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RESEARCH

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Development of antagonistic yeasts for controlling black mold disease of onion

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Abstract

Background The present study aimed to examine the biocontrol efficacy of 28 yeast isolates against *Aspergillus niger*, the common pathogen of onion black mold disease. The antagonistic potential of yeast isolates against *A. niger* growth was investigated using a dual culture technique.

Results Five yeast isolates, including 8 and 11, showed the highest inhibition effect on the mycelial growth of *A. niger*. Molecular analysis using 16S RNA identified strains 8 (AUN-AH14) and 11 (AUN-AH23) as *Galactomyces geotrichum* (JQ713185.1) and *G. geotrichum* (DQ849321.1), respectively. The culture filtrates of AUN-AH14 and AUN-AH23 at percentage concentrations of 20, 40, 60, and 80 were tested against the growth of *A. niger*. As a result, the lowest dry weight of the pathogen was obtained with culture filtrates of the two strains at concentrations of 60 and 80%.. Treatment of onion bulbs with formulation of antagonistic yeast isolates AUN-AH14 and AUN-AH23 and their corresponding cultural filtrates at 80% concentration significantly reduced the severity of black mold disease relative to the controls.

Conclusions The most significant reduction in black mold severity was proved in yeast formulations of AUN-AH14 and AUN-AH23, followed by their cultural filtrates (CF-AUN-AH14 and CF-AUN-AH23). The shelf life of formulated yeast isolates began to gradually deteriorate after 5 months of storage at 4 °C.

Keywords Antagonistic yeasts, *Aspergillus niger*, Cultural filtrate, Formulations, Onion

Background

Onion (*Allium cepa* L.) is one of the most important commercial vegetable crops used all over the world (Gateri et al. 2018). Egypt is a leading onion producer in Africa, and onion is one of the key export crops in the country (Fangary and Adam 2020). Post-harvest storage and onion marketing is critical to ensuring consumers demands are met all year-round (Mohammed et al. 2015). However, 35–40% of post-harvest onions were lost due to disease during storage, leading to huge commercial losses (Yadav et al. 2015). *Aspergillus niger* is one of the most common pathogens infecting onion bulbs causing black mould disease and severe crop loss (Özer and Arın 2014). Mold infection can potentially initiate the accumulation of mycotoxins, which can pose serious human health risks, such as neurological problems, liver cancer, and lung cancer (Kumar et al. 2017).

Fungicides have been extensively used to control pathogen which pose negative effects to human health and the environment (Sallam et al. 2017). Thus, numerous trials on the use of biological control strategies as alternatives have been widely conducted (Wang et al. 2021). Antagonistic fungi (yeast isolates) have been identified as potential biocontrols of fungal diseases in fruits (Vega 2018). Yeasts have particular benefits over other biocontrols due to their simple nutritional needs and ability to be cultivated in large quantities on low-cost substrate

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media. In addition, some yeast species are safe both to human health, environment, host plant and are unlikely to develop resistance. Pathogen development can be inhibited by several antagonistic mechanisms, such as by use of toxins or poisons, antibiosis, antibiotics, and extracellular digestive enzymes (Mohamad et al. 2015). Consequently, biochemical studies are still required to discover the chemical structures of bioactive compounds in antagonistic yeast filtrates. These could be used as a safe and ecologically friendly disease prevention technique in sustainable agriculture (Hameed et al. 2018). The effectiveness of biocontrol use in plant disease control depends on its delivery system. Therefore, ideal formulations of biocontrols which are easily manufactured and stable, with high density of viable propagules and with a prolonged shelf life, should be developed (Jayaraj et al. 2006).

The goal of this study was to screen the in vitro antagonistic capacity of yeasts against *A. niger*, identify yeast isolates using 16 s-rRNA, analyze yeast cultural filtrates, using GC–MS, and investigate the most effective carrier formulations of antagonistic yeasts and their corresponding cultural filtrates on the in vivo severity of onion black mold disease. In addition, the effect of storage period on bioactivity of formulated antagonistic yeasts was examined.

Methods

Isolation of antagonistic yeasts

Soil samples were collected from several sites on a farm at Assiut University, Faculty of Agriculture, Egypt. Isolation was carried out on an autoclaved yeast extract peptone dextrose agar plates supplemented with 100 mg/mL penicillin–streptomycin solutions to inhibit bacterial growth, as described by Hesham et al. (2018). Individual colonies were chosen at random based on their morphological traits, purified, and stored at 4 °C in slant media for subsequent analysis.

