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Isolation of a Novel *Kluyveromyces marxianus* Strain QKM-4 and Evidence of Its Volatilome Production and Binding Potentialities in the Biocontrol of Toxigenic Fungi and Their Mycotoxins

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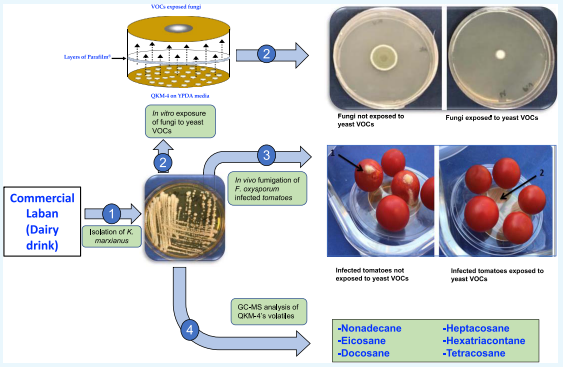
ABSTRACT: To overcome the economic losses associated with fungi and their toxic metabolites, environmentally safe and efficient approaches are needed. To this end, biological control using yeasts and safe bacterial strains and their products are being explored to replace synthetic fungicides. In the present study, the biocontrol effect of a yeast strain of *Kluyveromyces marxianus*, QKM-4, against the growth and mycotoxin synthesis potential of key toxigenic fungi was evaluated. *In vitro* assays were performed to find the application of yeast volatile organic compounds (VOCs) against fungal contamination on important agricultural commodities. The removal of ochratoxin A (OTA) and deoxynivalenol (DON) by living and heat-inactivated yeast cells was also explored. VOCs produced by strain QKM-4 were able to significantly limit the fungal growth of 17 fungal species belonging to genera *Aspergillus*, *Penicillium*, and *Fusarium*. Yeast VOCs were able to reduce OTA biosynthesis potential of *Penicillium verrucosum* and *Aspergillus carbonarius* by 99.6 and 98.7%, respectively. *In vivo* application of QKM-4 VOCs against *Fusarium oxysporum* and *A. carbonarius* infection on tomatoes and grapes, respectively, determined a complete inhibition of fungal spore germination. GC/MS-based analysis of yeast VOCs identified long-chain alkanes, including nonadecane, eicosane, docosane, heptacosane, hexatriacontane, and tetracosane. *In vitro* testing of the mycotoxin-binding potential of the living and heat-inactivated QKM-4 cells showed a reduction of OTA and DON up to 58 and 49%, respectively, from artificially contaminated buffers. Our findings clearly demonstrate the strong antifungal potential of *K. marxianus* QKM-4 and propose this strain as a strong candidate for application in agriculture to safeguard food and feed products.



1. INTRODUCTION

Mycotoxins, the secondary metabolites of toxigenic fungi, are mainly produced by *Aspergillus*, *Penicillium*, and *Fusarium*.[1,2] Ochratoxin A (OTA), a contaminant of several fruits, vegetables, and cereals, is produced by *Aspergillus carbonarius*, *Aspergillus ochraceus*, *Aspergillus westerdijkiae*, and some *Penicillium* spp. and is known to induce severe nephrotoxicity along with teratogenicity, hepatotoxicity, and immunosuppression.[3,4] Based on the strong evidence on the carcinogenic activity of OTA, the International Agency for Research on Cancer (IARC) has placed OTA in class 2B carcinogens.[5] Likewise, deoxynivalenol (DON), previously known as vomitoxin, is an important toxin of *Fusarium*, mainly *Fusarium culmorum* and *Fusarium graminearum*, and is a frequently reported contaminant of cereal grains.[6] The toxicological effects of DON range from mild gastrointestinal disturbances to severe neurological disorders.[6]

The preharvest fungal attack on cereal crops and/or on food and feed products during post-harvest storage are critical stages of mycotoxin accumulation and thus entry into the human food chain.[7] Several preharvest agricultural procedures including crop rotations, use of resistant crop varieties, and adaptation of precision agriculture technologies (use of satellites, autosprinklers, etc.) have shown promising role in limiting the fungal infection on sensitive crops. Other strategies such as use of chemical fungicides, although far more effective in minimizing fungal spread, are major drivers in the selection of resistant fungal populations, and their introduction into human food chain should be avoided as well.[8]

Biological control of phytopathogenic and toxigenic fungi in agriculture is a relatively recent and promising avenue in terms of environmental safety, acceptability, and applicability.[9−11]

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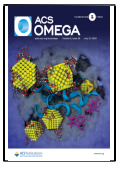

