yeasts have simple nutritional requirements, and are consequently effortlessly produced in great-scales. Furthermore, they do not host pathogenic or toxic abilities, in contradiction of filamentous fungi (Çorbacı and Uçar, 2017a; Mukherjee et al. 2020)

The current study was conducted to examine the production, detection, and testing the killer activities of mycocin produced by *Torulasporea delbrueckii* and *Wickerhamomyces anomalus* against fungi by using simple and economical tools under controlled conditions of fermentations.

Methods

Maintenance of microorganisms

Torulasporea delbrueckii and *Wickerhamomyces anomalus* strains were isolated and identified previously from soil samples by Abu-Mejdad (2019) to be used for producing killer toxins. Yeast cells were grown and maintained in slant medium of Yeast Extract-Peptone Dextrose agar (YEPD) containing 2% dextrose, 2% peptone, 1% yeast extract, and 2% agar at 4 °C (Himedia, India). Pathogenic strains (*Candida albicans*, *Aspergillus niger*, *Aspergillus flavus*) were obtained from the Mycology Lab., College of Science, University of Basrah, Basrah, Iraq. They were originally isolated from clinical samples and their identification and characterization were done according to morphological, biochemical, and molecular methods by using ITS1-ITS2 rDNA region, following Mirhendi et al. (2006). All isolates were sub-cultured and maintained on YEPD for further use.

Effects of pH, incubation temperature, stimulators, rpm, and period of incubation on killer toxin production

Inoculum preparation and production medium

Three discs with 6 mm in diameter of tested yeasts (*Torulasporea delbrueckii* and *Wickerhamomyces anomalus*) were transferred from the periphery of 2 days old culture to produce toxins and then added into the Yeast Extract Peptone Dextrose broth (YEPD). After inoculation, the flasks were incubated in different conditions.

In order to detect the optimum conditions for killer toxin production by *Torulasporea delbrueckii* and *Wickerhamomyces anomalus*, different factors were examined. Briefly, YEPD was prepared under various pH values (6.5, 6, 5.5, 5, and 4.5), different periods of incubation (7, 6, 5, 4, and 3) days, various rotations of liquid culture broth (200, 180, 160, 150, and 120) rpm, different temperatures of incubation during fermentation (15, 20, 25, 30, and 37) °C, and different stimulators were added to broth culture (Tween80 2% (BDH, England), glycerol 2% (BDH, England), DMSO 2% (Merk, Germany), ammonium sulfate 2% (Merk, Germany) and SDS 2% (Fisher Biotech., USA) for extracting crude toxins. The solution was evaporated at room temperature until dry and sterilized by Millipore filter paper 0.45 µ. The crude extract then kept in closed vials and reserved until used for susceptibility test of killer toxins (Al-Qaysi et al. 2017a).

Primary susceptibility test of extracted crude killer toxins as antifungal (killer assay)

Crude toxins extraction

The test of primary screening of crude killer toxins was performed in different conditions against three clinical fungal isolates *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*. The period of incubation was 2 days for *candida albicans* and 5 days for the filamentous fungi.

A 100 µl from the suspension of yeast (*Candida albicans*) and filamentous fungi (*Aspergillus niger* and *Aspergillus flavus*) was added to the methylene blue plates for fungi, then spread by L-shaped glass spreader. The plates were left to dry for 15 min at room temperature and then wells with 6 mm diameter were made using a sterile cork-borer. After that, a 100 µl of crude killer toxins at concentration 200 mg/ml D.W. was added individually to each well and incubated at 37 °C for 2 and 5 days for yeast and filamentous fungi, respectively. The results were calculated by measuring the inhibition zone diameter in millimeters. The positive control used in this experiment was Nystatin at concentration 200 mg/ml, which performed according to Serban et al. (2011).

Purification of killer toxins

The methods of purifying killer toxins were conducted according to Taguchi (1995) and Villalba et al. (2016).

