

Evaluation of the effectiveness of the yeast *Saccharomyces cerevisiae* in inhibiting of *Aspergillus piperis* and reducing ochratoxin A in orange fruits

Mohammed A. Zahir, Muhsen A.A. Muhsen

*Department of Plant Protection, College of Agriculture, University of Karbala, Karbala, Iraq Corresponding author e-mail: mohammed.zahir@s.uokerbala.edu.iq https://doi.org/ 10.59658/jkas.v12i3.4346

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Abstract

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This study aimed to evaluate the efficiency of the yeast Saccharomyces cerevisiae in inhibiting the growth of the fungus Aspergillus piperis isolated from orange fruits, and in reducing the resulting toxin Ochratoxin A (OTA) under laboratory and storage conditions. The results demonstrated the dominance of A. piperis in samples of local and imported orange fruits collected from Muthanna and Karbala governorates. Associated fungi were isolated and identified, and the most virulent was identified using a pathogenicity test. The results showed that the most virulent fungal isolate was A. piperis. Its identity was confirmed using molecular analysis of the ITS region sequence and registered in the NCBI database. This isolate also demonstrated a high capacity to produce OTA, reaching a concentration of 95.7 ng/g in sorghum media. When testing the bioactivity of yeast, different concentrations of S. cerevisiae showed remarkable effectiveness in inhibiting fungal growth, with the highest inhibition rate reaching 83.33% at the sixth dilution. In the storage experiment, treating the fruits with yeast contributed to a significant reduction in the OTA concentration from 125.33 to 59.70 ng/g, a significant difference. These results confirm the effectiveness of yeast as a promising bioagent for reducing post-harvest rot and toxic fungal contamination in orange fruits

Keywords: Ochratoxin A (OTA) · A. piperis · S. cerevisiae

Introduction

Oranges are considered one of the most important horticultural crops globally due to their nutritional and economic value. However, they are highly susceptible to post-harvest fungal infections, leading to financial losses ranging from 10% to 30% under normal conditions, and can reach more than 50% under improper storage conditions [1] .These losses are attributed to several pathogenic fungi such as *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus ochraceus*, *Aspergillus carbonarius And Aspergillus fumigatus* which are the most common in terms of frequency, presence and occurrence in many rotting fruits including orange [2] . Even less studied species such as *Aspergillus piperis* have recently been observed and the



environmental conditions prevailing in citrus orchards, marketing locations and storage areas play a significant role in determining the type of fungus that infects orange fruits [3], raising new environmental and health concerns [4]. The reliance on chemical pesticides (such as imidazalil and thiabendazole) has contributed to fungal resistance and the formation of harmful residues on fruits prompting the search for natural and sustainable alternatives [2]. Among the most prominent of these alternatives are antagonistic yeasts, which are easy to cultivate and effective in competing for space and food, as well as in secreting antagonistic compounds such as enzymes and volatile organic compounds [5] .Studies on citrus fruits, particularly oranges and lemons, indicate that yeast species such as Meyerozyma guilliermondii, Pseudozyma sp., and Saccharomyces cerevisiae (LCBG-49) are effective in inhibiting fungal growth by up to 95%, both in vitro and during storage [6]. These activities, yeasts have become a promising option for integrated postharvest disease management. However, efficacy studies against Aspergillus fungi associated with citrus, including A. piperis, are still limited, although there is preliminary evidence of their pathogenicity and fruit contamination [7].

This study aimed to evaluate the efficiency of the yeast *Saccharomyces cerevisiae* in inhibiting the growth of *A. piperis* isolated from orange fruits, and to reveal its ability to produce ochratoxin A, as well as to test the ability of the yeast to reduce this toxin from orange fruits under storage conditions.

Materials and Methods Sample Collection.

Three samples of local and imported orange fruits were collected from cold stores specialized in selling fruits and vegetables in the Muthanna and Karbala governorates. The study design was based on three replicates for each sample, with the weight of each replicate ranging between 2–3 kg. Each sample was placed in clean, sterile polyethylene bags, then transported directly to the laboratory and stored in a refrigerator (at 4°C) until testing.

