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ISOLATION AND CHARACTERIZATION OF KLUYVEROMYCES MARXIANUS FOR ANTIFUNGAL VOLATILOMES PRODUCTION

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ABSTRACT

Aim: In current study, volatile organic compounds released from yeast *Kluyveromyces marxianus* were investigated for their inhibitory activity against plant-pathogenic fungi *Aspergillus niger*.

Methods and Results: A screening revealed that volatile organic compounds from yeast *K. marxianus* AHT-4 remarkably inhibited the colony formation of plant-pathogenic fungi while also inhibiting the conidial germination of phytopathogenic *Aspergillus niger*. The volatile organic compounds were isolated from the culture filtrate of *K. marxianus* and different alkanes were identified as major compounds.

Conclusions: A mixer of isolated alkanes, nonadecanes, eicosane, docosane and tetracosane, was used to determine the antifungal activties.

Significance and Impact of the Study: This study provides significance antimicrobial activity of yeast volatile compounds which have novel biological control for inhibiting various fruit and vegetable rotting fungal diseases. It's provided a best alternate way to overcome the disadvantages associated with chemical fungicides used against phyto-pathogenic fungi.

INTRODUCTION

Secondary metabolites of some toxigenic fungi, the myotoxins are mainly produced by *Fusarium, Penicillium* and *Aspergillus* (Balendres *et al.* 2019). A neurotoxin called Ochratoxin A (OTA), a contaminant of some cereals, vegetables and fruits, is produced by *Aspergillus westerdijkiae, A carbonarius, A ochraceus,* and few *Penicillium* species. OTA have severe immunosuppresion, hepatotoxicity and nephrotoxicity (Zahoor-ul-Hassan *et al.* 2012; Heussner *et al.* 2015). Different fungal attacks on fruits and vegetables during post-harvest storage are critical stages of accumulation of myotoxins in feed products and thus entry into the human food chain (Goncalves *et al.* 2019). To improve the production of crops and other feed products, spread of phyto-pathogenic fungi is controlled. For this purpose different methods are applied such as chemical fungicides. But these chemicals effect the human food supply, so their use in agriculture is avoided (Chandler and D. 2018).

To overcome the disadvantages associated with chemical fungicides, biological control of plant pathogenic and toxic fungi is used. Some fungal species including yeast are used to control certain plant diseases caused by other fungi such as *Aspergilus* and *Fusarium* species. Bio-control of phytopathogenic and toxic fungi is promising having applicability, environmental safety and acceptability (Tsitsigiannis *et al.* 2012; Costa *et al.* 2013; Kohl *et al.* 2019). Some bacteria, fungi and yeast inhibit the growth of myotoxin producing fungi by secreting volatile organic compounds and other molecules such as certain enzymes (Tilocca *et al.* 2020). Use of yeast *Kluyveromyces marxianus* as a bio-control agent against plant pathogenic fungi is based on its non-pathogenic nature. *K. marxianus* is a GRAS (generally regarded as safe) organism which doesn't produce myotoxins like other filamentous fungi. Secondarily, yeast have simple requirements for their ideal growth (Fonseca *et al.* 2013; Alasmar *et al.* 2020). Yeast have different mechanisms as agents of biological control *i.e* these can inhibit growth of plant rotting species of fungi by producing volatile organic compounds, active secretions of different enzymes and also by resistance induction mechanism in plant cells against myotoxins or phyto-pathogenic fungi (Wisniewski *et al.* 2012). Yeast produce different kinds of antifungal volatile compounds, and the nature of these organic compounds depends on yeast strains and composition of culture media. Generally these volatile compounds include aldehydes, ketones, phenols and some hydrocarbons or their derivatives (Morath *et al.* 2012; Freimoser *et al.* 2019).