Susceptibility test of purified killer toxins extracted as antifungal

The susceptibility test of the purified toxins was performed as described in (Al-Hilfy and Abu-Mejdad, 2014). Briefly, a 100 µl was taken from the suspension of pathogenic fungal strains, and the suspension of fungi then added to the Sabourauds agar and spread by L-shaped glass spreader. The plates were left to dry for 15 min at room temperature, and then wells with 6 mm

diameter were made using a stirle cork-borer. After that, a 100 µl of killer toxins (TK, WK) at concentration 200 mg/ml DW was added individually to each well and incubated at 37 °C for 5 days. The results were read by measuring the inhibition zone diameter in mm.

Statistical analysis

The antifungal activity was analyzed statistically using one-way ANOVA in SPSS program version 20 to determine the level of significant difference ($P < 0.05$) in the effect of two purified toxins against clinical strains.

Results

Production of killer toxins by *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* under different conditions

The effect of various pH value on the activity of killer toxins were examined by using an agar diffusion well bioassay. The optimum inhibitory for crude killer toxins, which extracted from the two subjected yeasts against all tested pathogenic agents was recorded at pH value of 4.5. Generally, the effect of killer toxins produced by *Wickerhamomyces anomalus* had a higher effect on all tested pathogenic fungi in comparison to the killer toxins produced by *Torulaspora delbrueckii*.

The results in Fig. 1 show the mean diameters of inhibition zones under different pH values for the crude

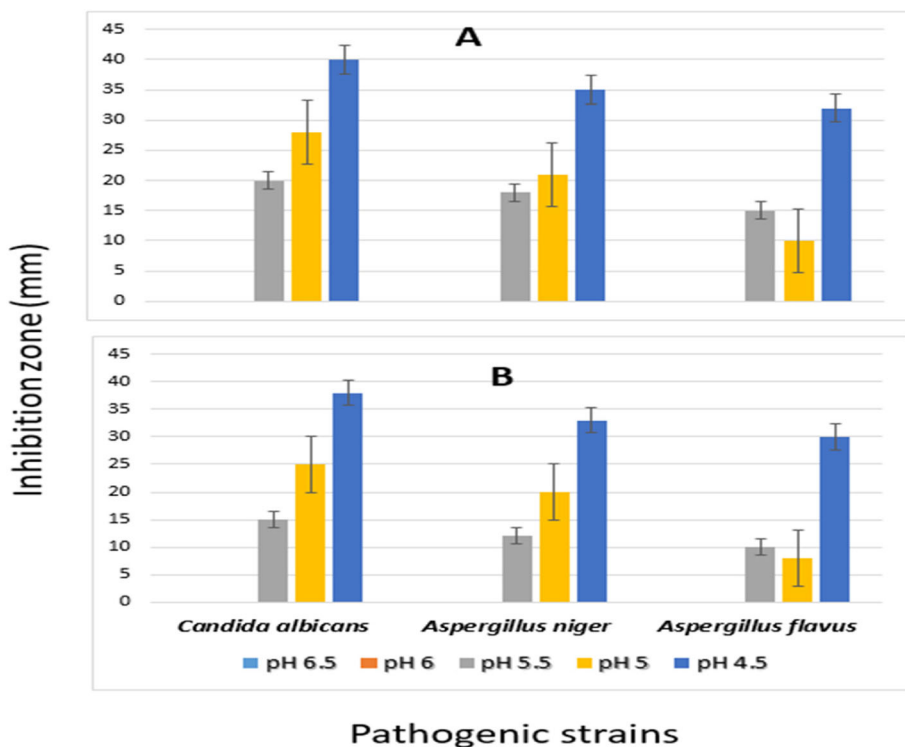


Fig. 1 Effects of five different pH of liquid fermentation broth on the production and killing activity of killer toxin produced by *Wickerhamomyces anomalus* (a) and *Torulaspora delbrueckii* (b) against pathogenic fungi. The killer activity was measured by the means of inhibition zone around the wells

killer toxins of *Wickerhamomyces anomalus* (Fig. 1a) and *Torulaspora delbrueckii* (Fig. 1b). The clear zone diameters of *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus* were 40, 35, and 32, and 38, 33, and 30 mm effected by killer toxins produced by *Wickerhamomyces anomalus* and *Torulaspora delbrueckii*, respectively.