Isolation of black mold pathogen

Onion bulbs infected with black mold disease were obtained from different markets in Assiut governorate. The infected parts of the bulb were cut into small pieces, surface sanitized in 1% sodium hypochlorite solution, rinsed three times with sterilized water, and then dried on autoclaved filter paper. Onion pieces were placed on plates (9 cm in diameter) containing PDA (Potato Dextrose Agar) medium and incubated at 25 °C for 3 days. To purify fungal colonies, repeated sub-culturing was carried out on sterile PDA. For subsequent analysis, a single colony of *A. niger* was collected and grown on PDA medium (Aryal 2019).

In vitro antagonistic capacity of yeast isolates against the growth of *A. niger*

A dual cultural approach was used to investigate the antagonistic potential of 28 yeast isolates on the growth of *A. niger* on PDA medium. Disks of *A. niger* (5 mm in diameter) were inoculated in the middle of Petri plates containing PDA media. Then, the examined yeasts were streaked in two opposite sides of the pathogen inoculum. In the control group, no yeast was added. Each treatment was arranged in three replicates. All plates were incubated at 27 °C for 5 days. The inhibitory activity of yeasts on the linear growth of pathogen (cm) was assessed by comparing them to control, using the following formula: Linear growth = growth in control (cm) − growth in treatment (cm)/ growth in control (cm), as described by Imran et al. (2021).

Molecular identification of antagonistic yeast isolates

Two yeast isolates, designated as AUN-AH14 and AUN-AH23, with the highest antagonistic effects were selected for molecular identification, as described below.

DNA extraction and amplification of the D1/D2 domain of 26S rRNA gene in selected yeasts

Total genomic DNA from the selected isolates was extracted according to Hesham, (2014). The D1/D2 domain region of 26S rDNA gene was amplified using the primers NL1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACGG-3′) according to Kurtzman and Robnett (1998). Next, PCR was performed in a final reaction volume of 50 µL containing GoTaq green master mix (Promega, Madison, WI, USA), 1 µL DNA sample, and 1 µL of each primer at a concentration of 0.5 mM (Sallam et al. 2019). The amplification program was set as follows: an initial denaturation at 95 °C for 5 min, followed by 36 cycles at 94 °C for 2 min, 52 °C for 1 min, 72 °C for 2 min, a final extension at 72 °C for 7 min, and a final hold at 4 °C. Then, a total of 5 µL of PCR products were analyzed using 1.5% 0.5 × TBE agarose gel electrophoresis, with a 100-bp DNA ladder as a marker. Ethidium bromide was used for gel staining, and photographs were taken under ultraviolet light.

Purification of PCR products and determination of the D1/D2 domain sequences of 26S rRNA gene

Accurate sized PCR products of ~ 600 bp were purified with Takara agarose gel DNA purification kit and then sequenced in both directions using an ABI 3730 automated sequencer at Macrogen Company (Seoul, Korea). Generated sequences were searched against the GenBank and aligned with known 26S rRNA gene

sequences using the BLAST function at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Phylogenetic trees were also constructed in MEGA v.4.0 using neighbor-joining algorithm, Jukes-Cantor distance estimation, and 1,000 bootstrap replicates, to confirm the taxonomic position of the yeast isolates. The nucleotide sequences from yeast isolates, AUN-AH14 and AUN-AH23, have been deposited in GenBank databases under accession numbers of MZ617254 and MZ617255, respectively.

In vitro screening of the activity of yeast cultural filtrates (CFs) against *A. niger*

The in vitro activities of cultural filtrates (CF) from AUN-AH14 and AUN-AH23 yeast isolates were tested against the growth of *A. niger*. The yeast isolates were inoculated in a 1-L Erlenmeyer flasks containing 500 mL sterile potato dextrose broth (PDB) with shaking at 150 rpm for 48 h. Cell suspensions were centrifuged at 10,000 rpm for 8 min. Pellets in each yeast CF were collected, sterilized with Zeits filter paper, and kept in autoclaved 500 mL flask until use (Gafni et al. 2015). Measured amounts of CF-AUN-AH14 and CF-AUN-AH23 were separately placed in a sterile Erlenmeyer flask containing 100 mL PDB medium, to make a concentrations at 20, 40, 60, and 80%. For control treatment, water was added to PDB medium instead of yeast CF. Each flask containing modified media was inoculated with a 5 mm disc of *A. niger* and then incubated at 27 °C ± 1 °C. After five days of incubation, the fungal mycelium of *A. niger* was collected using filter paper (Whatman No. 1). Then, the mycelial pellets were washed in distilled water several times before being dried overnight at 70 °C for estimating dry weight.