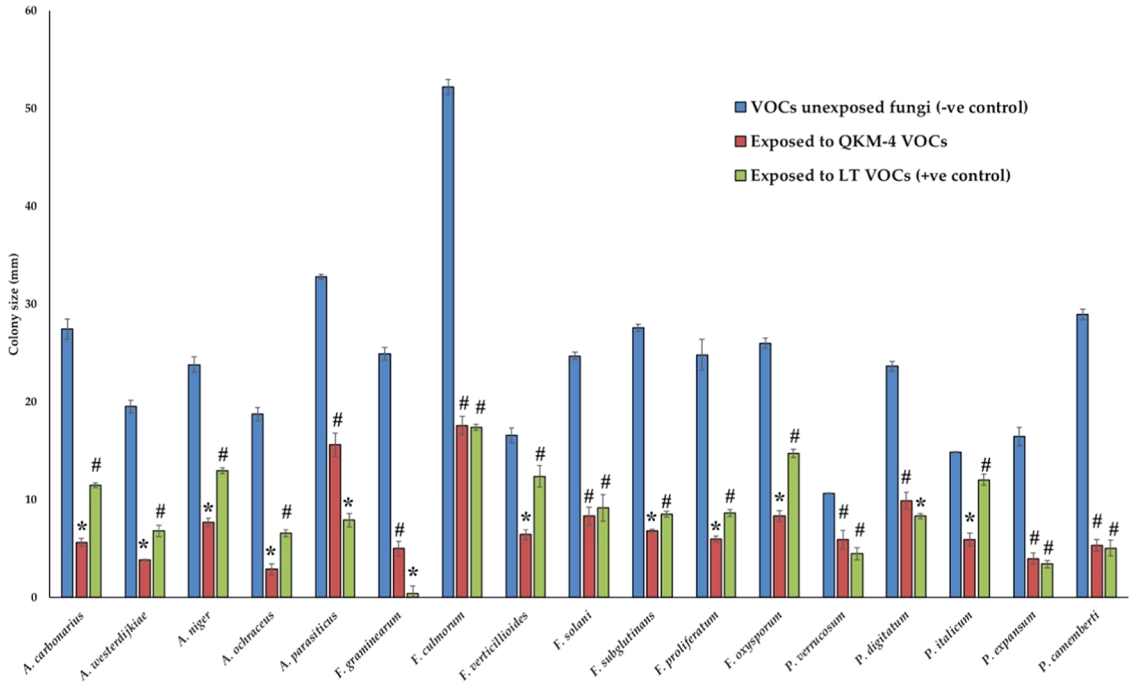

Figure 1. Spectrum of QKM-4 antifungal activity against 17 mycotoxigenic fungi. PDA plates were point-inoculated with the fungal spore suspension at their center and exposed to either VOCs produced by growing QKM-4 cells or *Lachancea thermotolerans* (LC) as a positive control. Colony diameters (mm) were measured at day 7 and compared with the negative control. Upon exposure to yeast VOCs, all of the tested fungal species showed significant reduction in the colony diameter. Each bar represents mean of nine values obtained from three independent experiments. The symbol hash (#) above the bars indicates statistically significant difference at $p \leq 0.05$, while the asterisk (*), at $p \leq 0.01$ as compared to VOC-unexposed fungi.

Several bacterial and yeast strains have been explored to inhibit the infection and toxigenic potentials of mycotoxin-producing fungi by either competing for nutrients and space, induction of resistance in crops against fungal diseases, and production of antimicrobial molecules (volatiles and diffusible compounds) or by direct antagonism with poorly understood mechanisms.[7,12,13]

The application of yeast strains as biological control agents against fungi has several advantages over the other microorganisms. Yeasts generally have simple growth requirements, can grow faster, even on dry surfaces, and are not known to produce toxic metabolites, like other filamentous molds.[14−17] Additionally, yeasts possess multiple biocontrol mechanisms, such as synthesis of active volatiles, secretion of enzymes, competition for nutrients and space, and resistance induction in plant hosts against phytopathogenic or toxigenic fungi.[18] The nature of antifungal volatile organic compounds (VOCs) produced by yeasts depends on several factors including the producing strain, composition of the media, and other environmental factors, but in general, these are blend of hydrocarbons, alcohols, phenols, aldehyde, ketones, and several other compounds.[19,20] Despite a huge list of effective yeast strains to be potentially used in agriculture settings, only a handful of them are registered and commercially available as plant protection products. This low number of developed yeast-based products is mainly due insufficiently explored mechanism of action, cost of registration, absence of commercial partners, and limited antagonistic potentials. *Candida oleophila* was the first yeast registered and commercialized for having potential to release antifungal enzymes such as proteases and VOCs. Similarly, *Aureobasidium*, *Cryptococcus*, *Metschnikowia*, and *Saccharomyces* (one member of each genus) have also been registered due to their antifungal VOCs or diffusible compounds.[20]

Many yeast strains have the potential to degrade/denature mycotoxin into nontoxic substances, while others have the capacity to bind different mycotoxins onto or within their cell wall, hence removing them from contaminated matrices.[21−23]

In the present study, an attempt was made to explore the antagonistic potential of *Kluyveromyces marxianus* QKM-4, isolated from an industrial laban (a traditional Arabic dairy drink), against a wide range of key toxigenic and phytopathogenic fungi. VOCs produced by this yeast were investigated on toxigenic *A. carbonarius* and *Penicillium verrucosum* fungi for their effects against ochratoxin A (OTA) biosynthesis. Further, to define a possible application of QKM-4's volatiles to preserve food products, tomatoes and grapes were exposed to growing yeast cells upon inoculation with two virulent strains of *Fusarium oxysporum* f.sp. lycopersici and *A. carbonarius*, respectively. Finally, the adsorption of ochratoxin A (OTA) and deoxynivalenol (DON) by living and heat-inactivated yeast cells was quantified in buffered solutions to record the mycotoxin-removal potential of QKM-4 from contaminated matrices. The selection of QKM-4 as a biocontrol antifungal agent was primarily based on its strong antifungal potential during initial screening experiments. Also, the isolation of QKM-4 from commercial food products (never reported for its adverse health effects), suggesting its possible nonpathogenic/toxigenic (although a thorough safety assessment is needed) activities, guided us to explore its biocontrol potential.