Two pH values (6 and 6.5) in the fermentation broth for the two yeasts showed no effect as antifungal on all the tested pathogenic fungi, as the values of inhibition zones were 0. The results also showed that the low acidity (4.5) reduced the killer activity, while the high acidity had increased it.

Five periods of incubation (7, 6, 5, 4, and 3) days were tested for the two subjected yeasts during fermentation process. The results showed that the 3days was the best incubation period in fermenting medium to produce killer toxins by *Wickerhamomyces anomalus* and *Torulaspora delbrueckii*. The effect of killer toxins produced by *Wickerhamomyces anomalus* was higher than *Torulaspora delbrueckii* when measuring the rate of inhibition diameters on the studied pathogenic fungi in the same period

The results in Fig. 2 show the effect of periods on killer toxin produced by the two yeasts. The 3-day period was the best. The killer toxins produced by *Wickerhamomyces anomalus* (Fig. 2a) during this period gave inhibition rates at 50, 43, and 40 mm, while toxins produced by *Torulaspora delbrueckii* (Fig. 2b) were at 46, 40, and 37 mm

toward *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*, respectively. On the other hand, a 7-day period for both tested yeasts gave the least inhibition diameters since the killer toxins produced by *Wickerhamomyces anomalus* during this period had inhibition rates of 15, 13, and 10 mm, while the toxins produced by *Torulaspora delbrueckii* were 10, 8, and 6 mm towards *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*, respectively.

A 5 rotation rate of 200, 180, 160, 150, and 120 rpm of liquid culture broth were used in this experiment. The results in the Fig. 3 also show that the rotation process (rpm) at a rate of 150 cycles per minute during the fermentation period for both tested yeasts gave raw killer toxins with high inhibitory efficacy. The killer toxins produced by *Wickerhamomyces anomalus* (Fig. 3a) gave inhibition rates at 45, 40, and 30 mm, while the toxins produced by *Torulaspora delbrueckii* Fig. 3b display the inhibition rates at 35, 32, and 25 mm toward *Candida albicans*, *Aspergillus niger* and *Aspergillus flavus*, respectively. In comparison, no inhibitory activity was shown for the killer toxins produced by the two yeasts against the tested isolates during the rotation process, which reached to 120 and 200, as shown in Fig. 3a, b.

A 5 temperature degrees (15, 20, 25, 30, and 37) °C were examined during this experiment. The results in Fig. 4 show that the 30 °C as an incubation temperature

