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Investigating the biological activity and biofilm inhibition of purified *Pichia kudriavzevii* killer toxin (PkKt) against pathogenic bacterial as a promising substance for food preservation

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Abstract

Killer toxins (PkKT) produced by yeasts exhibit antimicrobial effects against pathogenic bacteria and fungi. This study aimed to extract, purify, and characterize the killer toxin from *Pichia kudriavzevii* killer toxin (PkKT) and evaluate its antimicrobial and anti-biofilm potential. PkKT was produced as a killer toxins and was purified through ammonium sulfate precipitation, ion exchange chromatography, and gel filtration. The purified PkKT showed a molecular weight of 36 kDa. Antimicrobial testing revealed significant inhibition against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Acinetobacter baumannii, Escherichia coli*, and *Candida albicans* using well diffusion assays. The minimum inhibitory concentration (MIC) ranged from 16-128 µg/mL, while the sub-MIC was determined to be 8-64 µg/mL depending on the pathogen. At sub-MIC levels, PkKT exhibited a 66-89% reduction of biofilm formation in all tested species. *S. aureus, S. pyogenes*, and *A. baumannii* showed the highest MIC of 128 and 64 µg/mL sub-MIC, while E. coli and *K. pneumoniae* were most susceptible with MIC of 16 µg/mL and sub-MIC of 8 µg/mL. In conclusion, the purified PkKT toxin displayed broad-spectrum antimicrobial and anti-biofilm activities against common healthcare-associated pathogens. These findings reveal the potential of PkKT as a therapeutic agent to prevent or treat biofilm-associated infections.

Keywords

antibiofilm; antimicrobial activity; gel filtration; ion exchange chromatography; *Pichia kudriavzevii*; protein precipitation; toxin;

Introduction

Bacterial infections remain a major global health concern, as many strains have developed resistance to commonly used antibiotics (1). This has created an urgent need to identify new antimicrobial agents. Recently, interest has grown in yeast-derived antimicrobial proteins and peptides as potential alternatives to traditional antibiotics (2). Because of their broad range of activity against many microorganisms, specific yeast-killer toxins are among the most promising classes of new antimicrobial medicines (3). The phenomenon of killer toxins was initially discovered to be widespread in *Saccharomyces cerevisiae*, however, it was later discovered to

be numerous other yeast genera. Killer yeast strains possess the capacity to generate extracellular poisons, which are fatal to microorganisms with sensitive cells (4, 5). Although strains may withstand their toxins, the toxins can be used in a variety of methods to destroy delicate cells. One yeast species that has shown promising results in this regard is *Pichia kudriavzevii* (6).

P. kudriavzevii is a species of yeast that has been isolated from various environmental sources, including soil, insects, and fermented foods (7). Research has demonstrated that some strains of this yeast species produce small proteins and peptides that exhibit antimicrobial effects against bacteria (8, 9). These antimicrobial compounds are thought to help the yeast compete against bacteria in their shared environments. Recent studies have focused specifically on characterizing antimicrobial proteins secreted by a strain of *P. kudriavzevii* isolated from different sources, referred to as *P. kudriavzevii* purified killer toxin (PkKT) (10).

When tested against an array of pathogenic bacterial strains, including *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus*, and *Pseudomonas aeruginosa*, the species PkKT showed significant inhibitory effects on bacterial growth. The antimicrobial activity was observed against both Gram-positive and Gram-negative bacteria (11, 12).

In addition to suppressing growth, PkKT was found to inhibit biofilm formation in bacterial species. Biofilms are multilayered communities of bacterial cells that adhere to surfaces and are encapsulated in a self-produced matrix (13). Biofilm formation enhances the survival capability of bacteria and makes them more resistant to antibiotics. PkKT was shown to reduce biofilm mass and thickness in species like *S. aureus* and *P. aeruginosa.* This suggests that it may have the potential to disrupt established biofilms (14).

The current study aimed to investigate the killing activity of purified killer toxin isolated from a local yeast strain of *P. kudriavzevii*.

Materials and Methods

2.1 Materials

Yeast extract peptone dextrose (YEPD) broth, nutrient agar, Mueller-Hinton agar, nutrient broth, brain heart infusion (BHI) broth, and Sabouraud dextrose agar were provided from Himedia (India), while DEAE-cellulose and Sephadex G-150 were purchased from Pharmacia (Sweden).

2.2 Yeast and Bacterial Isolates

Pichia kudriavzevii, Saccharomyces cerevisiae, Candida albicans, Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Klebsiella pneumoniae, Listeria monocytogenes, Acinetobacter baumannii, and *Escherichia coli* strains were obtained from Biotechnology Research Center-Al-Nahrain University

2.3 Isolation and Killer Activity of PkKT

P. kudriavzevii was grown overnight in 100 mL of YEPD broth in a shaker incubator (180 rpm) at 30°C. The fresh cultures were used to inoculate 1 L of killer toxin production YEPD broth suspended in 500-mL conical flasks filled with 250 mL broth (10% v/v). The inoculated flasks were incubated at 30°C for 72 hours with shaking at 180 rpm. After incubation, the fermented broth was centrifuged at 10,000 × g for 15 min at 4°C. The pellet was discarded, and the supernatant, containing the crude killer toxin, was collected. (10). Protein content was determined using the Bradford method (15).