2. RESULTS AND DISCUSSION

2.1. Identification of the Antagonistic Yeast Strain and Its Spectrum of Antifungal Activities. The strain

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ACS Omega 2020, 5, 17637−17645

QKM-4, isolated from a local industrial laban (local dairy milk), was identified, with 100% similarity, as *K. marxianus* by comparing the sequence of its amplified rDNA intergenic sequence (NCBI accession number: MT158676) flanking the 5.8S rDNA, using NCBI BLAST software similarity analysis.

The antifungal activity of VOCs produced by QKM-4 yeast strains was initially explored against key mycotoxigenic or phytopathogenic fungi. In total, 17 fungal species were exposed to yeast VOCs by a coincubation method, where the yeast colonies had no direct contact with developing fungal colonies. All of the exposed fungal species showed a significant reduction in their colony size as compared to unexposed control fungi (Figure 1). Among the tested *Aspergillus* spp., *A. ochraceus* showed the highest sensitivity to QKM-4 volatiles with 85% reduction in the colony size, while the colony diameter reduction noted for other *Aspergillus* spp. was as follows: *A. westerdijkiae* (80%), *A. carbonarius* (79%), *Aspergillus niger* (68%), and *Aspergillus parasiticus* (52%). Upon exposure to QKM-4′s volatiles, *F. graminearum* showed 80% reduction in the colony diameter, followed by *Fusarium proliferatum* (76%), *Fusarium subglutinans* (75%), *F. culmorum* (66%), *Fusarium solani* (66%), and *Fusarium verticillioides* (61%). Likewise, among *Penicillium* species, *Penicillium camemberti* showed the highest reduction (82%), followed by *Paspalum expansum* (76%), *Penicillium italicum* (60%), *Pediomelum digitatum* (58%), and *P. verrucosum* (44%). Colony morphologies of selected fungi are shown in Figure 2.

Reduction in the fungal colony sizes due to bacterial and yeast volatiles has been reported in several studies.[24−28] The underlying antagonistic mechanisms of the yeasts on fungal growth are mainly associated with the nature of active compounds in yeast volatiles. Disruption of the endomembrane system of target fungi by yeast's caryolan-1-ol by altering sphingolipid synthesis was observed as the accepted mechanism of action.[29]

2.2. Effect of QKM-4 Volatiles on the OTA Synthesis by Toxigenic *A. carbonarius* and *P. verrucosum*. The exposure to QKM-4 volatiles resulted in significant reduction (31.4 ± 0.89 μg/kg) in the OTA synthesis by *A. carbonarius* as compared to unexposed cultures (2422.6 ± 4.04 μg/kg). Likewise, the synthesis of OTA by *P. verrucosum* was also significantly decreased (0.12 ± 0.03 μg/kg) by yeast's VOCs as compared to the unexposed fungus (31.1 ± 0.54 μg/kg; Figure 3).

Inhibition (or reduction) of mycotoxin biosynthetic potential by toxigenic fungi due to yeast VOCs has been previously reported.[24,25,30] Farbo et al.[25] showed that a low fermenting strain of *L. thermotolerans* produced 2-phenylethanol as the main antifungal volatile compound, which significantly altered the gene expression of two ochratoxigenic species of *Aspergillus* (*A. carbonarius* and *A. niger*). The downregulation of key genes involved in mycotoxin biosynthesis pathways, such as polyketide synthase (pks) and monooxygenase and nonribosomal peptide synthase, was observed. To the best of our knowledge, there is no information about *K. marxianus* antifungal VOCs, although Golbev[31] reported the synthesis of a nonvolatile fungicidal peptide (mycocin) by a *K. marxianus* isolate obtained from chal (dairy beverage).

2.3. *K. marxianus* QKM-4 VOCs Inhibit *F. oxysporum* Infection in Tomatoes and *A.carbonarius* Infection in Grapes. To explore the possible application of QKM-4 in preventing fungal infection on the agricultural commodities and extending their shelf life, tomatoes and grapes were artificially inoculated with fungal spores, followed by exposure to QKM-4 volatiles. The germination of fungal spores on the surface of tomatoes was completely inhibited in the presence of yeast VOCs (Figure 4A). On the other hand, in the absence of yeast volatiles, there was a visible fungal growth on 40% of the tomatoes damaging the skin and pulp of the fruit (Figure 4B).