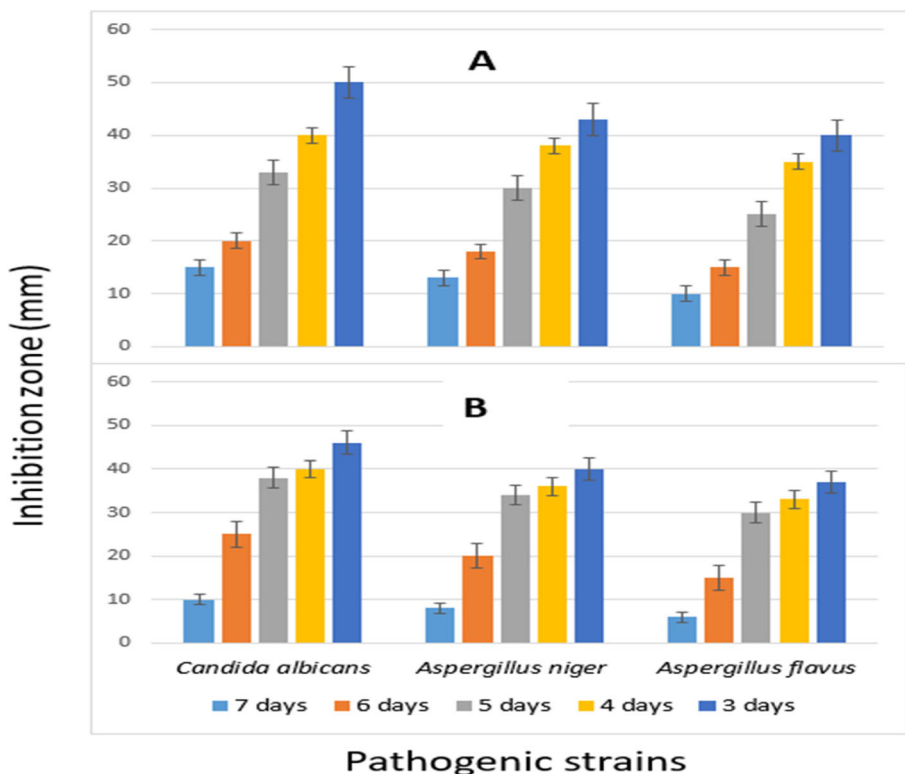


Fig. 2 Effects of fermentation period on the production and killing activity of killer toxin produced by *Wickerhamomyces anomalus* (a) and *Torulaspora delbrueckii* (b) against pathogenic fungi. The killer activity was measured by the means of inhibition zone around the wells

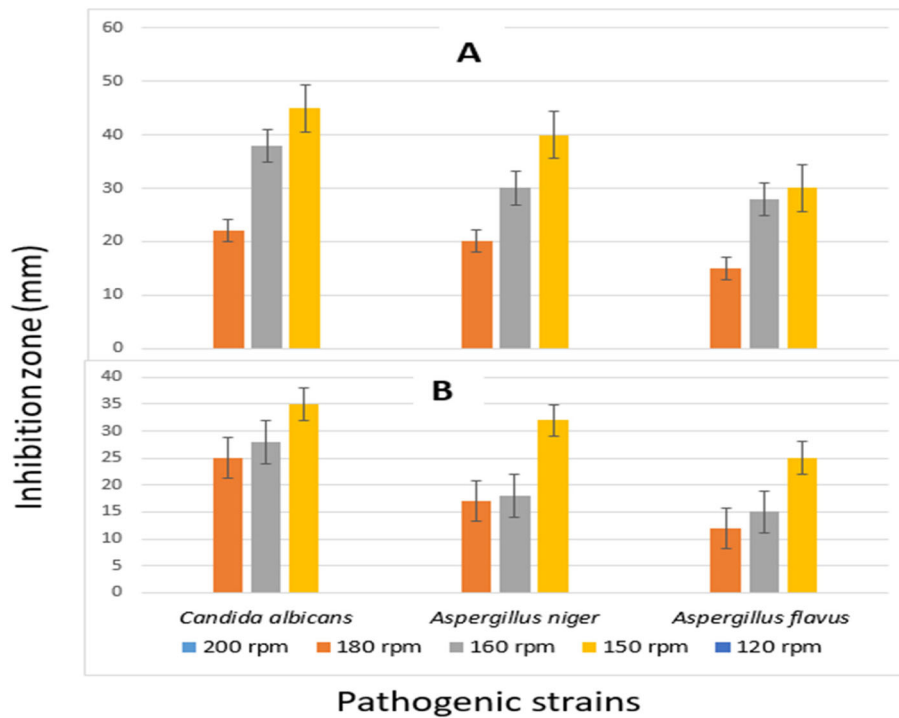


Fig. 3 Effects of the five different rotations per minutes during fermentation period on the production and killing activity of killer toxin produced by *Wickerhamomyces anomalus* (a) and *Torulaspora delbrueckii* (b) against pathogenic fungi. The killer activity was measured by the means of inhibition zone around the wells

