

Isolation and Identification of Killer Yeasts from Various Food Sources and their Potential Antimicrobial Activity

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ABSTRACT

Background: Killer yeasts produce protein toxins that are lethal to sensitive yeast strains, providing them with a competitive advantage in microbial environments. Initially discovered in brewing, these yeasts have been isolated from various food sources, including dairy products, fruits, and plants. Their antimicrobial properties make them potential candidates for applications in food preservation, fermentation control, and biotechnological processes. **Objective:** This study aimed to isolate and identify killer yeasts from diverse Iraqi food sources and evaluate their antimicrobial activity using molecular and laboratory-based microbiological techniques. **Methods:** Yeast isolates were obtained from meat, fruits, milk, and yogurt samples collected from different regions of Iraq. The samples were cultured on Sabouraud Dextrose Agar (SDA), and the resulting isolates were microscopically examined. Molecular identification was performed using polymerase chain reaction (PCR) amplification and sequencing of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA). The isolates were cultivated in a killer toxin production medium and tested for killer activity against *Saccharomyces cerevisiae* using agar diffusion assays. **Results:** A total of 16 yeast isolates were successfully obtained and identified by ITS sequencing. The identified species included *Candida metapsilosis*, *Lodderomyces elongisporus*, *Rhodotorula mucilaginosa*, *Hanseniaspora uvarum*, *Pichia kudriavzevii*, *Rhodotorula glutinis*, and *Naganishia diffluens*. All isolates demonstrated killer activity, with *Pichia kudriavzevii* exhibiting the highest toxin production level at 15 AU/mL. **Conclusion:** This study highlights the widespread occurrence of killer yeasts in various Iraqi food sources. The identification and characterization of these yeasts provide valuable insights into their potential applications in food safety, biopreservation, and microbial fermentation modulation. Further investigations into their antimicrobial mechanisms and interactions with pathogenic microorganisms could contribute to novel strategies in both food microbiology and clinical microbiology.

Keywords: Killer Toxin, Yeast, *Pichia kudriavzevii*, Sabouraud Dextrose Agar

INTRODUCTION

Yeasts are unicellular eukaryotic fungi that can be found in a wide range of environmental niches [1]. In the context of food microbiology, yeasts play important roles in the fermentation of foods and beverages [2]. Certain species and strains of yeasts have also been found to produce and secrete protein toxins that are lethal to other sensitive yeasts and fungi [3]. These killer yeast strains were first discovered in the brewing industry, where they exhibited inhibition of other yeasts during fermentation. Since then, killer yeasts have been isolated from other habitats including dairy products [4], fruits [5], nectars [6] and meats [7]. The anti-yeast toxins are encoded by virus associated double-stranded RNA in the cytoplasm, imparting a competitive advantage to the killer strains over sensitive species [8]. At least four distinct killer toxin types (K1, K2, K28, Klus) have been identified and characterized based on their killing profiles against other yeasts [9]. The toxins are commonly proteinaceous, acting as ionophores that disrupt membrane integrity and cause leakiness in sensitive cells [10]. Killer activity has been observed in yeast genera including *Saccharomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Kluyveromyces*, *Pichia* and *Zygosaccharomyces* [11, 12]. However, the distribution and activity can vary substantially between strains. A multitude of factors can modulate killer yeast phenotype and toxin production, including temperature, pH, sodium chloride levels, glucose depletion, nitrogen sources and divalent cations [13, 14]. While killer yeasts have been isolated from various foods and beverages, few studies have conducted targeted screening across diverse food sources. Many foods material harbor complex yeast microbiota, providing niches for interaction and competition between killer,

sensitive and neutral strains [15]. Surveying for killer yeasts across different foods could reveal prevalence patterns based on substrate, pH, nutrients or microbial ecology. This study aims to isolate killer yeasts from a wide selection of varied foods including fruits, dairy, and meats. We hypothesize that killer activity will be commonly present but unevenly distributed across food sources. Characterizing killer yeasts from foods will provide insights into toxin diversity, microbial interactions, and potential applications for biopreservation.