There is a large number of yeast strains available against plant rotting fungi. But only few of them are approved for commercial applications. The low number of developed yeast based products is due to limited antagonistic potentials, and insufficiently explored mechanism of action. *Candida oleophila* was the first registered and commercialized yeast against phyto-pathogenic fungi. It inhibits the growth of plant rotting fungi by secreting different enzymes such as proteases and yeast volatile organic compounds (VOCs). Likewise little other yeast such as *Sacchromyces*, *Cryptococcus* and *Metschnikowia* also produce antifungal volatile compounds (Freimoser *et al.* 2019). It is an intrinsic property of many yeast strains to neutralize or detoxify the myotoxins into harmless substances. Other yeasts bind myotoxins to their cell wall and remove them from contaminated environments (Farbo *et al.* 2016; Jouany *et al.* 2005; Fruhauf *et al.* 2012).

In this study, the antagonistic features of *Kluyveromyces marxianus* AHT-4 against other plant pathogenic fungi was explored. AHT-4, isolated from yogurt, produces a wide range of VOCs against *Aspergilus niger*. The VOCs produced by this fungus are more effective against orchatoxin A, a contaminant of fruits and vegetables. Furthermore, to explain the possible preservative applications of AHT-4′s volatile compounds, tomatoes and oranges were

exposed to growing *K. marxianus* yeast cells upon inoculation with virulent strains of *A. niger*. *K. Marxianus* AHT-4 was selected as a bio-control antifungal agent due to its strong antifungal applications against plant pathogenic fungus *A. niger.* Also the isolation of AHT-4 from the commercial feed products suggested that it is non-pathogenic.

Materials and methods

K. marxianus isolation and cultures for assay

This strain was isolated from yogurt by spreading few drops of curd on PDA plates. These plates were kept for incubation at 37˚C for 3-5 days. The grown yeast was further transferred on yeast peptone agar media (YPM). White opaque colonies of local yeast strain were grown on these YPM plates. These colonies were picked and further streaked on *Kluyveromyces* differential media plates (KDM). These plates were kept at 37˚C for 5 days. The desired yeast *K. marxianus* strain was isolated on the basis of morphological (color, structure and shape) characteristics (Sytar, O. *et al.* 2021). The yeast isolates were cultured and maintained in yeast maintenance medium called YPD medium (De Clercq, V. *et al*. 2021).

Aspergilus niger isolates preparation

To explore the anti-fungal activity of *K. marxianus*, fungal strains belonging to the genera, *Aspergillus niger* was exposed to *K. marxianus* volatilomes in co-culture experimental assays (Fahim, S. and K. Mahmoud. 2021). *A. niger* was inoculated on yeast peptone agar media plates and kept in incubator at 30˚C for 7 days. The growth of fungus was observed on yeast peptone media plate.

Determination of the antifungal activity of *K. marxianus* volatilomes on *Aspregilus niger*

Anti-pathogenic activity of yeast *K. marxianus* volatilomes was checked on *A. niger* cultures. After 7 days, a liquid culture media containing *K. marxianus* and its volatilomes was poured on one *A. niger* plate while the other *A. niger* plate was not exposed to the *K. marxianus* and used as control. Both the plates were kept it in incubator at 30˚C for next 7 days. After 7 days, colony size and growth of fungus was observed on both plates (Saleh, A.E. *et al.* 2021).

Determination of the antifungal activity of *K. marxianus* volatilomes on tomatoes and oranges

Fruits and vegetable rotting fungus *A. niger*'s spores and hyphae were placed on tomatoes and oranges. This fungus started bio-degradation of tomatoes and oranges. The antifungal activity of volatilomes produced from isolated *K. marxianus* AHT-4 strain was explored on tomatoes and oranges contaminated with *A. niger.*

Results

Assay to determine the anti-fungal activity of *K. marxianus* volatile compounds on *Aspregilus niger*