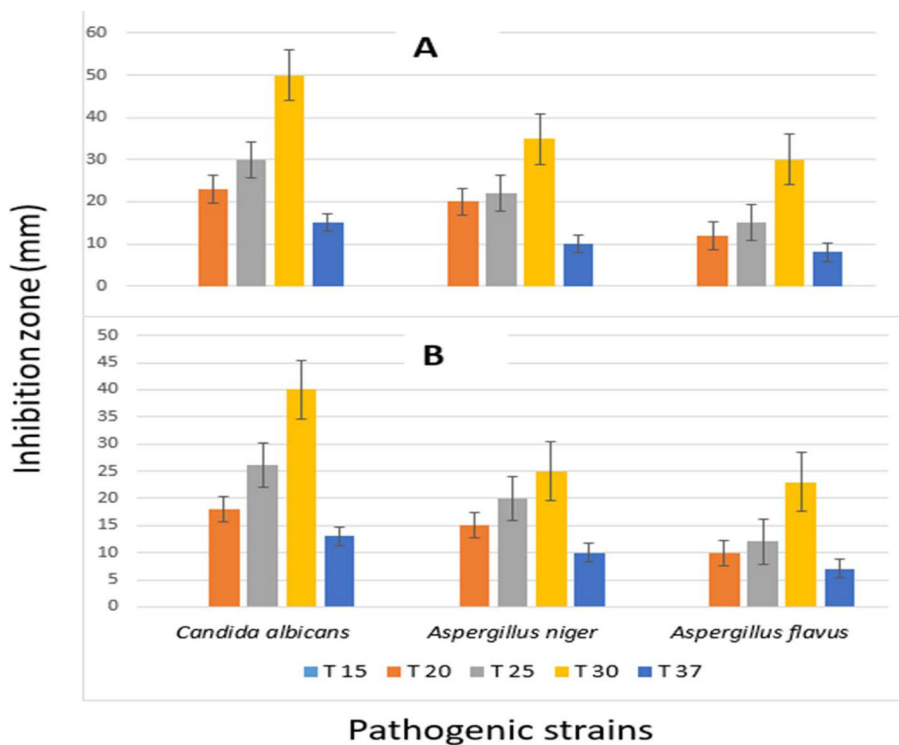


Fig. 4 Effects of the five different temperature during fermentation period on the production and killing activity of killer toxin produced by *Wickerhamomyces anomalus* (a) and *Torulaspora delbrueckii* (b) against pathogenic fungi. The killer activity was measured by the means of inhibition zone around the wells

during the fermentation period of both tested yeasts gave raw killer toxins with high inhibitory efficacy. The killer toxins produced by *Wickerhamomyces anomalus*, Fig. 4a, showed inhibition rates at 50, 35, and 30 mm, while the toxins produced by *Torulasporea delbrueckii* (Fig. 4b) were at 40, 25, and 23 mm toward *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*, respectively. Oppositely, no inhibitory activity was shown by the killer toxins produced by the two yeasts with regard to the tested isolates during the temperature of 15 °C, as shown in Fig. 4a, b.

A 5 stimulators (Tween 80 2%, glycerol 2%, DMSO 2%, Ammonium sulfate 2% and SDS 2% in liquid culture medium were added to broth culture in this experiment. Accordingly, the results in Fig. 5a, b showed that the SDS was the best stimulator for producing killer toxins in the fermentation medium of *Wickerhamomyces anomalus* and *Torulasporea delbrueckii*.

The effect of killer toxins produced by the yeast *Wickerhamomyces anomalus* was higher than *Torulasporea delbrueckii* when measuring the rates of inhibition diameters on the studied pathogenic fungi.

The results in Fig. 5 illustrate that the effect of stimulators on the production of killer toxins from the subjected yeasts. The SDS was the best in giving killer toxins produced by *Wickerhamomyces anomalus*, Fig. 5a,

during this period, as the inhibition diameters were 46, 40, and 34 mm, while toxins produced by *Torulasporea delbrueckii* (Fig. 5b) had inhibition diameters at 40, 38, and 35 mm towards *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*, respectively.