Isolation and identification of fungi associated with orange fruits.

Contaminated fungi were isolated from the damaged fruits by taking pieces of fruit tissue after preparing the PDA culture medium, sterilized in an autoclave at 121°C and a pressure of 15 pounds square per inch for 20 minutes, with the antibiotic Chloramphenicol added to it at a rate of 250 mg per liter. After cultivating the fruit pieces, the plates were incubated at 25 ± 2 °C for seven days, then they were re-purified by taking a 0.5 cm diameter disc from the edge of the fungal colony and placing it in a Petri dish containing the PDA culture medium. The plates were incubated again in the incubator at 25 ± 2 °C for seven days. The fungal isolates were identified to the genus level according to the approved taxonomic keys [8]. After that, the fungal samples were purified in a sterile environment, and the samples were stored for testing.

The incidence and frequency of fungal isolates were calculated according to the following equations:





Occurrence % =
$$\frac{\text{Number of samples in which the fungus appeared}}{\text{Total number of samples}} x100$$
frequency % = $\frac{\text{Number of isolates of a single fungus}}{\text{Total number of fungal isolates in all samples}} x100$

Pathogenicity testing of fungal isolates associated with orange fruits.

Pathogenicity testing was performed on five fungal isolates selected from among the fungi isolated in this study from orange fruits. This was done to reduce their number and select the most virulent ones for use in subsequent field tests and experiments. The pathogenicity test was conducted in the refrigerated storage room at the College of Agriculture, University of Karbala. Healthy orange fruits were placed in metal trays lined with parchment paper, which served as the experimental unit. Each experimental unit contained three healthy orange fruits. The fruits were sterilized for 3 minutes in 1% NaOCl solution and rinsed in sterile distilled water (SDW). The fungal inoculum (fungal suspension) was prepared by growing the fungi on PDA medium for 7 days at 25 + 2°C. The fungal spores were then harvested by adding 10 ml of sterile distilled water to each plate on which the fungus was grown. A sterile glass rod was then passed over the surface of the colonies to facilitate the separation of spores from the conidiophores. The spore suspension was then collected, and its numbers were counted using a counting slide. The spore concentration of the fungal species was adjusted to 106 spores/ml. Artificial inoculation of orange fruits with fungal isolates was carried out using a single method consisting of superficial scratching of the peel without reaching the pulp or grains, followed by spraying with a conidia suspension of each fungal isolate at a concentration of 5×10^4 conidia/ml, at a rate of 1 ml per fruit. The fruits were distributed into three replicates, each containing three identical fruits. A control treatment was carried out using the same method, spraying the fruits with 1 ml of sterile distilled water (SDW) after scratching, with the same number of replicates. The fruits were stored in a storage room at 22 ± 2 °C. After 21 days, observations were recorded, and the infection results were analyzed to assess the efficiency of the fungal isolates in causing disease.

Preparation of Nutrient Yeast Dextrose Broth (NYDB) Medium and Activation of S. cerevisiae.

NYDB medium was prepared for the activation and growth of S. cerevisiae by dissolving 50 grams of the medium components in 1000 ml of distilled water. The solution was then sterilized using an autoclave at 121° C and a pressure of 1.5 kg/cm^2 for 20 minutes. After sterilization, the flasks were inoculated with a commercial yeast preparation (S. cerevisiae) and incubated at $25 \pm 2^{\circ}$ C for two days.



Molecular identification of the Aspergillus sp.

Fungal isolate FL3 was targeted based on its high virulence in orange fruit rot and its ability to produce the mycotoxin Ochratoxin A. The isolate was identified by DNA sequencing and compared with the genomes of global isolates. The isolate was identified by DNA sequencing. PCR products were sent to Macrogen in South Korea to determine the nucleotide sequence of the Internal Transcribed Spacer (ITS) gene region. After receiving the nucleotide sequences of the fungal isolates, The sequences were analyzed using the Basic Local Aligment Search Tool (BLAST) program to compare them with the data available in the National Center for Biotecnology Information (NCBI) in the electronic GenBank that belong to the identical fungal isolates that were identified globally [9]. The fungal isolates that did not match any of the nucleotide sequences were registered at NCBI. Nucleotide analyses were performed using MEGA X version 11 to analyze the isolates and draw a kinship tree between each of these isolates and similar isolates registered at NCBI. The phylogenetic tree of the type Neighbor joining was built from the molecular nucleotide sequence of the ITS region of each isolate [10].