Determination of effective concentrations of antagonistic fungi and their corresponding CF

The highly antagonistic yeast isolates of AUN-AH14 and AUN-AH23 and their CFs were used in this experiment. Yeast isolates were cultured in 500-mL conical flasks with 250 mL sterilized PDB medium supplemented with 2.5 g/L carboxy methyl cellulose to induce high growth. Next, cultures were incubated for 48 h with shaking at 150 rpm. The cells were centrifuged for 8 min at 10,000 g., in cell density of each yeast isolate adjusted to 5×10^8 colony-forming unit (CFU)/mL using a spectrophotometer at 620 nm. The above procedure was repeated for yeast cells CF preparations. Formulation of yeast isolates was developed, as described by Sallam et al. (2009). A mixture of 1 kg talc powder and 10 g carboxymethyl cellulose was used to prepare the powder formulation, and calcium carbonate was added to adjust pH to seven. Adjusted yeast suspensions and their corresponding CFs at 80% were added separately to the powder formulation and then carefully mixed under sterile conditions. The mixture was left to dry to a constant moisture content of 35%, ground with electric mill, and packed in polyethylene bags then, stored at 4 °C until use.

In vivo effects of different yeast formulations on black mold disease severity in onion

Onion bulbs were initially surface sterilized with 1% sodium hypochlorite for 2 min and then left to dry under natural conditions. The bulbs were separated and treated as follows: Bulbs were dusted with each formulated yeasts isolate, including AUN-AH14 and AUN-AH23. Similarly, bulbs were treated with formulated CFs of CF-AUN-AH14 or CF-AUN-AH23. Untreated bulbs were used as control samples. After 24 h, all tested bulbs were sprayed with *A. niger* (4×10^6 CFU/ml), while onions treated only with *A. niger* were used as positive control. Each experimental group was carried out in triplicates. Percentage of disease severity (DS%) was calculated after ten days of incubation with the equation, DS = (A-B)/A* 100, as described by Badawy et al. (2011), where A is weight of healthy onions without rotting and B is onion weight with signs of rotting.

Effect of bioactivity of differently formulated yeast isolates on black mold during onion storage

The bioactivity of formulated yeast isolates during onion storage for 1–9 months at 4 °C was assessed. About 1 g of each formulation was suspended in 9 mL sterilized water, mixed thoroughly, and then serially diluted to 10^6. From each dilution, 0.1 mL was diffused in Petri plates containing PDA medium. The plates with formulated yeasts were incubated at 25 + 1 °C and observed regularly for colony development, which were counted after 24 h. The CFU/g) of each yeast isolate was calculated from the first to the ninth month by multiplying the mean number of colonies grown in each treatment with the sample dilution factor (10^6) (Jayaraj et al. 2006).

Statistical analysis

With statistical package statistics (ver. 8.1), experimental data were analyzed. Least significant difference (LSD) test at $P = 0.05$ was performed for disease severity, yield, and plant growth characteristics means to concede the differences among the means of various treatments. For all data, standard error (SE) was calculated and expressed as mean ± SE.

Results

Preliminary in vitro test for antagonistic effect of yeast isolates against the growth of *Aspergillus niger*

The antagonistic capacities of 28 yeast isolates against the growth of *A. niger* were screened using in vitro analysis. The results demonstrated that the tested isolates

demonstrated varying degrees of inhibition against *A. niger* growth (Table 1). Notably, the highest inhibition against pathogenic growth was observed in isolates of 8, 11, 15, 21, and 27.

Molecular identification of antagonistic yeast isolates using 16S-rRNA

Sequence homology searching was used to determine the identity and phylogenetic position of yeast isolates of 8 and 11, which showed highest antagonistic effects. The BLAST alignment of 26S rRNA gene sequences from isolates 8 and 11 with the published 26S rRNA sequences from GenBank indicated that *Galactomyces geotrichum* shared 100% identity with the two isolates. Sequencing the DNA-fragments of AUN-AH14 and AUN-AH23, from yeast isolates, 8 and 11, showed a 100% symmetry with different strains of *G. geotrichum* (JQ713185.1) and *G. geotrichum* (DQ849321.1), respectively, which formed a distinct cluster in the phylogenetic tree (Fig. 1). Phylogenetic tree was constructed using the sequences of AUN-AH14 and AUN-AH23 along with other sequences of the same genus retrieved from the GenBank.