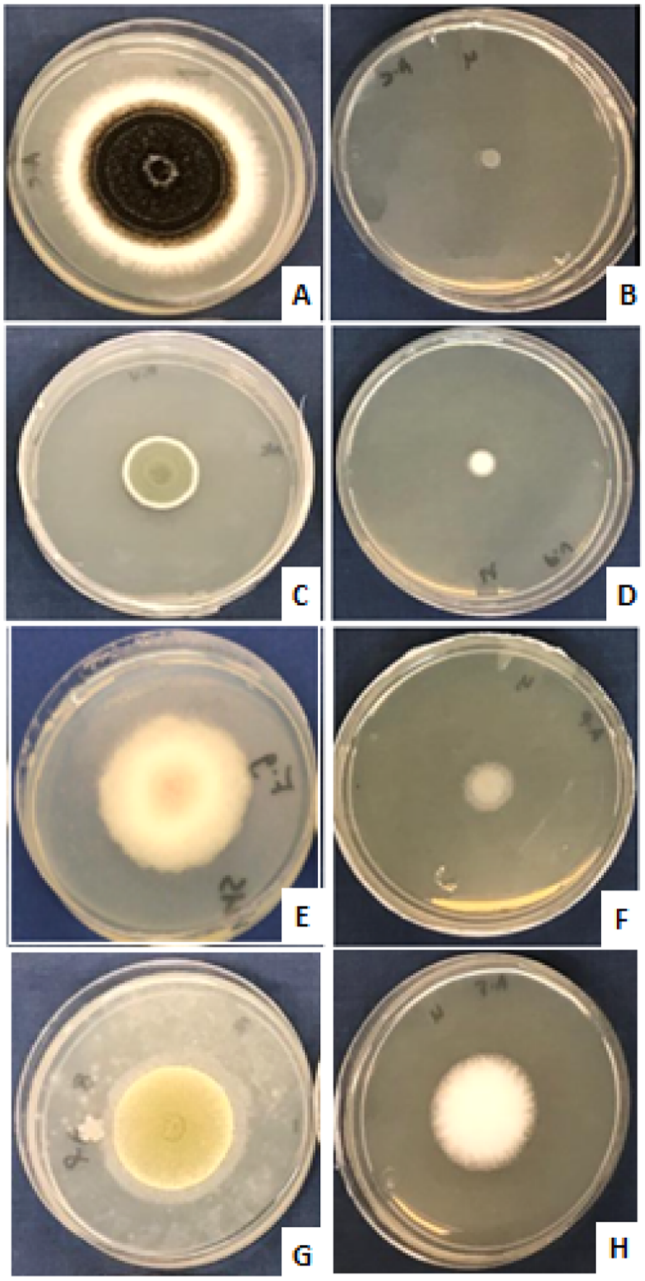

Figure 2. Colony morphology of *A. carbonarius*: (A) not exposed to yeast VOCs and (B) exposed to VOCs from QKM-4 cells (10^4/mL). Colony morphology of *P. verrucosum*: (C) not exposed to yeast VOCs and (D) exposed to VOCs from $\times 10^{-3}$ dilution of QKM-4 cells. Colony morphology of *F. graminearum*: (E) not exposed to VOCs of QKM-4 cells and (F) exposed to VOCs from (10^4 cells/mL) QKM-4 cells. Colony morphology of *A. parasiticus*: (G) not exposed to yeast VOCs and (H) exposed to VOCs from (10^4 cells/mL) QKM-4 cells. There is a visible effect of QKM-4 volatiles on the colony sizes and sporulation. Plates are representative of three independent experiments, with minimum of three replicates in each experiment.

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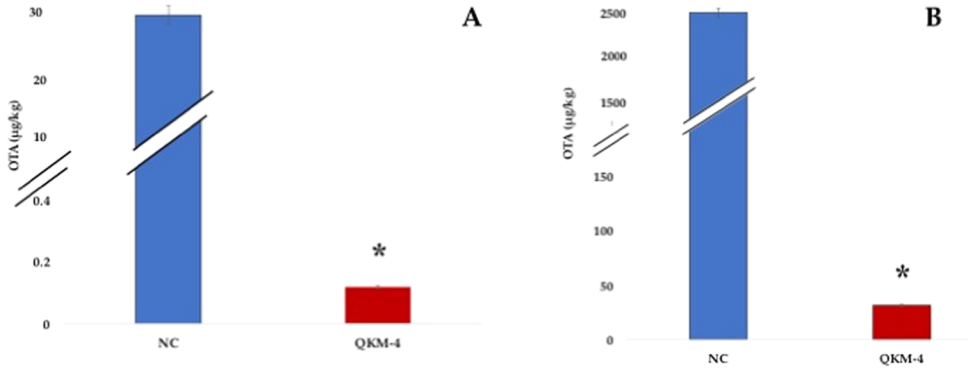

Figure 3. Effect of QKM-4 volatiles on the mycotoxin synthesis of toxigenic fungi. (A) *P. verrucosum* strain in a control environment synthesized 31.09 μg/kg OTA in media, while there was a significant reduction (99%) in OTA production upon exposure to QKM-4 volatiles. (B) Toxigenic potential of *A. carbonarius* was significantly reduced by yeast VOCs. Each bar represents the mean of nine values obtained from three independent experiments, followed by SD. The asterisks (*) above the bars represent statistically significant difference from control at $p \leq 0.05$.