MATERIALS AND METHODS

Sample Collection

Three different types of samples (meat, fruits, and dairy products) were collected in order to isolate various yeast species. A total of 20 raw meat samples were collected from butcher shops located in different areas in Baghdad, Iraq (Al-Mansour, Karada, Al-Bayaa, Al-Qadisiya). Approximately, 250 g of raw meat was collected. Raw meat samples were kept in sterilized plastic container immersed with ice for sample preservation. In the laboratory, 1 g of raw meat was suspended in a 100 mL flask containing 9 mL peptone water. The suspension was mixed thoroughly, and serial dilutions were prepared and plated onto Sabouraud Dextrose Agar plates supplemented with chloramphenicol (0.1 g/L) by the spreading method. The plates were incubated at 30°C for 48 h. Different types of partially rotten fruits were selected for the isolation of yeast species (Apple, Orange, Pear, Blueberry, Grape, and Strawberry). The fruits were selected from the local markets. Fruits were carefully washed with distilled water and allowed to dry at room

temperature. A small portion of the rotten fruit (1 g) was cut into slices using a sterile scalpel blade and vigorously vortexed in 9 mL of sterile peptone water. Aliquots of 100 µL of the suspension at desired dilution was then plated onto Sabouraud Dextrose Agar plates supplemented with chloramphenicol (0.1 g/L) by spreading method. The plates were incubated at 30°C for 48 h [16]. With respect to dairy food, samples of gout milk (5 samples) and local yogurt (5 samples) were collected in countryside areas around Baghdad. An aliquot of 1 mL of sample was added to 9 mL of peptone water under aseptic conditions. The suspension was mixed thoroughly and aliquots of 100 µL of the suspension at desired dilution was then plated onto Sabouraud Dextrose Agar plates supplemented with chloramphenicol (0.1 g/L) by spreading method. The plates were incubated at 30°C for 48 h. The suspected colonies were subjected to further identification.

Identification and Characterization of Yeast

Morphological Characterization

Based on colony formation and growth on solid media, the morphological characteristics of a selected yeast isolates were

investigated. Additionally, distinct texture characteristics, color elevation, colony surface, and edges were noted.

Microscopic Examination

Yeasts isolates grown in Sabouraud Dextrose Agar plates were further examined under microscope to determine yeast cell shape, budding and fission ability using lactophenol cotton blue staining protocol [17].

Molecular Characterization

Pure yeast isolates were diagnosed by molecular techniques involving DNA extraction using ABIopure™ Total DNA Extraction Kit (ABIopure, USA), ITS1 (5'TCCGTAGGTGAACCTGCGG'3) and ITS4 (5'TCCTCCGCTTATTGATATGC'3) amplification via polymerase chain reaction (Thermo Fisher Scientific, USA) according to the amplification program shown in Table (1), and finally DNA sequencing using ABI3730XL automated DNA sequences. The sequencing data were analyzed with the information in gene bank of the National Center for Biotechnology Information (NCBI) using Geneious software.

Table 1: PCR Amplification Program.

Step	Temp. (°C)	Time	No. of Cycles
Initial Denaturation	95	5 min	1
Denaturation	95	30 sec	
Annealing	55	30 sec	30
Extension	72	30 sec	
Final Extension	72	7 min	1
Hold	10	10	

Extraction of Killer Toxin

Killer toxin from isolated yeasts was extracted based on method described by [18]. Each yeast isolate was grown overnight in 100 mL of Yeast extract peptone dextrose (YEPD) broth in shaker incubator (180 rpm) at 30°C. The fresh cultures were used to inoculate 1 L of YEPD broth supplemented with 50 g/L of glycerol (pH: 5.0) 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 x g for 15 min at 4°C. The pellet was discarded, and the supernatant (crude killer toxin) was collected.

Detection of Killer Toxin Activity

The biological activity of extracted crude killer toxin was estimated using well plate assay against standard *Saccharomyces cerevisiae* strain. The crude toxin extracted from each yeast isolates 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 zone of inhibition was measured in mm. Killer activity was expressed in terms of arbitrary units (AU) multiply by the inverse of specified dilution (AU/mL). One AU was defined as the amount of killer toxin preparation which produces an inhibition zone of 10 mm [19].