Table 1 Preliminary test for 28 antagonistic yeast isolates against growth of *Aspergillus niger* in vitro

Yeast isolates	Inhibition growth (cm) of *A. niger*
1	3.3 + 0.36 BCDEFGH
2	3.3 + 0.24 BCDEFGH
3	3.0 0.49 EFGHIJ
4	3.1 + 0.09 CDEFGHIJ
5	2.9 + 0.50 GHIJ
6	3.6 + 0.19 B
7	3.1 + 0.10 DEFGHIJ
8	2.8 + 0.05 JK
9	3.5 + 0.09 BC
10	3.6 + 0.10 B
11	2.5 + 0.9 K
12	3.4 + 0.14 BCDEF
13	3.4 + 0.09 BCDE
14	3.1 + 0.10 CDEFGHIJ
15	2.9 + 0.00 HIJK
16	2.9 + 0.05 IJK
17	3.2 + 0.23 BCDEFGHI
18	3.3 + 0.31 BCDEFGH
19	3.1 + 0.5 CDEFGHIJ
20	3.6 + 0.14 B
21	2.9 + 0.0 HIJK
22	3.0 + 0.46 FGHIJ
23	3.3 + 0.10 BCDEFG
24	3.3 + 0.29 BCDEFG
25	3.6 + 0.19 B
26	3.5 + 0.37 BCD
27	2.9 + 26 I JK
28	3.3 + 24 BCDEFGH
Control	9.0 + 0.0 A

Values within the same column that are associated with different letters indicate significant differences ($P \leq 0.05$). Four replicates were used in this experiment

The in vitro antagonistic effects of CF of yeast isolates on *A. niger*

The in vitro activities of CF of CF-AUN-AH14 and CF-AUN-AH23 isolates were tested against the growth of *A. niger*. The results showed that filtrates at the concentrations of 20, 40, 60, and 80% were able to reduce the dry weight of *A. niger* (Table 2). Substantial differences in the dry weight reduction of *A. niger* were observed in the filtrates of each yeast isolate. CF-AUN-AH14 showed the lowest dry weight of *A. niger,* followed by CF-AUN-AH23. Notably, the results also revealed that the increase in concentration of cultural filtrate was correlated with the decrease in *A. niger* dry weight. The lowest dry weight was observed at 60 and 80% filtrate concentrations with no significant differences.

In vivo effect of different formulation of antagonistic yeast isolates and CF on onion black mold disease

Treatments with different formulations of AUN-AH14 and AUN-AH23, CF-AUN-AH14, or CF-AUN-AH23 at 80% concentration prior to onion bulb inoculation with *A. niger* significantly reduced DS compared to controls (Table 3). Formulations using yeast isolates displayed the highest reduction of DS as compared to formulations of their corresponding CFs. Nonsignificant difference in the reduction of DS was observed individual formulated yeast isolates. In addition, formulations of CF-AUN-AH14 and CF-AUN-AH23 demonstrated the same decreasing effect on the severity of onion black mold disease.

Effect of storage on the antagonistic efficiency of formulated yeasts against *A. niger*

The antagonistic bioactivity of formulated yeasts during a 9-month storage period at 4 °C was shown in (Table 4). Extending the storage time steadily reduced the antagonistic efficiency of fungal propagules. After 5 months of storage, the CFU decreased in AUN-AH14 and AUN-AH23 from 1.7×10^6 and 1.0×10^6 CFU/g to2.6×10^5 and 1.8×10^5 CFU/g, respectively. The highest reductions of yeast propagules in AUN-AH14 and AUN-AH23 were observed during the ninth month. This suggested that these formulations could be stored safely for nine months.