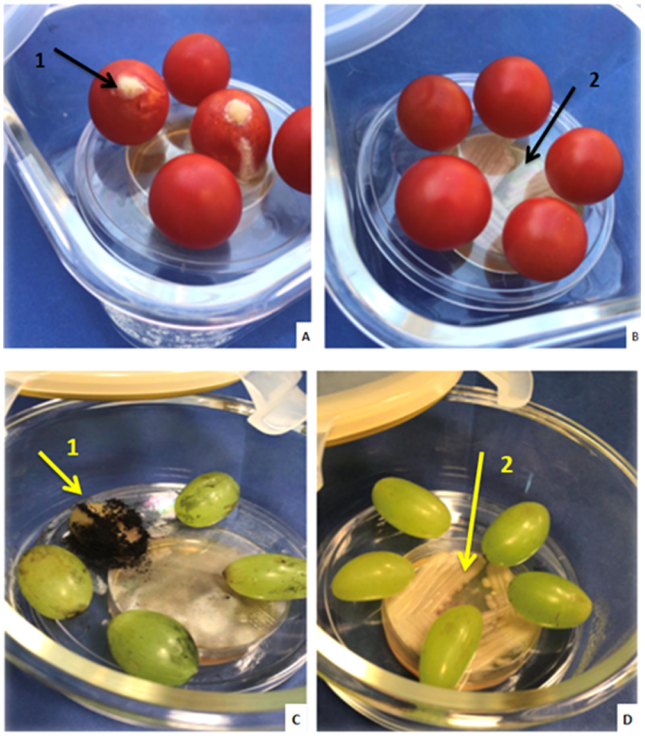

Figure 4. *In vivo* biocontrol of *F. oxysporum* and *A. carbonarius* contaminating the surface of tomatoes and grapes, respectively, by the application of QKM-4 VOCs. (A) Five tomatoes contaminated with *F. oxysporum*, which were not exposed to QKM-4 VOCs. Arrow 1 shows the surface of tomato fruits infected by *F. oxysporum* and covered by mycelium. (B) Five tomatoes infected with *F. oxysporum* and exposed to yeast's VOCs. Arrow 2 shows the plate of yeast extract peptone agar (YPDA) streaked with QKM-4 strain. (C) Five grape berries contaminated with *A. carbonarius*, which were not exposed to QKM-4 VOCs. Arrow 1 shows the surface of grape berries infected by *A. carbonarius* spores and covered by mycelium. (D) Five grape berries infected with *A. carbonarius* and exposed to yeast's VOCs. Arrow 2 shows the plate of YPDA streaked with yeast.

Likewise, in the presence of QKM-4 VOCs, *A. carbonarius* did not showed any growth on the surface of grape berries and they were fresh until the end of the experiment (day 10). All of the grape berries (100%) in the group without QKM-4's volatiles developed fungal infection (Figure 4C).

In line with the present study, *F. oxysporum* on the surface of artificially contaminated tomatoes was completely inhibited by the *L. thermotolerans* volatiles for up to 33 days.[24] In contrast to our findings, Fiori et al.[32] reported a significant inhibition of *A. carbonarius* infection on grape berries by living yeast cells, whereas no such effects were observed using yeast VOCs. Mewa-Ngongang et al.[33] in a similar study reported the protective role of two antagonistic yeasts (*Candida pyralidae*and *Pichia kluyveri*) against the growth of pathogenic fungi on apples and grapes. The inhibitory activity of yeast strains was characterized by a blend of VOCs having organic acids, alcohols, and esters. Likewise, the post-harvest infection of strawberries by *Botrytis cinerea* was significantly inhibited by VOCs produced by *Galactomyces candidum* JYC1146.[34] These findings support our hypothesis of a feasible application of yeast VOCs for the protection of food commodities against toxigenic microorganisms in a contained environment.

2.4. Composition of the Antifungal Volatile Blend Produced by QKM-4. The chemical nature of yeast's volatiles depends on several factors including the producing strains, composition of media, and other environmental factors. GC-based analysis has confirmed a list of compounds consisting of derivatives of hydrocarbons, alcohols, aldehydes, ketones, cyclohexanes, and benzenes. In the present study, headspace volatile analysis of QKM-4 molecules showed a blend of alkanes including nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36), and tetracosane (C24) (Table 1). The absence of these

Table 1. List of Compounds Detected in QKM-4 Volatiles

S. No	name of compounds	retention time (min)	peak area (%)
1	nonadecane	37.63	8.00
2	eicosane	39.20	8.52
3	docosane	40.77	10.91
4	heptacosane	42.17	10.95
5	hexatriacontane	43.58	10.59
6	tetracosane	44.94	9.59

compounds in the volatiles of negative control flasks (YPD media without yeast) confirmed that these compounds are synthesized by the yeast. The synthesis and antifungal activities of alkanes by biocontrol agents such as yeasts and bacteria have already been reported in several studies.[35−38] In line with the present study, eicosane in the volatiles of *Streptomyces strain*

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was found to be the major bioactive compound against *Rhizoctonia solani*infection of the tobacco leaf.[37] VOC analysis of the antifungal strain of *Bacillus atrophaeus* (strain CAB-1) highlighted the presence of several alkanes such as heptadecane, tetrapentacontane, eicosane, silane, and others.[35] Likewise, a broad-spectrum antimicrobial activity of *Pseudomonas putida* was associated with its potential to produce alkanes such as heneicosane and tetratriacontane along with other volatile compounds.[36] The presence of dodecane and eicosane as major bioactive volatiles produced by *Streptomyces* against microbial agents augment our findings.[38] The presence of already published work on the antifungal activities of alkanes refrained us to perform further testing with synthetic alkanes. In the recent studies, the antagonistic potential of microbial volatiles against plant pathogens as well as toxigenic fungi is being explored at a high pace.[12,35−38] However, this research domain is still in infancy in terms of elucidating precise mechanism of inhibition, largely due to potentially interacting blends of VOC's produced by microbial strains.