For killer toxin activity estimation, crude toxin at specified dilution was prepared and poured into wells of diameter 7 mm (~ 50 µL /well) cut on Sabouraud Dextrose Agar plates pre-spread with S. cerevisiae culture. Plates were incubated at 30°C for 24 h, and the zone of inhibition was measured in mm. Killer activity was expressed in terms of arbitrary units (AU) multiplied by the inverse of specified dilution (AU/mL). One AU was defined as the amount of killer toxin preparation that produces an inhibition zone of 10 mm (16).

2.4 Purification of PkKT

The crude PkKT was subjected to purification processes involving salt precipitation, ion exchange chromatography, and gel filtration. The crude PkKT was precipitated by 30% ammonium sulphate and the mixture was mixed overnight using a magnetic stirrer at 4°C. PkKT was harvested by centrifugation (10,000 rpm) at 4°C for 10 min. The precipitate was dissolved in an appropriate volume of sodium acetate buffer and dialyzed against distilled water at 4°C for 24 h using dialysis bags (3, 10 kDa) (17). PkKT was then added to the DEAE-cellulose column (3 x 20 cm) and equilibrated with sodium chloride/hydroxide solution (0.25 M). After that, the column was washed with an equivalent volume of the same buffer and attached proteins were stepwise eluted with gradual concentrations of sodium chloride (0.1- 1 M). The flow rate through the column was 3 mL and the absorbance of each fraction was measured at 280 nm using a UV-visible spectrophotometer (Cecil CE2041, Italy). Fractions with high killing activity were collected and loaded into the Sephadex G-150 column (2 x 40 cm) equilibrated with potassium phosphate buffer (pH: 7), and eluted at a flow rate of 3 mL/fraction. After that, the absorbance of each fraction was also measured at 280 nm (18). The molecular weight of purified PkKT was determined using a series of standard proteins with known molecular weight.

2.4.1 Characterization of Purified Killer Toxin

Purified killer toxin characteristics were determined as follows:

2.4.2 The Effect of pH on Killer Toxin Activity and Stability

The purified killer toxin was mixed with sodium acetate buffer (Prepared in 2.2.7.1A) at different pH values (3.5, 4, 4.5, 5, and 5.5). The activity of killer toxin was measured. On the other hand, the stability of the killer toxin was estimated by incubating the killer toxin in the buffer of several pHs (3.5-5.5) for 30 min at 30°C, then the toxin activity was estimated, and the remaining activity was

calculated.

2.4.3 The Effect of Temperature on Killer Toxin Activity and Stability

The killer toxin activity was measured at several temperatures (25, 30, 35, and 37°C). For thermal stability, the purified toxin was pre-incubated at the same temperature for 30 min and the activity and remaining activity were determined.

2.5 *Antimicrobial Activity of PkKT*

The agar well diffusion method was used to detect the antimicrobial activity of PkKT against selected microbial isolates at a concentration of 512.0 µg/mL (19). In brief, an aliquot of 0.1 mL of freshly prepared microbial isolate was spread on the surface of nutrient agar at 37°C for 24 h. A single colony was introduced to a test tube containing 5 mL of sterile normal saline to produce a mildly turbid microbial suspension comparable to the standard turbidity solution of 1.5 × 108 CFU/mL. A portion of the bacterial suspension was carefully transferred and evenly dispersed on Mueller-Hinton agar medium using a sterile cotton swab, and it was left for 10 min. In the preceding agar layer, five millimeter-diameter wells were formed (3 wells/plate). An Aliquot of 0.1 mL of purified killer toxin was added to each well using a micropipette. The diameter of the inhibitory zones was measured after overnight incubation at 37°C on the plates. Normal saline water was used as the negative control.

2.6 Broth Dilution Method

The minimum inhibitory concentration (MIC) value of purified PkKT against microbial isolates was determined using the broth dilution method (20). A fresh microbial culture was prepared by growing microbial isolates overnight in nutrient broth (5 mL) at 37°C. After incubation, the microbial suspension was adjusted to a concentration of 1.5 × 108 CFU/mL using sterile normal saline water. A serial dilution series of purified killer toxins (0, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1000 µg/mL) was prepared in test tubes containing nutrient broth (total volume 3 mL). An Aliquot of 0.3 mL of adjusted microbial suspension was used to inoculate each tube. The tubes were incubated at 37°C for 24 h. After incubation, the lowest concentration that completely inhibits microbial growth (no turbidity) was considered as MIC.

2.7 Antibiofilm Activity of Purified PkKT

The antibiofilm activity of PkKT was estimated at MIC and sub-MIC concentrations against microbial pathogens that are capable of forming biofilms using the microtiter plate method (21). An Aliquot of 180 µL of BHI broth containing PkKT at MIC and sub-MIC was added to each well. Then, 20 µL of microbial suspension (1.5 x 108 CFU/mL) was added to each well and incubated at 37°C for 24 h. After incubation, the medium was taken from the wells and washed three times with normal saline to remove the unattached microbial cells and left to dry at room temperature for 15 min. The wells were then filled with 200 µL of crystal violet solution (5 mg/mL) and allowed to sit for 20 min. The unbound dye was removed by washing the plate three times with normal saline and allowing the plate to dry at room temperature for 15 min. Finally, 200 µL of 95% ethanol was added to each well and the optical density was measured using a microtiter plate reader (Bio-Rad, Germany) at 630 nm.

Statistical analysis

The antibacterial activity of PkKT was demonstrated against G+ve and G-ve bacteria. Data obtained were analyzed using the ANNOVA test through MiniTab statistical analysis software version 20.4.