Activation of the yeast S.cervisiae and testing its effectiveness in inhibiting the growth of the fungal isolate Aspergillus piperis

S.cervisiae yeast a traditional product of Russian origin was obtained from local markets and grown on Nutrient Yeast Dextrose Broth (NYDB) medium. The method involved adding 1 ml of yeast suspension grown on NYDB liquid medium for 3 days (from the 6th, 8th and 10th dilutions) to a Petri dish containing PSA culture medium and rotating the dish to spread the yeast suspension. Then, a 0.5 cm diameter disc of 7-day-old pathogenic fungal culture was placed in the center of each dish. Three dishes were used for each treatment and three dishes were left without yeast as a control. The dishes were incubated (25±2°C for 7 days), and the growth rate of pathogenic fungi and the percentage of inhibition were calculated to select the best fungal inhibitor concentration [11].

Yeast activation and testing of its efficiency in inhibiting the growth of *Aspergillus piperis*.

Commercial yeast (S. cerevisiae) was obtained from local markets (of Russian origin) and grown on liquid NYDB medium. For testing, 1 ml of yeast suspension grown for three days on NYDB medium (at dilutions of 10^{-6} , 10^{-8} , and 10^{-10}) was added to Petri dishes containing Potato Sucrose Agar (PSA) medium. The suspension was then distributed in a circular motion to ensure homogeneous dispersion. A 0.5 cm diameter fungal disc taken from a 7-day-old pure culture of A. piperis was placed in the center of each dish. Three dishes were used for each treatment, with three additional dishes designated as a control group without yeast addition. The plates were incubated at $25 \pm 2^{\circ}$ C for seven days, then the growth rate of the pathogenic fungus was measured and the percentage of inhibition was calculated, with the aim of determining the best inhibitory concentration for fungal growth [11].



Testing the efficiency of A. piperis isolate on the production of Ochratoxin A

The Aspergillus sp2 isolate was grown on sorghum medium for 21 days at $25\pm2^{\circ}\text{C}$ with periodic stirring to ensure homogeneous growth, in three replicates. After incubation, the grains were dried and crushed, and 10 g of each sample was taken for toxin extraction using a 1:9 methanol:KCl (4%) solution, shaken for 45 min. After 24 h, the extract was filtered and mixed with 30 ml of 30% ammonium sulfate, then filtered again. The extract was purified through a chromatography column filled with activated silica gel and anhydrous sodium sulfate. The extract was then added to the column, followed by washing with chloroform. The lower layer was separated using a separating funnel. The final product was filtered over anhydrous sodium sulfate, then the solvent was evaporated and the filtrate was stored at -20°C until analysis. Quantitative and qualitative analyses were performed using an HPLC equipped with a C18 column and a fluorescent detector (λ ex=333 nm, λ em=460 nm), with a mobile phase of acetonitrile/water/acetic acid (99:99:2) and a flow rate of 1 ml/min, according to the protocol of [12].

Evaluation of the efficiency of the yeast Saccharomyces cerevisiae in reducing ochratoxin A in orange fruits.

For the storage experiment, healthy orange fruits of similar size and free of mechanical damage or visible injuries were used. The fruits were distributed into three replicates, each containing six fruits. The fruits were treated with a Saccharomyces cerevisiae yeast suspension, in addition to artificial inoculation with a pathogenic fungal isolate producing ochratoxin A, previously identified in the study. The fruits were stored under refrigerated storage conditions (temperature, relative humidity) for 51 days. After the storage period, samples were collected, and chemical analysis was performed to detect ochratoxin A levels using a sequential step similar to that applied in the previous paragraph, to evaluate the efficiency of the yeast in reducing fungal and toxic contamination in the fruits.