Results and Discussion

A total of 36 samples were collected from different sources for isolation and identification of different types of yeasts. Samples were characterized as follows: 20 meat samples, 6 fruit (Apple, Orange, Pear, Blueberry, Grape, and Strawberry) samples, 5 gout milk samples and 5 local yogurt samples. All samples were processed in the lab and

cultured onto Sabouraud Dextrose Agar plates and incubated for 48 h at 30°C. After incubation, results showed that 7 isolated meat samples were confirmed successful growth of yeasts without bacterial contamination, while the other 13 meat samples either failed to grow or showed contaminated mixed cultures. Regarding fruit samples, only samples from orange, pear and apple were showed uniform colonies on Sabouraud Dextrose Agar plates. In addition, 6 isolates (3 from gout milk and 3 from local yogurt) were showed non-contaminated growth on Sabouraud Dextrose Agar plates. Sabouraud Dextrose Agar is a specialized growth medium designed to selectively isolate fungal organisms, particularly yeasts, from different specimens. It contains dextrose which provides carbon sources to support fungal growth. The acidic pH (typically 5.6) helps suppress bacteria that may be present in the sample while still allowing yeasts and certain molds to grow. Additionally, Sabouraud Dextrose Agar contains antibiotics such as chloramphenicol, gentamicin or tetracycline which inhibit the growth of Gram-positive and Gram-negative bacteria [20]. The isolated yeasts from different sources were morphologically and microscopically examined depending on the colonial shape and form when re-cultured on the surface of Sabouraud Dextrose Agar plates, in addition to the microscopic appearance of yeast cells. The colonies of 5 yeast isolated from meat source were white to cream in color with glabrous and smooth edges. Under microscope these isolates were ellipsoid and/or spherical to elongate cell with multilateral budding blastoconidia. Such characteristics are most likely attributed to *Candida* and *Lodderomyces*. Colonies of *Candida* in Sabouraud Dextrose Agar are typically large, smooth, glossy, and cream-colored or white, while under microscope *Candida* appeared as budding yeasts with oval bicellate cells and narrow-based buds. Some species showed as chains of elongated yeast cells [21]. On the other hand, the genus *Lodderomyces* consists of yeast species closely related to *Candida* that

on Sabouraud Dextrose Agar, colonies appear cream-colored, smooth, and mucoid, similar to *Candida* species. Microscopically, *Lodderomyces* reveal oval yeasts cells reproducing by multilateral budding [22]. The other 2 isolates of yeast that isolated from meat samples exhibited a sticky orange to red colonies with smooth surface and neat edges on Sabouraud Dextrose Agar plates. Under a light microscope, the cells appeared as round to oval budding yeasts. These results are in concordance with prior investigation that identify this type of yeasts to *Rhodotorula*, which showed that this type of yeast form moist, mucoid colonies that appear pink, coral, or orange due to carotenoid pigments. Microscopically, *Rhodotorula* yeasts can be identified by their oval budding cells and lack of pseudohyphae formation [23].

Three yeast isolates from orange, pear and apple were successfully grown on Sabouraud Dextrose Agar plates. Morphologically, 2 isolates from orange and apple showed a waxy, creamy-white color colonies with smooth edges. The lemon shape of the cells with spheroidal to ovoidal appearance under microscopic observation. These characteristics suggest the identification of *Hanseniaspora*. *Hanseniaspora* is a yeast genus containing several species recognized as opportunistic pathogens capable of causing invasive infections. When grown on selective fungal culture media such as Sabouraud Dextrose Agar, *Hanseniaspora* colonies have a moist, mucous appearance, smooth texture, and off-white to creamy coloration. Microscopic morphology under high magnification reveals ovoidal yeast cells that reproduce by bipolar budding - a distinguishing feature of *Hanseniaspora*, with buds emerging from both poles of the cell [24]. Yeast isolated from pear showed a cream colored to yellowish colonies with smooth edges. Small cells with elongated to ovoid shape were the most unique features for this yeast which suggest identifying as *Pichia*. It's well known that *Pichia* form white to cream colored colonies that are typically smooth, moist and mucoid in texture, similar to other yeasts with oval shape under