Results

3.1 PkKT Activity and Purification Steps

The capability of *P. kudriavzevii* to produce PkKT was assessed using a well plate assay against standard *S. cerevisiae* strain. Results showed that *P. kudriavzevii* was able to produce PkKT with a maximum activity of 15 AU/mL. *P. kudriavzevii* is known to produce killer toxins, which are proteins or glycoproteins that can kill sensitive strains of the same or related yeast species. The toxins act by binding to specific cell wall receptors on sensitive cells, creating pores that disrupt the integrity of the cell membrane and cause ion leakage, eventually leading to cell death (22).

Recent research has shown that different strains of *P. kudriavzevii* can vary significantly in their PkKT production and activity. In comparative studies, some strains such as RY55 showed robust PkKT activity, evidenced by large zones of growth inhibition against sensitive indicator strains, 32 AU/mL after 72 hrs incubation (10). The diameter of the clear zone of *S. cerevisiae*, which denotes the presence of PkKT, revealed that *P. kudriavzevii* has the highest activity. The production of primary and secondary metabolites results in killing action (12). Acetic acid, pyruvic acid, and lactic acid are examples of the organic acids that *P. kudriavzevii* can create as primary metabolites. It has been demonstrated that the lactic and acetic acids produced by yeast metabolism can inhibit the growth of other microbes. Additionally, *P. kudriavzevii* can create ethanol as a secondary metabolite, resulting in the formation of a clear zone in the *S. cerevisiae* (11). Differences in the PkKT genes and expression levels, efficiency of toxin secretion, and composition of cell wall receptors in target strains are all factors that can potentially affect killer activity (23).

Crude killer toxin isolated from *P. kudriavzevii* was purified via three purification methods, salt precipitation, ion-exchange chromatography, and gel filtration. Results in Table (1), show that the crude PkKT activity (69.0 AU/mL) and specific activity (138 AU/mg) were increased after ammonium sulphate saturation at a concentration of 30% (w/v) with a 2.07-fold increase in purification and 51.8% yield.

PkKT obtained by ammonium sulphate precipitation was partially purified in ion exchange chromatography using DEAE-Cellulose resin. Results in Table (1) and Fig (1) show that one sharp peak was obtained from the washing

Table (1): Purification steps of killer toxin produced by *P. kudriavzevii.*

Purification Step	Volume (mL)	Killer Toxin Activity (AU/mL)	Protein Concentration (mg/mL)	Specific Activity (AU/mg)	Total Activity (AU)	Purification (Folds)	Yield (%)
Crude Killer Toxin	65	26.6	0.4	66.5	1729	1	100
Ammonium Sulphate Precipitation 30%	13	69	0.5	138	897	2.07	51.8
DEAE-Cellulose	21	40	0.2	200	840	3	48.5
Sephadex- G150	18	46	0.09	511	828	7.6	47.8

step and the other peak was observed at the elution step with gradient concentrations of sodium chloride. All peaks were assayed to detect the activity of PkKT. The wash peak contained most of the PkKT activity (40 AU/mL) at fractions numbered (20 to 28). PkKT-specific activity was increased to

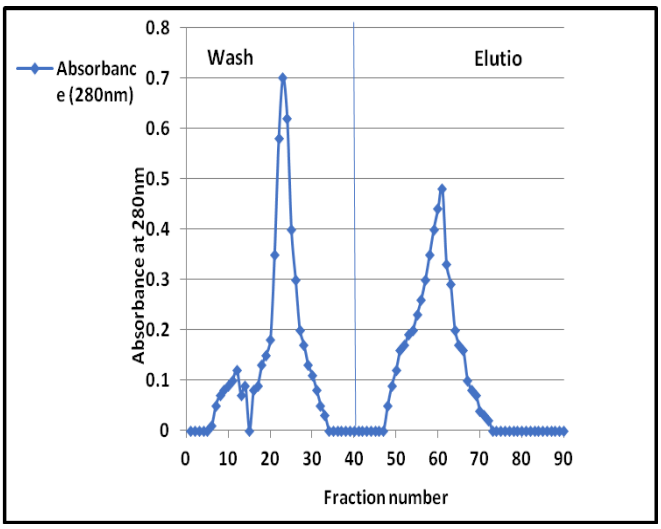

Fig. 1. Ion exchange chromatography of PkKT using DEAE-cellulose column (3 × 20 cm).

200 AU/mg protein with 3.0-fold purification fold 48.5% yield.

Partially purified PkKT resulting from DEAE-Cellulose was subjected to the final step of purification by gel filtration chromatography using Sephadex- G150. As observed in Table (1) and Fig. (2), a sharp peak was obtained after the elution step and the activity of PkKT

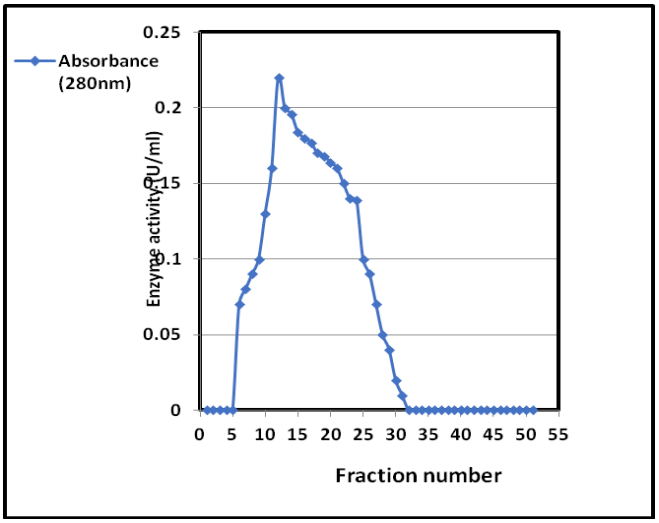

Fig. 2. Gel filtration chromatography of PkKT using Sephadex G-150 column (2 × 40 cm).

was entirely associated with this peak. The specific activity was substantially increased to 511.0 AU/mL with a 7.6 purification fold and 47.8% yield.