For comparison, the Tween 80 stimulating substance for both tested yeasts gave the lowest inhibition diameters of 20, 18, and 15 mm for the killer toxins produced by *Wickerhamomyces anomalus*, while toxins produced by *Torulasporea delbrueckii* had 18, 15, and 14 mm towards *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus*, respectively, as shown in Fig. 5a, b.

Note: The results of isolation and purification of these proteins have been published by the same authors of the current study (Abu-Mejdad et al. 2020).

Killer assay of TK and WK

The antifungal activity of TK and WK compounds at 200 mg/ml was tested against *Candida albicans*, *Aspergillus niger*, and *A. flavus*. The results showed that the WK compound had the higher inhibitory effect against all tested isolates compared to the TK. The results were also compared with the positive control compounds (Nystatine) as an antifungal compound, both were used at 200 mg/ml concentration as shown in Table 1 and Figs. 6 and 7.

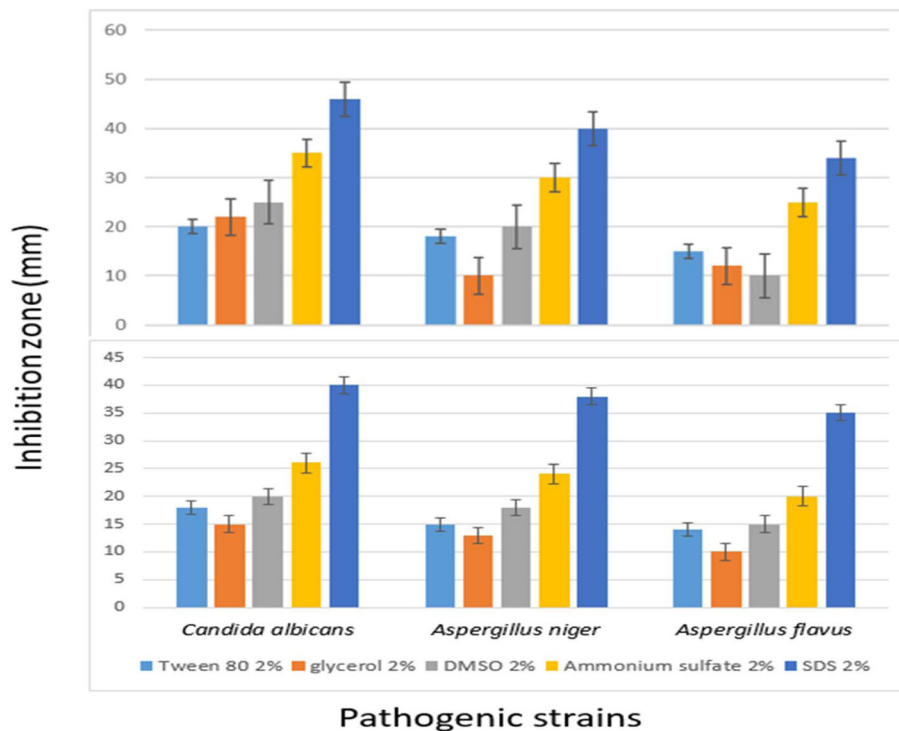


Fig. 5 Effects of the five stimulators added to YPD broth separately in concentration 2% on the production and killing activity of killer toxin produced by *Wickerhamomyces anomalus* (a) and *Torulasporea delbrueckii* (b) against pathogenic fungi. The killer activity was measured by the means of inhibition zone around the wells

Table 1 Inhibition zone diameters (killer assay) of WK and TK against fungal clinical isolates

No.	Fungal species	Inhibition zone (mm)	
		WK (mean ± SD)	TK (mean ± SD)
1	<i>Candida albicans</i>	35 ± 2	30 ± 2
2	<i>Aspergillus niger</i>	28 ± 2	20 ± 2
3	<i>Aspergillus flavus</i>	25 ± 2	18 ± 2
4	Nystatine	0	0

The WK and TK toxins with a concentration of 200 mg/ml were tested against three clinical isolates. In general, the results showed that the effect of WK was higher than TK.