Results and Discussion

Isolation and identification of fungi associated with orange fruits

The results of Table 1 showed the distribution of fungal isolates isolated from infected orange fruits, which were collected from different locations within the Muthanna and Karbala governorates. The total number of fungal isolates reached 177, of which Aspergillus piperis recorded the highest frequency with 72 isolates, followed by A. flavus with 68 isolates, indicating the widespread prevalence of these two species in the storage and marketing environments of oranges within the studied areas. The geographical distribution of the isolates showed that Al-Hussainiya district in Karbala governorate recorded the highest number of isolates with 78 isolates, followed by the city center with 54 isolates, and then Al-Khader district in Muthanna governorate with 45 isolates. This may be attributed to the variation in storage conditions, humidity levels, and infection levels in markets or warehouses between different regions. On the



other hand, other fungal species appeared in relatively more minor numbers, as Penicillium spp. were recorded with 22 isolates and Alternaria spp. 14 isolates were found, In contrast only one Rhizopus spp. was found in Al-Khader district, indicating its rarity among the fungal isolates in this study, or its influence on isolation conditions and the surrounding environment. The isolates were distributed among five fungal genera, with a clear dominance of the genus Aspergillus, of which sp1 and sp2 represented approximately 76.1% of the total isolates, highlighting its potential role in causing fungal spoilage of orange fruits. At the governorate level, Karbala Governorate recorded the most significant percentage of isolates (74.6%), compared to Muthanna Governorate (25.4%), reflecting environmental or administrative differences that may affect the intensity of infection and the spread of fungi, The results of this study, which recorded the presence of multiple Aspergillus species in orange fruits, are consistent with those reported by [13]. [14] noted the prominent role of Aspergillus species, especially A. niger and A. flavus, in causing postharvest rot in citrus fruits, with varying severity depending on the fungus species, cultivar, and environmental conditions. A previous study showed that postharvest fungi in citrus, such as Aspergillus niger and A. flavus, cause significant losses in quality and market value. Biological control using antagonistic organisms or plant extracts, including essential oils from Eucalyptus camaldulensis, Citrus aurantium, and C. sinensis, is a promising option as a safe and environmentally friendly alternative to chemical fungicides, as it achieved high growth inhibition rates for these fungi [15].

Table (1): The fungi isolated from orange fruits infected with fruit rot disease.

	The ranging	Al-Muthanna	Karbala		Total
Seq	Treatments	Al-Khidhir District	Al-Husseiniya District	Central site	number of fungal isolates
1	Aspergillus sp 1	29	21	18	68
2	Aspergillus sp 2	13	35	24	72
3	Penicillim sp	1	9	12	22
4	Alternaria sp	1	13	0	14
5	Rhizopus sp	1	0	0	1
To	tal number	45	78	54	177

The results (Table 2) also showed that isolates differed in their occurrence and frequency rates. Aspergillus sp1 and Aspergillus sp2 isolates outnumbered each other in frequency, recording the highest occurrence rates of 38.41% and 40.67%, respectively, with an occurrence rate of 100% for each, indicating their widespread



distribution and close association with infected fruits. Penicillium sp isolates followed with a frequency of 12.43% and a full occurrence rate of 100%, reflecting their potential role in fruit rot. Alternaria sp was found in only two-thirds of the samples (66.66%), with a relatively low frequency (7.91%), indicating a lower prevalence. In contrast, Rhizopus sp was the least frequent and appeared, with a frequency of 0.75% and only one-third of the samples, indicating a secondary or limited role in infection. These results reflect the diversity of the fungal community and the severity of infection, with Aspergillus species emerging as the fungi most associated with orange fruit rot, These results are consistent with a previous study showing that Aspergillus flavus and A. niger were the most frequently isolated fungi from rotting orange fruits. A. flavus was characterized by its high pathogenicity, causing rapid fruit decay. Its presence was also associated with aflatoxin contamination [16]. [17] also indicated in his study that the genus Aspergillus is one of the most important fungal causes of postharvest rot in agricultural crops, including citrus fruits, and was the most frequently isolated in infected samples. Some of these species were characterized by their high pathogenicity, leading to rapid fruit decay, in addition to their ability to produce mycotoxins within the tissues, which contributed to fruit contamination and reduced quality and market value.