The molecular weight of purified PkKT was determined using a Sephadex- G150 column. The linear relationship between the molecular weight of each standard protein and the recovery volume of each protein relative to

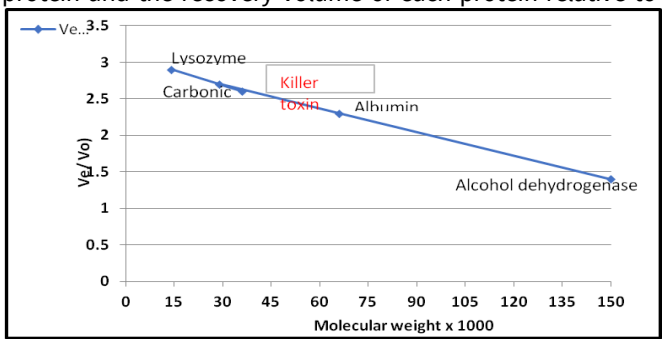

Figure (3): Standard curve for estimating the molecular weight of PkKT by gel filtration method using Sephadex G-150 column (2 x 40 cm).

the void volume (Ve /Vo) was plotted (Fig. 3) and results showed that the molecular weight of killer toxin was 36,000 Dalton.

Discussion

Killer toxins have been isolated from different yeast genera using techniques such as salt precipitation, ion exchange chromatography, gel filtration, etc. (10). Ammonium sulfate is added to the crude PkKT to a saturation point, causing the toxin protein to precipitate out of the solution while leaving behind soluble impurities. This initial step removes some contaminating proteins, carbohydrates, lipids, and other molecules based on differential solubility and for this reason, the specific activity of PkKT was increased by 2.07-fold. This result is in concordance with other previous findings (24, 25).

Ion exchange chromatography using DEAE-Cellulose resin helps further purify the toxin by exploiting differences in charge. The negatively charged PkKT was suitable to apply to the anion exchange DEAE-Cellulose resin, which allowed killer toxin molecules to repel the resin and pass through the gel while other components with positive charges bound to the resin (10). The isoelectric point of different yeast killer toxins ranged from pH: 4.3 - to 4.5. Most proteins have a negative net charge at high pH levels, where they resist the negative matrix charge in anion exchangers (26). Using negatively charged DEAE-Cellulose with buffer at pH: 7 successfully improved the purity and activity of PkKT by 3.0-fold compared to crude preparation.

Fig. (2) demonstrates that the single peak reflects the higher biological activity because of pure PkKT. Impurities elimination, salt removal, and gel filtration resulted in a good specific activity compared to the crude extract with a 7.6-fold increase in activity. Two two-step purification methods including gel filtration are routinely used to purify yeast-killer toxins. Concentration by ammonium sulphate and Sephacryl S-200 gel filtration step were used for killer toxin purification extracted from Bakery yeast [27]. In addition, the molecular weight of 36 KDa of purified PkKT was estimated by gel filtration. Killer toxins have different molecular weights in different yeast strains. For instance, the molecular weights of killer toxins from several yeast genera ranged from 18 to 300 kDa. Killer toxin extracted from *P. kudriavzevii* RY55 possesses a molecular weight of 39.8 kDa (28), another study identified a killer toxin from *P. kluyveri* with a molecular weight of 54 kDa (29).

3.2 Antimicrobial Activity of Purified PkKT

The antimicrobial activity of PkKT obtained from the final purification step was examined against several pathogenic microorganisms *C. albicans, S. aureus, S. pyogenes, P. aeruginosa, K. pneumoniae, L. monocytogenes, Acinetobacter baumannii,* and *E. coli* using well diffusion method. Results in Table (2), show that all the tested pathogenic microorganisms were highly susceptible to the proteins collected from the washing step indicating the existence of PkKT. On the other hand, antimicrobial activity was not found in the elution step, indicating that no PkKT was bound to the ion exchange resin, and all the killer toxin was already passed in the washing step. The antimicrobial activity of purified PkKT showed minor variation in the producing zone of inhibition ranging from 14±1.4 (*K. pneumoniae*) to 18.0±3.0 mm (*S. aureus*) with no significant differences among tested pathogens.

Table (2): Analysis of variance (ANOVA) for *PkKT* against different types of bacterial pathogens.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Klebsiella pneumoniae	9.94	1	9.94	58.15	<0.0001
Pseudomonas aeruginosa	5.67	1	5.67	33.17	<0.0001
Listeria monocytogenes	0.1650	1	0.1650	0.9656	0.3365
Candida albicans	3.49	1	3.49	20.44	0.0002
Streptococcus pyogenes	1.640×10^{-6}	1	1.640×10^{-6}	9.597×10^{-6}	0.9976
Escherichia coli	0.0046	1	0.0046	0.0269	0.8712
Staphylococcus aureus	4.90	1	4.90	28.66	<0.0001
Acinetobacter baumannii	0.9233	1	0.9233	5.40	0.0297

$R^2 = 0.8703$, Adjusted $R^2 = 0.8172$, Predicted $R^2 = 0.662$, Significant ≤ 0.050

Yeast produces toxins that exhibit antimicrobial activity against other yeasts and bacteria. These toxins are known as yeast-killer toxins or mycotoxins (30). They are secreted proteins or glycoproteins that can kill sensitive microorganisms. The killer phenomenon was first discovered in *S. cerevisiae* but has since been found in other yeast species (16, 28). Research has shown that yeast -killer toxins have a wide range of antimicrobial activity against medically important pathogens including *Candida albicans, C. neoformans, S. aureus,* and *P. aeruginosa* (5, 6).