Discussion

Different pH values 4, 4.5, 5.5, 6, and 6.5 were used in the culture media showed that the concentration of pH 4.5 had the highest growth rate. While, the growth was decreased with the increasing of pH values, this result is concordant with Banjara et al. (2016), who tested the killer activity of 23 strains against *C. albicans* and *C. tropicalis*, and noticed that there was no killer activity for any strain at pH ≥ 6.5 or at ≥ 35 °C.

The temperature was an another factor to impact the production of enzymes, since the growth at 22, 25, 30, and 37 °C gave varied amount of production enzymes for the killer toxins with a maximum level of growth rate observed at 30 °C. This result is similar to the finding

reported by Al-Qaysi et al. (2017b), who studied the effects of temperature and the production of killer toxins against several pathogenic microorganisms, such as *C. albicans*, *C. neoformans* *Staphylococcus aureus*, and others. Their results confirmed that the ideal inhibitory influence of killer toxin was at ≥ 25 °C.

The incubation time in fermentation media was monitored at 72, 96, and 168 h post inoculation. The result is in concord with the finding of Thammasittirong et al. (2013), who reported that the time was accelerated the vegetative phase of yeasts and encouraged them to reach the production phase at 72 h. To guarantee the efficient production and supply of oxygen to yeast, the rotation rate was tested at 120, 150, 180, and 200 rpm. The production rate was stimulated at highest level with 150 rpm. Culture media was also supplemented with sodium dodecyl sulphate (SDS) at concentration of 2%, which enhanced the growth rate. This may be attributed to the efficiency of this medium that contains nitrogen and carbon sources, which are used by the yeasts, similar explanation was giving by Çorbacı and Uçar (2017b). Besides, the addition of SDS and the detergent that destroys yeast wall and release killer toxin intracellular production. According to Grzegorzcyk et al. (2017), crude killer toxins have the ability to counteract the growth of bacteria and yeasts through pore formation by hydrolytic enzymes production. The toxin binds to its specific receptors on the surfaces of the bacterial and yeast cell walls, then forms pores to release the contents

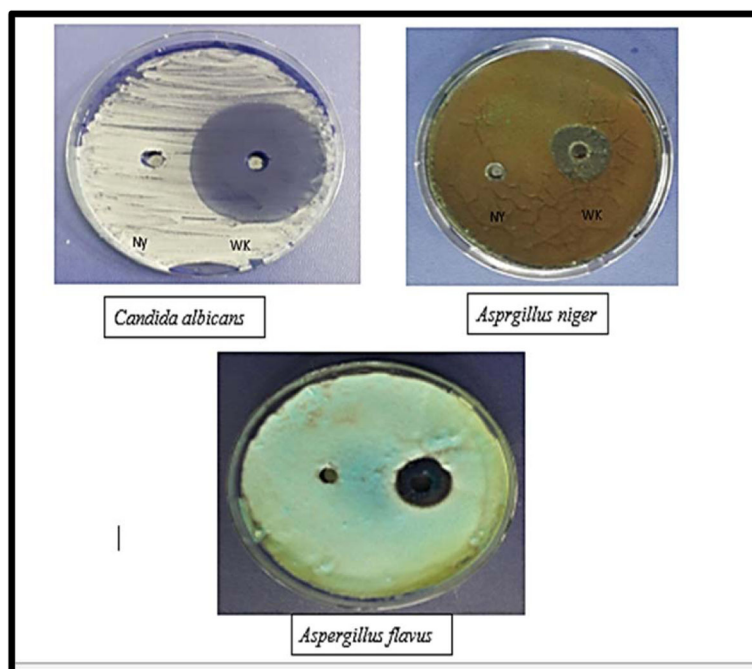


Fig. 6 The killer assay of WK for *Wickerhamomyces anomalus* against *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus* at concentration of 200 mg/ml