To check the antifungal activity of yeast volatile organic compounds, produced by *K. marxianus* strain, *A. niger* was inoculated on yeast peptone agar media plates and kept in incubator at 30˚C for 7 days. The growth of fungus was observed regularly, after 7 days, *K. marxianus* culture producing volatile organic compounds (VOCs) was poured on one of the *A. niger* plates (fig. 1 plate B) while the other *A. niger* plate (fig. 1 plate A) was not exposed to the *K. marxianus* and thus used as control. Both the plates were kept it in incubator at 30˚C for next 7 days. The colony size of *A. niger* was restricted on plate B while the normal growth was observed on plate A. It proved that the volatilomes produced by the *K. marxianus* inhibited the growth of *A. niger* on the culture plate B. Hence, a result deduced that the VOCs produced from *K. marxianus* possess an antifungal activity.

A

B

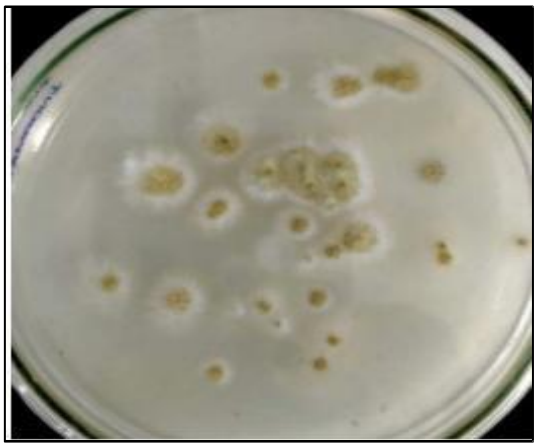

Fig. 1:
Plate A was not exposed to *K. marxianus* and its volatilomes while plate B was exposed to the *K. marxiauns* and its volatilomes.

Assay to determine the antifungal activity of *K. marxianus* volatile compounds on tomatoes and oranges

Preparation of tomatoes and oranges

Before infection with *A. niger* spore suspension, tomatoes were washed using tap water followed by disinfection with 70% alcohol and washed again with sterile dH_2O. The same treatment was given to oranges. Tomatoes and oranges were placed in separate sterilized boxes.

activity of *K. marxianus* volatilomes on tomatoes and oranges

Some spores and hyphae of *A. niger* were placed on the tomatoes and oranges. This fungus started rotting tomatoes and oranges. The antifungal activity of volatilomes produced from isolated *K. marxianus* AHT-4 strain was explored on tomatoes and oranges contaminated with *A. niger.*

Figure 2 set A, tomatoes contaminated with *A. niger* were exposed with the plates having no growth of isolated *K. marxianus*. While the set B had contaminated tomatoes and was exposed to three days old *K. marxianus* culture plates. Both the sets were sealed with parafilm and placed in the incubator for 15 days at 30˚C. The growth of *A. niger* was observed regularly, after 15 days, in set A fungus continued rotting tomatoes. While in set C growth of *A. niger* inhibited due to volatilomes produced by *K. marxianus* AHT-4.

Anti-fungal activity of *K. marxianus* AHT-4 volatilomes was also checked on oranges contaminated with *A. niger.* Orange fruit containing fungus was taken in an isolated and sterilized jar, and when fungus started bio-degradation, in set D orange rotted rapidly. Set E was exposed to *K. marxianus* AHT-4 and its VOCs. Both sets were placed in incubator at 30˚C. Normally, orange fruit decay in 3-5 days when exposed to the fungus, but when orange with the fungus was exposed to *K. marxianus* and its volatilomes, growth of *A. niger* was inhibited.