PkKT are proteins or glycoproteins that act by disrupting the cell membrane integrity of sensitive microorganisms (31). Research has shown that PkKT has a wide range of antimicrobial activity against both Gram-positive and Gram-negative bacteria of medical significance (32).

Studies have demonstrated that PkKT inhibits the growth of common Gram-positive pathogens such as *S. aureus, S. pyogenes,* and *L. monocytogenes* (10). The toxin creates pores in the bacterial cell membrane, leading to ion leakage and cell death (33). For Gram-negative bacteria like *E. coli, P. aeruginosa,* and *K. pneumoniae*, the PkKT can pass through the outer membrane and cause cytoplasmic membrane damage (34). This broad-spectrum activity against Gram-positive and negative pathogenic bacteria makes PkKT a promising natural antimicrobial agent. Further investigations on the functional characterization and mechanisms of action of the toxin could provide insight into potential therapeutic applications. Overall, considering the findings of the experiment, PkKT represents an interesting alternative strategy to combat multidrug-resistant infections caused by bacterial pathogens (35).

PkKT demonstrates potent fungicidal effects against certain *Candida* species. The toxins bind to specific receptors on the Candida cell wall and membrane, creating pores that disrupt membrane integrity. This leads to ion leakage, loss of metabolites, and apoptosis (36). Research shows PkKT exhibits candidacidal activity against clinically important species like *C. albicans, C. glabrata,* and *C. tropicalis* (37). The strength of activity depends on both the Candida strain and PkKT type. Further work is exploring combinations of PkKT with antifungal drugs to improve efficacy against drug-resistant Candida infections. Overall, PkKT represents promising natural antimicrobials to combat Candida (38).

3.3 MIC of Purified PkKT

The MIC of purified PkKT against pathogenic microorganisms was detected using the broth dilution method. The MIC and sub-MIC of PkKT were identified for each pathogen. The MIC and sub-MIC in *S. pyogenes, S. aureus,* and *A. baumannii* were 64 and 128 µg/mL, respectively. The MIC and sub-MIC effect of PkKT against *L. monocytogenes* and *C. albicans* were 32 and 64 µg/mL, respectively. *P. aeruginosa* was completely killed at a

concentration of 32 µg/mL, the concentration 16 µg/mL was considered as sub-MIC. Both *E. coli* and *K. pneumoniae* exhibited the most extreme sensitivity to killer toxin among all pathogens, with a killing concentration of 16 µg/mL and sub-MIC of 8 µg/mL.

Determining the MIC provides important information about the potency and potential applications of PkKT. The MIC is defined as the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism after overnight incubation. Research studies have used broth dilution methods to establish MIC values for PkKT against different microbial strains. Reported MICs range from 0.24 to 500 µg/mL depending on the toxin variant, producer strain, target organism, and assay conditions (39). Lower MICs indicate greater antimicrobial potency (40). Hence, according to the study, the MIC of PkKT ranged from 16 to 128 µg/mL, indicating the potent antimicrobial effect of this toxin against all types of pathogenic microorganisms. The MIC of *P. kluyveri* against food and beverage spoilage yeasts was found to be from 2 to 5 µg/mL (29). Killer toxin from *P. pastoris* was used to control bacterial contaminations in the food industry with a MIC of 1 AU/mL (35).

3.4 Antibiofilm of Purified PkKT

The anti-biofilm capability of purified PkKT was estimated using the sub-MIC concentration of killer toxin designated for each pathogen. After completing the treatment of microbial isolates with sub-MIC of killer toxin, the results showed bacterial sensitivity to PkKT and reduction in biofilm formation (Fig. 4). Biofilm formation in all tested microbial pathogens was significantly reduced and the levels of biofilm inhibition varied among the pathogens and ranged from 66.67 ± 5.76% to 88.93 ± 1.04%. *P. aeruginosa, L. monocytogenes,* and *E. coli* showed no significant differences in the rate of biofilm reduction upon

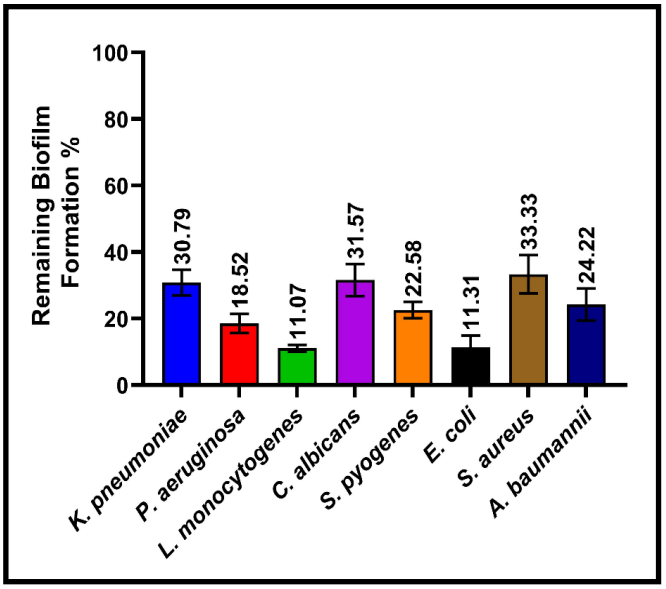

Figure (4): Mean percentage of remaining biofilm formation in different microbial pathogens after treatment with purified PkKT at sub-MIC.

treatment with purified PkKT and these bacterial species were gathered in one group that is significantly ($p < 0.05$) from other microbial species concerning biofilm inhibition rate.