Table (2): The percentage of occurrence and frequency of fungi isolated from infected orange fruits.

Seq	Treatments	Total number of fungal isolates	% occurrence	% frequency
1	Aspergillus sp 1	68	100%	38.418
2	Aspergillus sp 2	72	100%	40.677
3	Penicillim	22	100%	12.429
4	Alternaria	14	66.66	7.909
5	Rhizopus	1	33.33	0.567

Pathogenicity testing of the studied fungal isolates.

The results (Table 3) showed apparent variation in the infection percentage and severity of infection resulting from the different fungal isolates in the scarification treatment. The isolate *Aspergillus sp* 2 (KFL3) recorded the highest infection percentage, reaching 100%, and the highest infection severity, reaching 90%, indicating its superior pathogenicity compared to the other fungal isolates studied, demonstrating its high ability to cause infection and fruit rot. This isolate was followed by Aspergillus sp 1 (MATB), with an infection percentage of 66% and an infection severity of 25.67%, which is significantly lower than the previous isolate in terms of severity despite the high infection rate. The isolates *Penicillium sp* (KDT8) and *Alternaria sp* (KDT6) recorded relatively low infection percentages, reaching 20.67%



and 33%, respectively, with similar infection severity rates, reaching 32% and 26.67%. at the same time, the isolate *Rhizopus sp* (MAT9) had the lowest infection percentage, which reached 11.33%, and an infection severity of 25.67%. These results indicate that there are significant differences between the isolates in terms of causing infection. The LSD values of 1.41 for infection and 3.90 for infection severity confirm that the isolate KFL3 is the most virulent, and it is recommended to adopt it in subsequent experiments to evaluate the effectiveness of biological or chemical treatments in reducing infection and rotting of orange fruits, These results are consistent with what was indicated by [18], which stated that Aspergillus spp. were among the most prevalent fungal pathogens on citrus fruits, and were characterized by a high pathogenicity, as evidenced by the rapid development of rot symptoms and the wide area of their spread on the fruit surface. These fungi recorded high infection rates compared to other isolated fungi, with some isolates causing complete fruit decomposition within a short period (3-5 days) of inoculation. These results reflect the pivotal role of Aspergillus species in increasing the severity of infection and the risk of postharvest rot, especially given their association with the production of mycotoxins, which add an additional dimension to health and food risks. A previous study showed that Aspergillus niger was one of the most prominent fungi isolated from decaying orange fruits and was characterized by high pathogenicity with an infection rate of 100% and a high level of decay intensity, which confirms its important role as a major cause of post-harvest rot and its potential danger as a source of food contamination [19].

Table (3): The percentage of infection and percentage of infection severity in the pathogenicity test of the selected fungal isolates.

Seq.	Treatments	% infection	% Disease severity
1	Aspergillus sp 1(MATB)	66.00	25.67
2	Aspergillus sp 2 (KFL3)	100	90.00
3	Penicillim SP (KDT8)	20.67	32.00
4	Alternaria SP (KDT6)	33.00	26. 67
5	Rhizopus SP (MAT9)	11.33	25.67
	$L.S.D_{0.05}$	1.41	3.90

Nucleotide sequence analysis of an A. piperis isolate

The results of nucleotide sequence analysis of the SP2 Aspergillus fungus isolated from orange fruits, which was characterized by its high virulence, confirmed that the isolate belongs to the fungus Aspergillus piperis. The fungal isolate was registered at the National Center for Biotechnology Information (NCBI) under the special code pv769987.1. The molecular nucleotide sequences achieved the highest percentage of identity, ranging from 99.82 to 99.46%, with the ITS gene region when compared with the equivalent nucleotide sequences retrieved from GenBank at the National Center for Biotechnology Information (NCBI) using the BLAST program. Nucleotide analyses



were also conducted using the MEGA program to analyze the isolates and draw a kinship tree between this isolate and similar isolates registered at the NCBI Center, which was built from the molecular nucleotide sequence of the ITS region belonging to each of the isolates Figure 1.