2.5. Removal of Mycotoxins by *K. marxianus* QKM-4 Living and Heat-Inactivated Cells. In the present study, living and heat-inactivated QKM-4 cells were used to test the potential to remove OTA and DON from a buffer solution at pH 5. The addition of living yeast cells resulted in the reduction of OTA by 24 and 11% from the supernatant of buffer solution artificially spiked with 0.9 and 1.8 μg/L, respectively (Figure 5). The addition of inactivated QKM-4

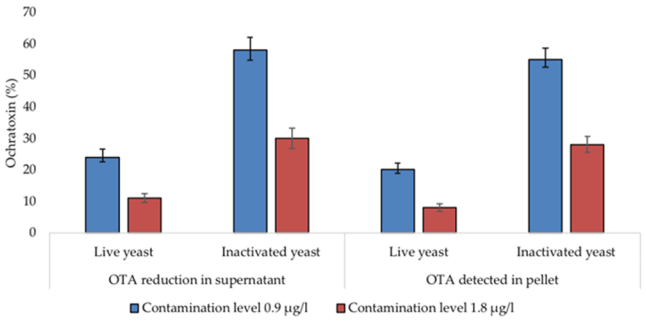

Figure 5. Absorption of OTA on living and heat-inactivated QKM-4 cells from a buffer solution at pH 5. Living or inactivated yeast cell were added to tubes having OTA at 0.9 or 1.8 μg/L and incubated for 1 h. Mycotoxin levels in the supernatant and pellet were tested by enzyme-linked immunosorbent assay (ELISA). Bars represent the mean of three independent experiments, with three replicates each time (n = 9).

cells to OTA-contaminated buffer solution showed a higher binding of 58 and 30% at contamination levels of 0.9 and 1.8 μg/L, respectively. The efficacy of inactivated yeast was significantly higher than that of the living QKM-4 cells, showing about 50% absorption of OTA (at the higher tested level). The amount of removed OTA from the supernatant was mainly detected in the pellet of the tube.

Post-harvest mycotoxin management of cereals and grains involves multiple strategies. Addition of mycotoxin-adsorbing substances to the contaminated substrates is one among several other approaches of decontamination. In commercial agriculture and livestock settings, materials such as clays (bentonites, zeolite, hydrated sodium calcium aluminosilicates, montmorillonite, and smectite), activated charcoals, yeast cell walls, and their derivatives are being used as mycotoxin binders. The choice of mycotoxin-adsorbing substance mainly depends on the spectrum of its activity against a range of target mycotoxins, high absorption potential, safety, irreversibility, and finally the proven *in vivo* studies. In fact, the spectrum of a mycotoxin binder depends on several factors such as the polarity of the target toxin, nature of binder, pH of the medium, and level of contamination. Several clays are very effective against aflatoxins but equally incompetent for other mycotoxins. In such situations, a blend of binders is the most appropriate choice to use.

The adsorption potential of QKM-4 was also tested against DON mycotoxin. At a low DON contamination level (40 μg/L), living and inactivated yeast cells showed 12 and 6.5% reduction in mycotoxin content in the supernatant. This reduction (or adsorption) reached 49 and 41% by living and inactivated yeast, respectively, when the buffer solution was spiked with a higher (80 μg/L) DON level. In all cases, the removed mycotoxin was detected in the pellet (Figure 6). In

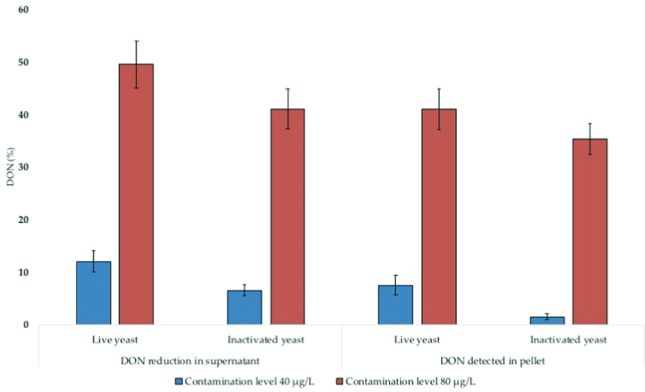

Figure 6. Adsorption of DON on live and inactivated yeast cells. QKM-4 at both physical states (live and inactivated) showed a significant adsorption of DON at its higher contamination level (80 μg/L) as compared to the low contamination level (40 μg/L). Each bar represents the mean of nine values, obtained from the three independent experiments.

line with the findings of the present study, adsorption of DON on the yeast cells has been reported by Zeidan et al.[24] and Devegowda et al.[39] However, in both these studies, the removal of DON by the live yeast cells was not more than 17%.

The adsorption of the mycotoxin on the microbial cell wall is a function of interacting toxin and cell surface functional groups such as polysaccharides, lipids, and proteins. These interactions may appear as reversable physical bindings, hydrogen binding, ionic exchange, or hydrophobic interactions, especially in case of OTA, where cell wall polysaccharides enable hydrophobic interactions with non-ionized OTA.[40] The high mycotoxin-binding efficacy noted in the present study by heat-inactivated yeast cells as compared to live yeast is likely the effect of heat-treatment-associated boosting of binding sites on yeast cell wall.[32]

3. CONCLUSIONS

The antifungal activity of *K. marxianus* QKM-4, isolated from laban (a traditional Arabic dairy drink), was tested against 17 key fungal species of agricultural importance. The VOCs produced by *K. marxianus* QKM-4 showed a significant inhibitory effect on the fungal growth as measured by the colony diameters. QKM-4 volatiles were further explored on *P. verrucosum* and *A. carbonarius* to find any effect on their OTA

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biosynthetic potential. Surprisingly, yeast VOCs were able to reduce OTA biosynthesis in *P. verrucosum*and in *A. carbonarius* by 99.6 and 98.7%, respectively. *In vivo* application of yeast VOCs against *F. oxysporum* and *A. carbonarius* infection on tomatoes and grape berries, respectively, showed a complete inhibition of fungal spore germination, suggesting a strong biopreservation potential. GC/MS-based analysis of yeast VOCs highlighted long-chain alkanes including nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36), and tetracosane (C24). Testing the mycotoxin-binding potential of the live and heat-inactivated *K. marxianus* QKM-4 cells showed the reduction of OTA and DON up to 58 and 49%, respectively. The findings of the present study clearly demonstrate a strong antifungal potential of *K. marxianus* QKM-4 for its possible application in the agriculture and food industry.