Biofilm formation enhances the tolerance of microorganisms to antimicrobials and environmental stresses (41). Several studies have explored the potential of yeast-killer toxins in preventing or disrupting biofilms formed by harmful bacteria and fungi (42). Killer toxins secreted by *Pichia*, *Williopsis*, and other yeasts demonstrate broad-spectrum antimicrobial effects. There is evidence that some killer toxins can prevent biofilm formation by susceptible organisms. For example, PkKT inhibited biofilm formation by *C. albicans, S. aureus,* and *P. aeruginosa* at sub-MIC levels (43). The anti-biofilm activity has been attributed to interference with microbial growth and adhesion.

In addition, some killer toxins exhibit biofilm-disrupting ability by damaging or dismantling established biofilms. *A Williamses* killer toxin reduced pre-formed biofilms of *Candida tropicalis* by 75-90% after 2 hours of treatment (44). This toxin likely degrades biofilm EPS (exopolysaccharide) components and kills embedded cells. The anti-biofilm effects of killer toxins appear to involve both microbicidal activity and EPS breakdown (45).

The results highly confirmed that PkKT was extremely active in preventing biofilm formation in all tested Gram-negative bacteria. Studies showed that a killer toxin from *Wickerhamomyces anomalous* inhibited biofilm formation by *P. aeruginosa* and *E. coli* at sub-inhibitory concentrations. This toxin reduced biofilm biomass by more than 60% and altered biofilm structure and thickness (16, 28).

Conclusion

The killer toxin purified from *P. kudriavzevii* exhibited potent antimicrobial and anti-biofilm activity against a range of pathogenic bacteria that are mostly found on fruits and can be isolated from food such as *Klebsiella pneumoniae, Pseudomonas aeruginosa, Listeria monocytogenes, Streptococcus pyogenes, Escherichia coli, S. aureus, and A. bumanni* and extended to exhibit activity against *Candida albicans*. All tested pathogens were highly susceptible, with a 66-89% reduction in biofilm formation when treated with sub-MIC toxin concentrations. These results demonstrate the promising therapeutic potential of this purified killer toxin as a novel antimicrobial and anti-biofilm agent. With a potential of wide spectrum inhibition, and MIC values ranging from 16-128 µg/mL, this natural product could be a promising substance that can be used in food preservation for its selectivity against competitive microorganisms and exerts no effect on humans.

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Not applicable

Authors' contributions

NNH handled microbial isolation, identification, cultivation, preservation, and drafting of the article. SAS conducted the planning of the research, and ZAT performed sample curation.

Compliance with ethical standards

Conflict of interest: : The authors declare that they have no conflicts of interest

Ethical issues: This research project was approved by the Ethics Committee at Mustaniriyah University with ref. no. E. B. 8- 2.1.23.

Consent for Publication

The results of this research were approved by all authors and agreed for publication.

Availability of data and material

Data, samples, and genomic materials used during this study were available and stored at the Biotechnology Research Center, Al-Nahrain University.

Competing interests

This research was conducted without conflict of interest among authors, funding agencies, or any other research group in other institutes.