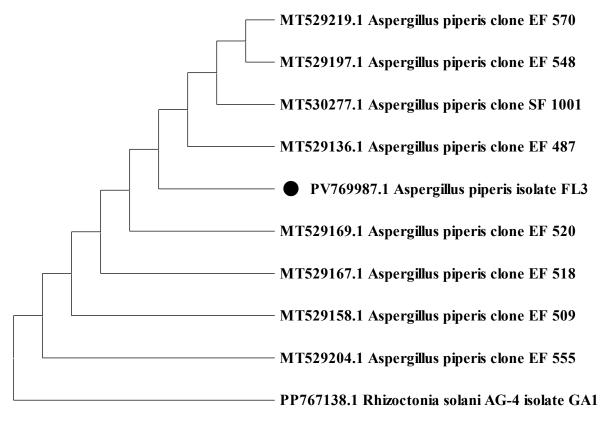


Figure (1): The phylogenetic tree of the fungus Aspergillus piperis isolate FL3 (circled in black) constructed from the ITS-rDNA sequences of the fungus and global strains of the same pathogen obtained from the GenBank data repository. Genetic distances were calculated using the neighbor-joining method.

The Role of Saccharomyces cerevisiae Yeast in Inhibiting and Preventing the Growth of Aspergillus Piperis

The results Table 4 showed that all *Saccharomyces cerevisiae* yeast treatments showed a significant effect on the growth of *Aspergillus piperis* compared to the control treatment of 0% inhibition. The highest inhibition rate was 83.33% at dilution 106, followed by dilutions 108 and 1010, with 68.44% and 62.96%, respectively, with significant differences according to the LSD value of 4.79. These results demonstrate that the inhibition effectiveness of yeast decreases with increasing dilution, indicating an inverse relationship between yeast concentration and its inhibitory capacity. A study showed that yeasts have a practical ability to inhibit the growth of *Aspergillus sp.* and reduce fruit rot disease in oranges, confirming their potential as a promising postharvest biocontrol agent [20]. These results are consistent with a previous study indicating that the use of high concentrations of S. cerevisiae led to a clear inhibition of Aspergillus



SP growth. The effect was more pronounced at 22°C compared to 25 and 32°C [21]. [22] indicated in a study on the mechanisms of yeast in inhibiting *Aspergillus flavus* growth and reducing mycotoxin production that it is due to several integrated mechanisms, including direct attachment to fungal hyphae and destruction of the cellular structure, secretion of the exochitinase enzyme capable of degrading the fungal cell wall, and production of active secondary metabolites such as 4-hydroxyphenethyl alcohol, which collectively contributed to achieving high rates of inhibition of growth, sporulation, and mycotoxin synthesis.

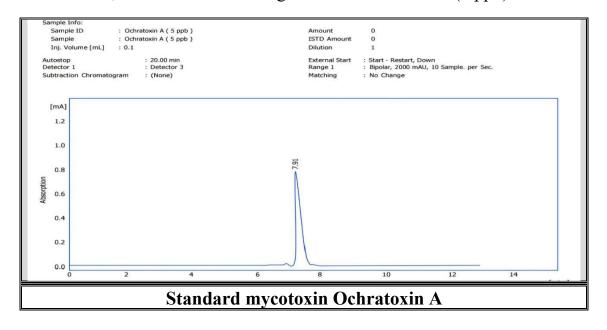
Table (4): The efficiency of several yeast dilutions in inhibiting the growth of

Aspergillus piperis in the laboratory.

Seq	Treatments	% inhibition.
1	Control	0.00
2	S. cerevisiae 10 ⁶	83.33
3	S. cerevisiae 10 ⁸	68.44
4	S. cerevisiae 10 ¹⁰	62.96
L.S.D _{0.05} 4.79		

Efficiency of the fungal isolate A. piperis in producing ochratoxin A in sorghum media.

Testing the ability of the fungal isolate Aspergillus piperis to produce ochratoxin A was demonstrated. As previously mentioned, the analysis conducted at the Ministry of Science and Technology demonstrated a high capacity for toxin production by the pathogenic fungal isolate (Figure 2). The average concentration of ochratoxin A replicates in the tested isolate was 95.7 nanograms per gram (ppb), compared to the control treatment, which recorded no significant concentration (0 ppb).