4. MATERIALS AND METHODS

4.1. Chemicals, Supplies, and Biological Strains. Yeast DNA was extracted using DNeasy plant mini kit (Qiagen, Valencia). ITS1/ITS4 primers were obtained from New England Biolab (Massachusetts). Acetate buffer (pH 5) used in the mycotoxin-binding part was prepared as described by Faucet-Marquis et al.[40] Mycotoxin standard solutions were obtained from Trilogy Analytical Laboratory (Washington, MO). ELISA kits for mycotoxin testing were purchased from R-Biopharm, Germany (RIDASCREEN). A microplate reader (Multiskan FC, Thermo Scientific, Waltham, MA) installed with Skanlt software (version 4.1. Thermo Scientific, MA, 2015) was used to obtain the absorbance of ELISA plates. The fungal strains used in this study were *A. carbonarius* (AC82), *A. niger* (AN8), *A. ochraceus* (CECT2948), *A. parasiticus* (AF82), *A. westerdijkiae* (AW82), *F. culmorum* (FCu11), *F. graminearum* (FGr14), *F. oxysporum*(Fox9), *F. solani*(FS05), *F. subglutinans* (FSuF12), *F. proliferatum* (FP08), *F. verticillioides* (FV04), *P. camemberti* (PC44), *P. expansum* (PE82), *P. digitatum* (PD43), *P. italicum* (PI48), and *P. verrucosum* (TF11).

4.2. Isolation and Molecular Identification of Local Yeast Strain. The yeast strain QKM-4 of *K. marxianus*, used in this study, was isolated from local Qatari dairy product laban, following the method described by Qvirist et al.[41] Briefly, QKM-4 was inoculated on yeast extract peptone agar (YPDA), prepared by adding yeast extract (10 g), peptone (20 g), dextrose (20 g), and agar (15 g) in 1 L of sterile distilled water. The isolate was purified by subculturing isolated colonies on YPDA plates. DNA extraction was performed using Qiagen plant DNA extraction kit. The yeast strain molecular identification was performed by the sequencing and NCBI BLAST software similarity analysis of a PCR fragment amplified (accession number MT158676) using the universal primers ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-′TCC TCC GCT TAT TGA TAT G-3′) corresponding to the intergenic sequences flanking the 5.8S ribosomal DNA as described by White et al.[42]

4.3. Investigation of Antagonistic Effects of QKM-4's Volatiles against Toxigenic Fungal Strains. To explore the antifungal activity of yeast, 17 fungal strains belonging to the genera *Aspergillus*, *Penicillium*, and *Fusarium* were exposed to QKM-4's volatiles in coculture experimental assays.[24] The yeast cells were spread on YPDA plates and incubated for 24 h at 28 °C. At the center of PDA plates, 20 μL of the spore suspension (10^4/mL) of test fungi was inoculated and sealed against the growing QKM-4 cell (Figure 7) or *L. thermotolerans* (as positive control). Sealed plates were incubated for 7 days at

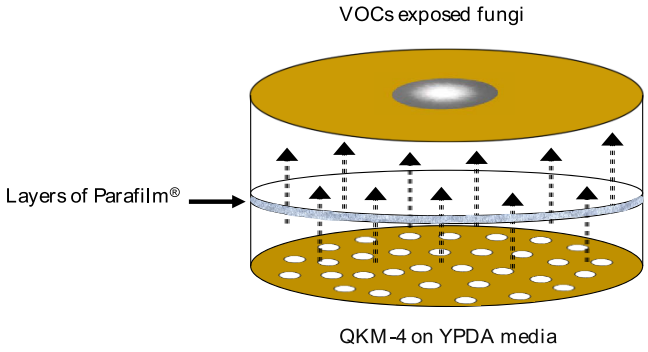

Figure 7. Diagrammatic representation of the coincubation experiment. In the lower plate, yeast cells were cultured on YPDA, while at the center of the PDA plate (above), fungal spores were inoculated. Two plates (after removing their covers) were sealed against each other by three layers of Parafilm along with an additional layer of sealing tape to avoid the leakage of volatiles. The VOCs emitted by QKM-4 (expressed by arrows) interact with germinating fungi to inhibit their growth.

28 °C to observe the effect of yeast VOCs on the fungal growth. In the control plates, fungal spores were incubated on PDA without exposing to QKM-4 volatiles and sealed against YPDA plates, in a similar manner as experimental plates. Colony diameters (mm) of VOCs exposed (T) and unexposed (C) were measured at day 7, and fungal growth inhibition percentage was calculated as

$$\text{fungal growth inhibition (\%)} = \frac{(C-T)}{C} \times 100 \quad (1)$$

where C is the colony size of control fungi (mm) and T is the colony size of VOC-exposed fungi (mm)

The experiment was repeated three times, with minimum of three plates of each fungi exposed to QKM-4 VOCs.