References

1. Terreni M, Taccani M, Pregnolato M. New antibiotics for multidrug-resistant bacterial strains: latest research developments and future perspectives. Molecules. 2021 May 2;26(9):2671. https://doi.org/10.3390/molecules26092671.
2. Naimah AK, Al-Manhel AJ, Al-Shawi MJ. Isolation, purification and characterization of antimicrobial peptides produced from *Saccharomyces boulardii*. International Journal of Peptide Research and Therapeutics. 2018 Sep;24:455-6. https://doi.org/10.1007/s10989-017-9632.
3. Liu GL, Chi Z, Wang GY, Wang ZP, Li Y, Chi ZM. Yeast killer toxins, molecular mechanisms of their action and their applications. Critical reviews in biotechnology. 2015 Apr 3;35(2):222-34. https://doi.org/10.3109/07388551.2013.833582
4. Anke T, Schüffler A. Physiology and Genetics, In: R. Schaffrath F. Meinhardt and R. Klassen (Eds.), Yeast killer toxins: Fundamentals and applications, Springer link, pp. 107-130, 2009. https://doi.org/10.1007/978-3-642-00286-1
5. Anke T, Schüffler A. Physiology and Genetics, In: R. Schaffrath F. Meinhardt and R. Klassen (Eds.). Yeast killer toxins: Fundamentals and applications, Springer link, pp. 87-118, 2009. https://doi.org/10.1007/978-3-642-00286-1
6. Mannazzu I, Domizio P, Carboni G, Zara S, Zara G, Comitini F, Budroni M, Ciani M. Yeast killer toxins: From ecological significance to application. Critical reviews in biotechnology. 2019 Jul 4;39(5):603-17. https://doi.org/10.1080/07388551.2019.1601679
7. Douglass AP, Offei B, Braun-Galleani S, Coughlan AY, Martos AA, Ortiz-Merino RA, Byrne KP, Wolfe KH. Population genomics shows no distinction between pathogenic Candida krusei and environmental *Pichia kudriavzevii*: one species, four names. PLoS pathogens. 2018 Jul 19;14(7):e1007138. https://doi.org/10.1371/journal.ppat.1007138
8. de Ullivarri MF, Mendoza LM, Raya RR. Characterization of the killer toxin KTCf20 from *Wickerhamomyces anomalus*, a potential biocontrol agent against wine spoilage yeasts. Biological control. 2018 Jun 1;121:223-8. https://doi.org/10.1016/j.biocontrol.2018.03.008
9. Delali KI, Chen O, Wang W, Yi L, Deng L, Zeng K. Evaluation of yeast isolates from kimchi with antagonistic activity against green mold in citrus and elucidating the action mechanisms of three yeast: *P. kudriavzevii*, *K. marxianus*, and *Y. lipolytica*. Postharvest Biology and Technology. 2021 Jun 1;176:111495. https://doi.org/10.1016/j.postharvbio.2021.111495
10. Bajaj BK, Raina S, Singh S. Killer toxin from a novel killer yeast *Pichia kudriavzevii* RY55 with idiosyncratic antibacterial activity. Journal of basic microbiology. 2013 Aug;53(8):645-56. https://doi.org/10.1002/jobm.201200187
11. Utama GL, Kurniawan MO, Natiqoh N, Balia RL. Species identification of stress resistance yeasts isolated from banana waste for ethanol production. InIOP Conference Series: Earth and Environmental Science 2019 Jul 1 (Vol. 306, No. 1, p. 012021). IOP Publishing. https://doi.org/10.1088/1755-1315/306/1/012021
12. Utama GL, Rahmah SA, Kayaputri IL, Balia RL. Antibacterial activities of indigenous yeasts isolated from pomegranate peels (*Punica granatum* L.). Journal of Advanced Pharmaceutical Technology and Research. 2022 Jan 1;13(1):56-60. https://doi.org/10.4103/japtr.japtr_86_21
13. Lata P, Kumari R, Sharma KB, Rangra S. In vitro evaluation of probiotic potential and enzymatic profiling of *Pichia kudriavzevii* Y33 isolated from traditional home-made mango pickle. Journal of Genetic Engineering and Biotechnology. 2022 Dec 1;20(1):132. https://doi.org/10.1186/s43141-022-00416-2
14. Magliani W, Conti S, Salati A, Vaccari S, Ravanetti L, Maffei DL, Polonelli L. Therapeutic potential of yeast killer toxin-like antibodies and mimotopes. FEMS Yeast Research. 2004 Oct 1;5 (1):11-8. https://doi.org/10.1016/j.femsyr.2004.06.010
15. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976 May 7;72(1-2):248-54. https://doi.org/10.1006/abio.1976.9999
16. Bajaj BK, Sharma S. Construction of killer industrial yeast *Saccharomyces cerevisiae* HAU-1 and its fermentation performance. Brazilian Journal of Microbiology. 2010;41:477-85. https://doi.org/10.1590/S1517-83822010000200030
17. Ragavan ML, Das N. Production and purification of killer toxin from probiotic yeasts and its effect on foodborne pathogens. Journal of microbiology, biotechnology and food sciences. 2020 Dec 1;10(3):350-3. https://doi.org/10.15414/jmbfs.2020.10.3.350-353
18. Abdulateef SA, Hussein MH, Al-Saffar AZ. In vitro cytotoxic and genotoxic of lipopolysaccharide isolated from klebsiella pneumoniae as1 on mcf-7 human breast tumor cell line. International Journal of. Drug Delivery Technology. 2021;11 (1):184-9.
19. Lewus CB, Kaiser AL, Montville TJ. Inhibition of food-borne bacterial pathogens by bacteriocins from lactic acid bacteria isolated from meat. Applied and Environmental Microbiology. 1991 Jun;57(6):1683-8. https://doi.org/10.1128/aem.57.6.1683-1688.1991
20. Cazella LN, Glamoclija J, Soković M, Gonçalves JE, Linde GA, Colauto NB, Gazim ZC. Antimicrobial activity of essential oil of *Baccharis dracunculifolia* DC (Asteraceae) aerial parts at flowering period. Frontiers in plant science. 2019 Jan 29;10:27. https://doi.org/10.3389/fpls.2019.00027
21. Singh AK, Prakash P, Achra A, Singh GP, Das A, Singh RK. Standardization and classification of in vitro biofilm formation by clinical isolates of *Staphylococcus aureus.* Journal of global infectious diseases. 2017 Jul 1;9(3):93-101. https://doi.org/10.4103/jgid.jgid_91_16

22. Shrivastava A, Pal M, Sharma RK. Pichia as yeast cell factory for production of industrially important bio-products: Current trends, challenges, and future prospects. Journal of Bioresources and Bioproducts. 2023 May 1;8(2):108-24. https://doi.org/10.1016/j.jobab.2023.01.007

23. Ciani M, Comitini F. Yeast interactions in multi-starter wine fermentation. Current Opinion in Food Science. 2015 Feb 1;1:1-6. https://doi.org/10.1016/j.cofs.2014.07.001

24. Moura VS, Pollettini FL, Ferraz LP, Mazzi MV, Kupper KC. Purification of a killer toxin from Aureobasidium pullulans for the biocontrol of phytopathogens. Journal of Basic Microbiology. 2021 Feb;61(2):77-87. https://doi.org/10.1002/jobm.202000164

25. Kulakovskaya E, Zvonarev A, Farofonova V. Characteristics of killer toxin of the yeast *Cryptococcus pinus*. Journal of Biosciences and Medicines. 2019 Mar 6;7(4):73-82. https://doi.org/10.4236/jbm.2019.74008

26. Jandera P. Comparison of various modes and phase systems for analytical HPLC: in Valkó, (Ed.), Handbook of Analytical Separations, Elsevier Science, pp. 1-91, 2020. https://doi.org/10.1016/B978-0-444-64070-3.00001-1

27. Alsoufi MA, Aziz RA. Use killer toxin extracted from bakery yeast for extending shelf life of fruits. Pakistan Journal of Biotechnology. 2017 Jan 25;14(1):23-7.