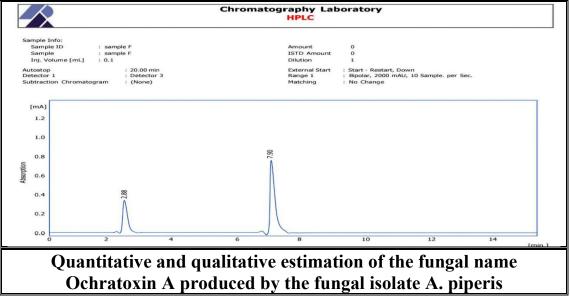


Figure (2): Shows the quantitative and qualitative estimation of the mycotoxin Ochratoxin A produced by the fungal isolate A. piperis in sorghum medium.

The role of the yeast S. cerevisiae in reducing the concentration of ochratoxin A in orange fruits.

The results of Table 5 and Figure 3 showed that treating orange fruits with the fungus Aspergillus piperis resulted in a significant increase in the concentration of ochratoxin A, reaching 125.33 ng/g, compared to the biological treatment using the yeast Saccharomyces cerevisiae, which significantly reduced the toxin level to 59.70 ng/g, with a significant difference at LSD = 5.22. This demonstrates the effectiveness of yeast in reducing mycotoxin production, enhancing its potential as a safe bioagent for combating fungal contaminants. [23] indicated that S. cerevisiae possesses a high capacity to reduce OTA by more than 60% when applied to contaminated fruits, attributing this to components of its cell wall, such as glucomannan, Previous studies have shown that Saccharomyces cerevisiae cell wall preparations, particularly aqueous or commercially available glucans, are capable of adsorbing more than 55% of ochratoxin A concentration, with maximum efficacy at near-neutral pH and a marked decrease in alkaline conditions, highlighting their potential role in reducing mycotoxin contamination [24]. [25] indicated that Saccharomyces cerevisiae yeast has a remarkable effectiveness in limiting the growth of toxin-producing fungi and reducing their levels. The volatile organic compounds produced by it were able to inhibit the growth of Aspergillus carbonarius by up to 100% and reduce the production of ochratoxin A by more than 99%, in addition to its effect in reducing the expression of genes associated with toxin synthesis. The yeast, alone or with Lactobacillus plantarum, achieved complete removal of aflatoxin B1 and ochratoxin A from food products, which supports its effectiveness as a biocontrol agent, consistent with the results of the current study in limiting the growth of Aspergillus and reducing its mycotoxicity.



Table (5): The efficiency of yeast in reducing Ochratoxin A in orange fruits.

Seq	Treatments	Inhibition percentage of Ochratoxin A
1	Aspergillus piperis	125.33
2	Aspergillus piperis + S. cerevisiae	59.70
L.S.D _{0.05} 5.22		

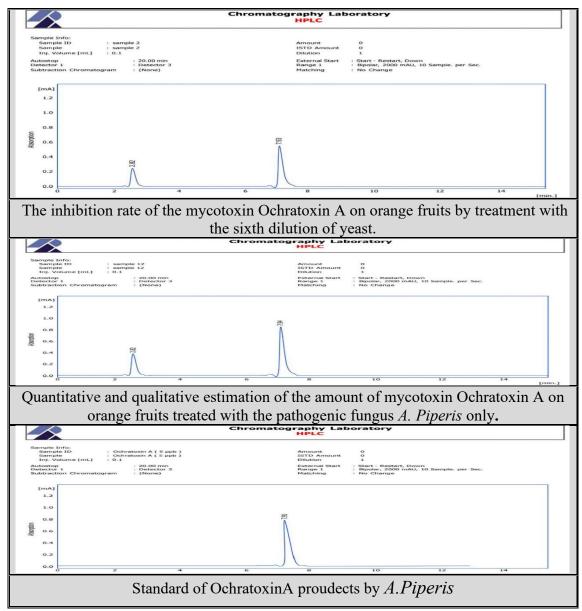


Figure (3): The role of yeast in reducing Ochratoxin A in orange fruits.

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