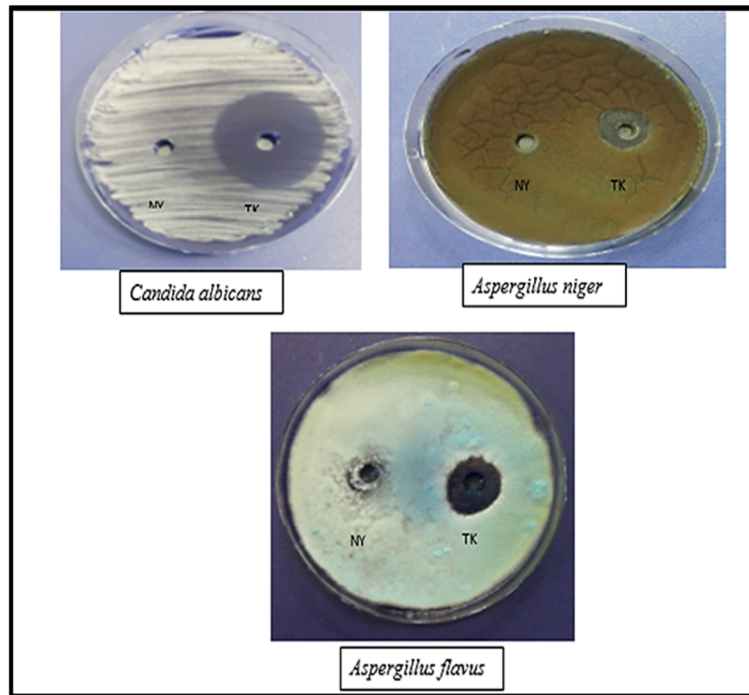


Fig. 7 The killer assay of TK for *Torulaspora delbrueckii* against *Candida albicans*, *Aspergillus niger*, and *Aspergillus flavus* at concentration of 200 mg/ml

inside the target cells leading to death. This can be attributed to the different mechanism actions reported by Becker and Schmitt (2017). In terms of K28, it has a specific primary receptor in the yeast cell wall, which allows rapid K28 absorption via an energy-independent binding mechanism. Afterward, α -1, 3- or α -1,2- mannose side-chains of a 185 kDa cell wall mannoprotein were identified as primary K28 receptor and cell wall binding sites. In contrast, Vadasz et al. (2000) stated that the ionophoric killer toxin (K2) has the ability of killing cells by causing shrinkage as a characteristic of cytosol efflux, which can be seen clearly by electron microscope. The cytosolic efflux and cell death are the results of initial interaction between yeast toxin and specific cell wall receptors, followed by the formation or activation of endogenous ion channels in the plasma membrane of target cells (Ahmed et al. 1999).

Conclusion

The results revealed that the *Torulaspora delbrueckii* and *Wickerhamomyces anomalus* secreted killer toxins. The optimal killer activity was obtained at low pH and mesophilic temperature. Moreover, the two yeast strains had a good role in reducing the radial growth of tested pathogenic fungi and the medical importance of two killer toxins against fungi was confirmed. Also, different type strains under different conditions in vitro showed variable inhibition zones because the conditions of incubation were very effective in producing the killer toxins.

Abbreviations

DMSO: Dimethylsulfoxide; G1: (GAP 1) phase; ITS: Internal transcript spacer; K1: Killer toxin 1; K2: Killer toxin 2; K28: Killer toxin 28; K-S strains: Killer-sensitive; PH: Potential hydrogen; rDNA: Ribosomal deoxy nucleic acid; S phase: (Synthesis) phase; SDS: Sodium dodecyl sulfate; TK: *Torulaspora delbrueckii* killer toxin; WK: *Wickerhamomyces anomalus* killer toxin; YEPD: Yeast Extract-Peptone Dextrose agar

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Authors' contributions

All work was done by NA and MM under supervision and advice of AA and AA. NA have drafted the work, and MM was a major contributor in substantively editing, revising, and writing the manuscript. All authors read and approved the final manuscript.

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Competing interests

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