28. Bajaj BK, Singh S. Biology of killer yeast and technological implications. Yeast Diversity in Human Welfare. 2017:163-90. https://doi.org/10.1007/978-981-10-2621-8_7

29. Labbani FZ, Turchetti B, Bennamoun L, Dakhmouche S, Roberti R, Corazzi L, Meraihi Z, Buzzini P. A novel killer protein from Pichia kluyveri isolated from an Algerian soil: purification and characterization of its in vitro activity against food and beverage spoilage yeasts. Antonie Van Leeuwenhoek. 2015 Apr;107:961-70. https://doi.org/10.1007/s10482-015-0388-4

30. Younis G, Awad A, Dawod RE, Yousef NE. Antimicrobial activity of yeasts against some pathogenic bacteria. Veterinary world. 2017 Aug;10(8):979. https://doi.org/10.14202/vetworld.2017.979-983

31. Belda I, Ruiz J, Alonso A, Marquina D, Santos A. The biology of Pichia membranifaciens killer toxins. Toxins. 2017 Mar 23;9(4):112. https://doi.org/10.3390/toxins9040112

32. Giovati L, Ciociola T, De Simone T, Conti S, Magliani W. *Wickerhamomyces* yeast killer toxins' medical applications. Toxins. 2021 Sep 15;13(9):655. https://doi.org/10.3390/toxins13090655

33. Gier S, Schmitt MJ, Breinig F. Analysis of yeast killer toxin K1 precursor processing via site-directed mutagenesis: implications for toxicity and immunity. Msphere. 2020 Feb 26;5(1):10-128. https://doi.org/10.1128/mSphere.00979-19

34. Abu-Mejdad NM, Al-Badran AI, Al-Saadoon AH, Minati MH. A new report on gene expression of three killer toxin genes with antimicrobial activity of two killer toxins in Iraq. Bulletin of the National Research Centre. 2020 Dec;44:1-9. https://doi.org/10.1186/s42269-020-00418-5

35. Carboni G, Fancello F, Zara G, Zara S, Ruiu L, Marova I, Pinna G, Budroni M, Mannazzu I. Production of a lyophilized ready-to-use yeast killer toxin with possible applications in the wine and food industries. International journal of food microbiology. 2020 Dec 16;335:108883.0. https://doi.org/10.1016/j.ijfoodmicro.2020.108883

36. Robledo-Leal E, Rivera-Morales LG, Sangorrin MP, González GM, Ramos-Alfano G, Adame-Rodriguez JM, Alcocer-Gonzalez JM, Arechiga-Carvajal ET, Rodriguez-Padilla C. Identification and susceptibility of clinical isolates of Candida spp. to killer toxins. Brazilian journal of biology. 2018 Feb 1;78(04):742-9.

37. Giovati L. Candidacidal Activity of a Novel Killer Toxin from *Wickerhamomyces anomalus* against Fluconazole-Susceptible and-Resistant Strains, Toxins. 2018;10:492. https://doi.org/10.3390/toxins10020068

38. Ahmadi F, Najafpour GD, Mohammadi M. Production of bio-fungicide from sugarcane bagasse using *Pichia membranifaciens* yeast and its activity against post-harvest pathogenic fungi. Biointerface Res Appl Chem. 2021;11:10435-45. https://doi.org/10.33263/BRIAC113.1043510445

39. Agarbati A, Ciani M, Esin S, Agnolucci M, Marcheggiani F, Tiano L, Comitini F. Comparative Zymocidial Effect of Three Different Killer Toxins against *Brettanomyces bruxellensis* Spoilage Yeasts. International Journal of Molecular Sciences. 2023 Jan 9;24(2):1309. https://doi.org/10.3390/ijms24021309

40. Kowalska-Krochmal B, Dudek-Wicher R. The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. Pathogens. 2021 Feb 4;10(2):165. https://doi.org/10.3390/pathogens10020165

41. Abebe GM. The role of bacterial biofilm in antibiotic resistance and food contamination. International journal of microbiology. 2020;2020(1):1705814. https://doi.org/10.1155/2020/1705814

42. Gouka L, Raaijmakers JM, Cordovez V. Ecology and functional potential of phyllosphere yeasts. Trends in Plant Science. 2022;27(11):1109-23. https://doi.org/10.1016/j.tplants.2022.06.007

43. Calazans GF, SILVA JC, Delabeneta MF, Paris AP, Yassuda Filho P, Auler ME, Menolli RA, Paula CR, Simão RD, Gandra RF. Antimicrobial activity of *Wickerhamomyces anomalus mycocins* against strains of *Staphylococcus aureus* isolated from meats. Food Science and Technology. 2020 Oct 9;41:388-94. https://doi.org/10.1590/fst.39319

44. Tan HW, Tay ST. Anti-Candida activity and biofilm inhibitory effects of secreted products of tropical environmental yeasts. Tropical Biomedicine 2011; 28(1):175–180.