4.4. Effect of QKM-4's VOCs on Ochratoxin A (OTA) Synthesis by *A. carbonarius* and *P. verrucosum*. To investigate the effects of QKM-4's volatiles on the OTA synthesis potential of fungi, toxigenic strains of *A. carbonarius* and *P. verrucosum* were exposed to yeast as explained in Section 4.3 above. Based on the local prevalence and OTA accumulation profiles of *A. carbonarius* and *P. verrucosum* on fruits and vegetables, these fungi were chosen in further antagonistic studies, although the sensitivity to QKM-4's volatiles was not as stronger as seen for other fungi in their respective genus. At day 10 of coincubation, colonized plugs of media were removed with a sterilized cork-borer, weighed, and subjected to OTA extraction.[43] Before analysis, samples were dried using a vacuum drier (SpeedVac) and resuspended in 0.13 M sodium hydrogen carbonate buffer. Samples were diluted at 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} with 0.13 M $NaHCO_3$ buffer solution and analyzed by ELISA. Levels of OTA in yeast VOC-exposed fungi were compared with those in unexposed control fungi. With each test fungus, the experiment was repeated thrice with a minimum of three replicates each time.

4.5. *In Vivo* Investigation of QKM-4's VOCs against Artificial Fungal Contamination in Tomatoes and Grapes. *In vivo* application of the yeast VOCs was investigated against *F. oxysporum* infection on cherry tomato fruits and *A. carbonarius* infection on grapes. The selection of *F. oxysporum*

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and *A. carbonarius* was based on their natural affinity to infect tomatoes and grapes, respectively. Before infection with 5 μL of 10^6/mL fungal spore suspension, tomatoes and grapes were washed using tap water followed by disinfection with 70% alcohol and washed again in sterile dH_2O. Five fruits (tomatoes and grapes) were placed on a sterilized platform in each glass box. In the treatment set, a Petri dish (60 mm × 15 mm) of YPDA with a 48 h old culture of QKM-4 was kept opened below the tomato fruits to allow the dispersal of yeast VOCs inside the glass box. However, the uninfected surface-sterilized fruits represented a negative control. A positive control was represented by tomatoes and grapes infected with the fungal spore suspension and not exposed to any yeast volatiles. All of the boxes were tightly covered with their lids, tightly sealed with Parafilm, and incubated at 26 °C for 10 days. The fungal infection rate was monitored periodically and compared with control groups.

4.6. GC−MS-Based Analysis of QKM-4 Volatiles. Headspace volatiles (VOCs) produced by yeast were adsorbed on activated charcoal and eluted with dichloromethane as described by Ul-Hassan et al.[13] with minor modifications. Briefly, the yeast cells were incubated at 30 °C with continuous shaking (0.6*g* approximately) in 200 mL of yeast extract peptone dextrose broth (YPDB). After 48 h of incubation, air was passed in flasks to remove the VOC and bind them with activated charcoal. Trapped volatiles were eluted with dichloromethane and filtered using syringe filters (0.2 μm). VOC's trapping on charcoal and collection were performed three times from both QKM-4 flasks and flasks with YPDA alone (control). VOCs were analyzed by GC/MS fitted with an MSD detector. The samples were separated on a fused silica column (0.32 mm i.d., 30 m length, 0.5 μm film thickness). The column's temperature was set at 50 °C for 5 min and then raised to 250 °C by increment of 5 °C each minute. The injection port was operated in the splitless mode. The MS conditions (EI mode) used were as follows: ionization voltage, 70 eV; mass range, m/z 30−550; and ion source temperature,180 °C. The mass spectra of detected peaks were compared with NIST, EPA, and NIH libraries to know the nature of compounds.

4.7. *In Vitro* Mycotoxin-Binding Experiments. To investigate the mycotoxin-binding potential of the novel yeast strain, living and heat-inactivated yeast cells were coincubated with mycotoxins in a buffer solution at pH 5. The living and heat-killed yeast cells were obtained by already established methods in our lab.[24] For the binding experiments, 1.5 mL Eppendorf tubes were filled with 5 mg of yeast cell wall powder or 20 μL of living yeast culture and incubated for 5 min with either 990 or 970 μL of buffer, respectively. Mycotoxin solutions (10 μL), obtained by diluting mycotoxin standards, were added to reach a final concentration of 0.9 and 1.8 μg/L for OTA or 40 and 80 μg/L of DON in each tube. After incubation for 1 h at 37 °C with end-to-end shaking (0.55*g* approximately), tubes were centrifuged at the maximum speed for 5 min. The supernatant was separated from the pellet and analyzed by ELISA for mycotoxin content. The adsorption potential of the yeast was calculated in percentage. Experiments were repeated three times with additional duplicate analysis of each sample.

4.8. Statistical Analysis. Data obtained in growth inhibition experiments are presented in percentage reduction of the VOC-treated fungal colony diameter as compared to control colonies. Analysis of variance (ANOVA) was followed by Fisher's least significant difference (FLSD) test on the data of colony diameters. Data obtained from mycotoxin synthesis inhibition experiments and mycotoxin binding by yeast was analyzed by one-way ANOVA, followed by FLSD. SPSS statistical software (version 23, USA, 2017) was used.

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Notes

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