A

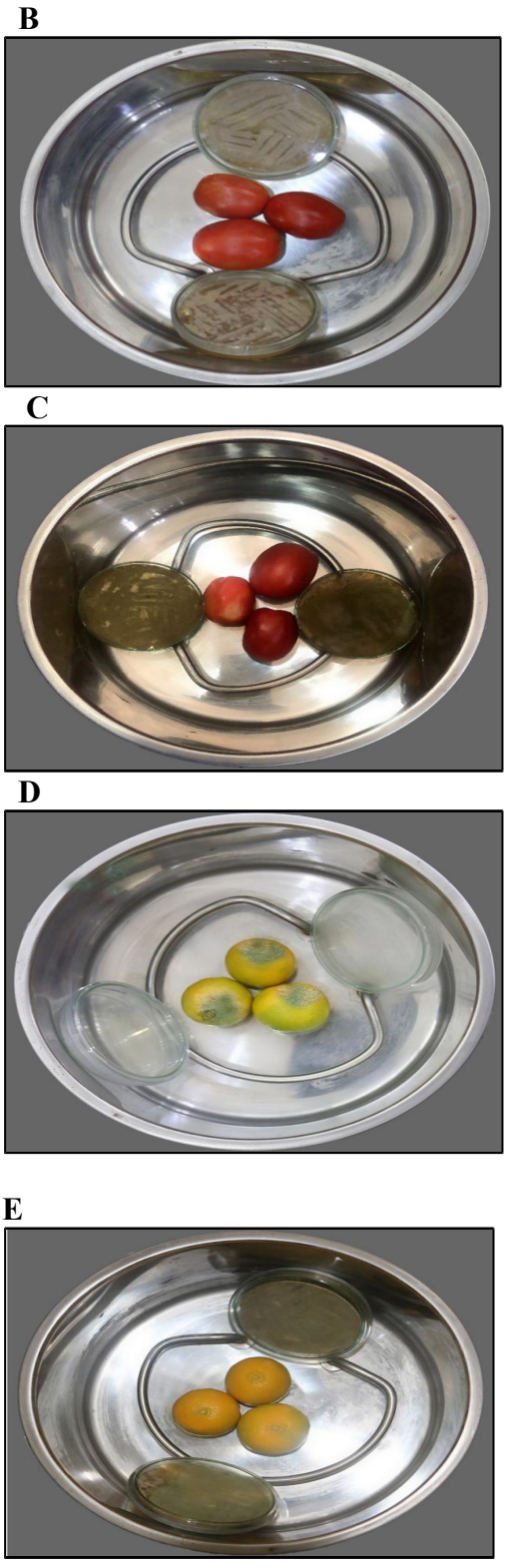

Fig. 2: Set A, tomatoes which were not exposed to *K. marxinus* AHT-4, in set B 3 days old AHT-4 culutre plates were placed and in set C tomatoes with plates of 15-day AHT-4 plates. Set D showed rapid bio-degradation of oranges, while in set E oranges were not decayed even after 15 days when exposed to AHT-4 and its VOCs.

Isolation of antifungal *K. marxianus* volatile compounds

K. marxianus produces a variety of different volatile organic compounds, the number and chemical nature of these volatilomes depend on the types of strains used, chemical composition of media and growth as will as ambient factors. A screening of these volatilomes by gas chromatography showed that different volatile compounds in pure and derivative forms were most abundant. Antifungal activities of these compounds were checked using colony test. 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 AHT-4 molecules showed a blend of alkanes including nonadecane (C19), eicosane (C20), docosane (C22), heptacosane (C27), hexatriacontane (C36), and tetracosane (C24).

Discussion

In the current study, the antimicrobial activity of volatilomes, produced by *K. marxianus* AHT-4, was screened. This fungus produces a variety of volatile organic compounds against phyto-pathogenic fungi. *K. marxianus'* VOCs inhibited growth of *Aspergilus niger*, a plant rotting fungus, in petri-plates as will as on contaminated tomatoes and oranges. A blend of molecules was isolated using gas chromatography and their antifungal activity was tested. Isomyl, isobutyle alcohols and alkanes such as nonadecanes, eicosane, docosane and tetracosane were detected during gas chromatographic analysis. *K. marxianus* volatilomes are further being utilized for the inhibition of the conidial germination of *A. niger* (Alasmar, R.M. 2020).