microscope [25]. Regarding the yeasts isolated from gout milk (3 isolates), all isolates were morphologically displayed the characteristics of *Naganishia* with cream-colored to pale brown mucoid colonies, while under microscope the cells showed globose to ovoid budding yeast. These features were in agreement with Oliveira et al, that *Naganishia* shows oval budding yeast cells without hyphae formation and off-white to pale brown colonies [26]. Finally, the colonies that resulted from culturing local yogurt samples indicated that only 3 samples were exhibited growth on Sabouraud Dextrose Agar plates with a sticky orange to red colonies, smooth surface and neat edges. The cells of these yeast were round to oval budding cells with pink pigment under microscope. Such characteristics are belonging to the genus *Rhodotorula* [27]. The 16 yeast isolates that isolated from different food sources were subjected to species-level molecular characterization based on the identification results. The isolates were subjected to polymerase chain reaction, ITS rDNA sequencing and then examined for homology with additional dependable sequences stored in GenBank. Results in Fig. (1), indicate that molecular diagnosis of yeast isolates revealed the identification of 7 isolates at species level. Out of 7 isolates collected from meat samples and confirmed morphological and microscopical examination, only 3 isolates were identified as *Candida metapsilosis*, *Lodderomyces elongisporus* and *Rhodotorula mucilaginosa*, while the remaining isolates were failed to identify. Two isolates collected from orange and pear were identified as *Hanseniaspora uvarum* and *Pichia kudriavzevii*, respectively. The yeast isolated from apple failed to identify. Six isolates that showed yeast morphological and microscopical characteristics and collected from gout milk (3 isolates) and local yogurt (3 isolates) were subjected to molecular identification, however only one isolate from each milk type was identified, *Rhodotorula glutinis* from local yogurt and *Naganishia diffluens* from gout milk. The remaining isolates failed to discriminate at species level.

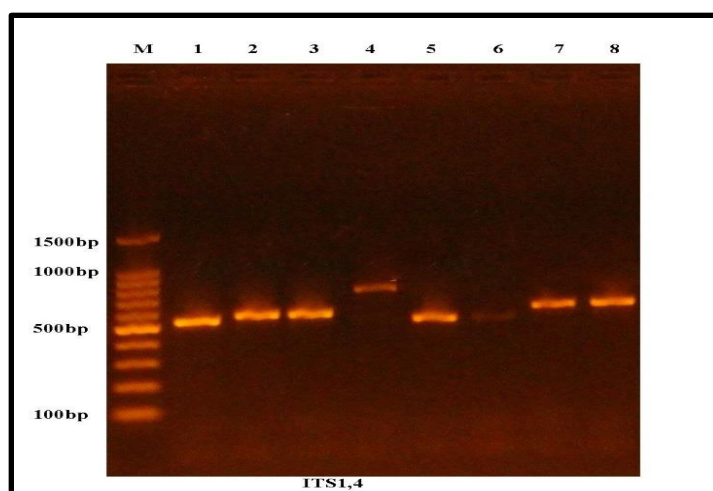


FIGURE 1: Amplification of *ITS* Gene of Unknown Yeast Species that were Fractionated On 1.5% Agarose Gel Electrophoresis Stained with Ethidium Bromide. M: 100 Bp Ladder Marker. Lanes 1-8 Resemble PCR Products.

Conventional microbiological techniques, particularly morphological and physiological methods, are frequently used to diagnose yeast infections; nevertheless, their sensitivity and specificity are insufficient to accurately distinguish between all yeast species. Clinical yeast isolates can now be more easily detected and identified thanks to nucleic acid-based techniques like PCR and DNA sequencing [28]. The internal transcribed spacer (ITS) region of ribosomal DNA

(rDNA) is a powerful target for molecular diagnosis of yeasts at the species level [29]. PCR amplification and sequencing of the ITS region provides several key advantages over conventional methods like microbiological culture and biochemical tests. First, ITS sequencing allows rapid and accurate identification of yeasts, often within 1-2 days, compared to days to weeks for culture-based methods. Additionally, it enables identification of species that are difficult or

impossible to culture [30]. The hypervariability of the ITS region also provides robust species-level resolution, permitting discrimination between closely related species with nearly identical phenotypes and biochemical properties [31]. By contrast, phenotypic methods frequently cannot differentiate cryptic or sibling species. Furthermore, ITS sequencing works directly from clinical samples, bypassing the need for isolation and culture [32].

The availability of curated ITS sequence databases covering most known yeast species also facilitates comparison and identification. Given these advantages, ITS rDNA analysis has become a standard tool for rapid, accurate molecular identification of clinically relevant yeasts [33]. The capability of isolated yeasts from different food sources for

production of killer toxin was estimated. *C. metapsilosis*, *L. elongisporus*, *R. mucilaginosa*, *H. uvarum*, *P. kudriavzevii*, *R. glutinis* and *N. diffluens* were cultivated for 72 hours in killer toxin production medium. After incubation, killer toxin activity for each isolate was estimated using well plate assay against standard *S. cerevisiae* strain. Results in Fig. (2), shows that all isolates were able to produce killer toxins in variant activities. *P. kudriavzevii* showed the higher killer toxin activity (15 AU/mL) compared with other isolates, while *N. diffluens* possessed the less killer toxin activity by 6.5 AU/mL. *C. metapsilosis*, *L. elongisporus*, *R. mucilaginosa* and *H. uvarum* exhibited killer activity above 10 AU/mL, while the activity of *R. glutinis* was below 10 AU/mL.

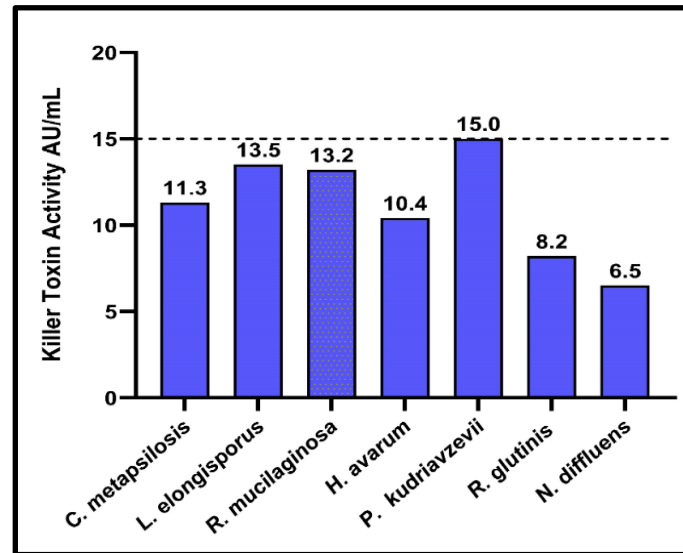


FIGURE 2: Killer Toxin Activity (in AU/mL) of Characterized Yeasts Isolated from Different Food Sources.

Killer toxins produced by yeasts such are an interesting area of study due to their potential applications as biocontrol agents against undesirable microorganisms. The results showed that *P. kudriavzevii* was the most potent isolate in producing killer toxin with higher activity. *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 [34]. Recent research has shown that different strains of *P. kudriavzevii* can vary significantly in their killer toxin production and activity. In comparative studies, some strains such as RY55 showed robust killer toxin activity, evidenced by large zones of growth inhibition against sensitive indicator strains, 32 AU/mL after 72 hrs incubation.

The diameter of the clear zone of *S. cerevisiae*, which denotes the presence of killer toxin produced by *P. kudriavzevii* isolate, revealed that *P. kudriavzevii* has the highest activity. The production of primary and secondary metabolites results in killing action [35]. 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* [36]. The factors that contribute to higher killer toxin activity in certain *P. kudriavzevii* strains are still being elucidated. Differences in the killer toxin 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.

Conclusion

In conclusion, this study successfully isolated and identified killer yeasts from varied food sources including meat, fruits, milk and yogurt. Molecular characterization revealed species identities including *P. kudriavzevii*, which displayed potent killer toxin activity of 15 AU/mL against *S. cerevisiae*. Isolating and screening yeasts from foods could reveal novel killer strains for applications in biopreservation and food fermentation. Further research should explore genetic factors influencing killer toxin production in potent isolates like *P. kudriavzevii*.

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