In the current study, the isolated yeast strains showed phenotypic growth that was similar to *K. marxianus* in colony and vegetative appearance that shared the same oval, spherical shape and budding as a

means of asexual reproduction (Powell, C.D; D.W. Kerruish. 2017). Yeasts growing on solid surface media form colonies with distinctive morphology, this is because individual species often form colonies of different size and appearance as reported by (Maryam *et al*. 2017; Sytar, O. *et al.* 2021). Yeast strains obtained were able to grow at 40˚C slightly higher than average temperature (25 ˚C). The survival of all the wild yeast strains at physiological temperature (room temperature) and subjected to mild heat shock temperature (35-40˚C) and then lethal heat shock temperature of 40-50˚C as reported by Bi, C.Y.T. *et al*. 2021; Li, G. *et al*. 2021). Prior heat treatment enables cells to survive after subsequent exposure to lethal temperature shocks (Obasi *et al*. 2014).

Different growth media have been designed for the differential isolation of yeasts such as *Candida parapsilosis*, *C. tropicalis*, *C. albicans*, and related species (ALyassree, H. and M. Alrufae Mohamed 2021). Using this selective and differential approach, isolation of *K. marxianus* from dairy food products was done. It is based on the enzyme called beta-galactosidase, which cleaves the chromogenic 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). A compound isopropyl β-D-1-thiogalactopyranoside (IPTG) stimulates the synthesis and increases the activity of beta-galactosidase (Saadat, Y.R., *et al.* 2020). In current study, six yeast strains, belonging to different species were isolated from curd and dairy products. Only the strains of *K. marxianus* produced shiny white colonies, thus allowing their differential detection. *K. marxianus* showed selectively growth by utilizing lactose, as carbon source at 40˚C temperature. Péter, G. *et al*. (2017) also showed growth curve at low temperature of 40˚C as a difference between *K. marxianus* and *K. lactis.* To improve the performance of KDM medium, tests with two supra-optimal incubation temperatures 40 ˚C and 42˚C were also performed. As a result, incubation at 40 or 42˚C could not be used as an additional selective or differential factor to discriminate between *K. marxianus* and *K. lactis,* both had the same color colonies on KDM. Variation in temperature treatment did not affect the colony color as an expression of b-galactosidase activity, but did effect growth (Madeira-Jr & Gombert. 2018).

Volatilomes produced by one of the six yeast strains, are effective against some fruit post-harvest pathogens. Mainly alcohols (ethyl alcohol, 3-methyl-1-butanol and phenyl ethyl alcohol) and esters (ethyl acetate and Isoamyl acetate) are reported to be main VOCs produced (Contarino *et al*. 2019). The anti-fungal activity of VOCs produced by *K. marxianus,* was initially explored against key myco-toxigenic or phyto-pathogenic fungus, *A. niger*. This fungus was exposed to *K. marxianus* VOCs by a co-incubation method, where the yeast colonies had no direct contact with developing fungal colonies. All of the exposed fungi showed a significant reduction in their colony size as compared to unexposed control fungi. Same studies showed that *Aspergillus ochraceus* has highest sensitivity to QKM-4 volatilomes with 85% reduction in the colony size, *A. westerdijkiae* had (80%), *A. carbonarius* (79%) and *Aspergillus niger* showed (68%) colony diameter reduction. Some other yeast species such as *Zygosaccharomyces, Debaryomyces and Williopsis* are also reported as bio-control agents against phyto-pathogenic fungi (Al-Qaysi SA *et al*. 2017; Liu SQ, Tsao M. 2010). Reduction in the fungal colony sizes due to bacterial and yeast volatiles has been reported in several studies. Yeast volatilomes have antagonistic mechanisms on fungal growth (Pretscher J *et al*. 2018).

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Author contributions

This is the work of M.phil student, which has been assisted by all authors under the supervision of Dr. Tayyaba Huma. For volatilome analysis, help was taken from Govt College University Faisalabad. Other authors helped in write up process.

Conflict of interest

The authors declare that they have no conflict of interest.

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