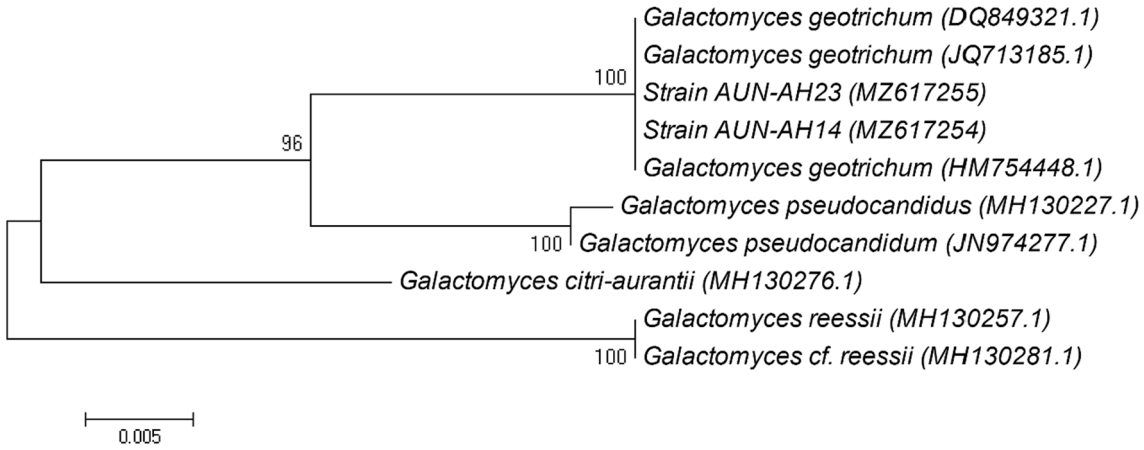

Fig.1 Phylogenetic relationships AUN-AH14 and AUN-AH23and 26S rRNA gene sequences from other published *Galactomyces* spp. by neighbor-joining method. The scale bar indicates 0.005 nucleotide substitutions per nucleotide position. Numbers at the nodes indicate bootstrap support (%) based on 1000 replicates. GenBank accession numbers are given in parentheses

Table 2 Effect of different concentrations of yeasts culture filtrates on the *Aspergillus niger* on dry weight in vitro

Treatments		Dry weight (g)	Mean
Yeasts cultural filtrate	Concentration of filtration yeasts (%)		
CF-AUN-AH14	20	0.21 + 0.068 e	0.15 a
	40	0.14 + 0.027 f	
	60	0.11 + 0.0 g	
	80	0.14 + 0.0 g	
CF-AUN-AH23	20	0.42 + 0.019 c	0.38 b
	40	0.42 + 0.071 c	
	60	0.34 + 0.027 d	
	80	0.33 + 0.014 d	
Control *(A. niger)*	20	0.67 + 0.034 a	0.530 c
	40	0.55 + 0.042 b	
	60	0.47 + 0.018 c	
	80	0.44 + 0.029 c	

Values within the same column that are associated with different letters indicate significant differences ($P \leq 0.05$) based on two-way ANOVA. Four replicates were used in this experiment

Table 3 Effect of formulations of antagonistic yeasts and their culture filtrates on disease severity of onion black mold disease

Treatments	Disease severity %
AUN-AH14	5.0 + 0 c
AUN-AH23	4.5. + 0 c
CF-AUN-AH14	14.3 + 7.18 b
CF-AUN-AH23	13.3 + 2.07 b
Control infected	96.0 + 0.322 a

Values within the same column that are associated with different letters indicate significant differences ($P \leq 0.05$). Four replicates were used in this experiment

Discussion

Black mold is one of the most commercially important onion bulb diseases which causes substantial post-harvest onion loss. Biological disease control approaches are regarded to be a safer alternative to pesticides; hence, finding new biocontrol agents is a crucial research goal. This study assessed the antagonistic capacity of 28 yeast isolates against *A. niger*, which cause black mold disease in onion. The growth of *A. niger* was inhibited by antagonistic yeast isolates with varied inhibitory effects. The highest inhibitory effect against *A. niger* growth was detected with yeast isolates of 8, 11, 15, 21, and 27. These findings supported prior findings of antagonistic yeast isolates suppressing *A. niger* colonization on grape berries (Bleve et al. 2006). Yeasts are among the most important biocontrol agents used to combat disease during post-harvest. Despite the ability of most yeasts to successful growth on agar plates, they displayed significant differences in their antifungal capacities (Hilber-Bodmer et al. 2017). The principal biocontrol mechanism of yeast is to compete with other microbes and their host for nutrients and habitat (Spadaro and Droby 2016). As a result, they produce enzymes, such as glucanases, chitinases, and proteases, which are involved in biocontrol activity to enhance their fitness during competition (Freimoser et al. 2019). In addition, these enzymes can break down cell walls of other microbes; thus, they could act as potential biocontrol agents (El-Tarabily and Sivasithamparam 2006).

The highly antagonistic yeast isolates 8 and 11 identified in this study were selected for in vitro molecular characterization using 26S rDNA. As a result, two yeast isolates, AUN-AH14 and AUN-AH23, were identified as *Galactomyces geotrichum* with accession numbers:

Table 4 Effect of storage period on viability of yeasts formulation

Treatments	Storage time cfu /ml (months)									
	Zero	One	Two	Three	Four	Five	Six	Seven	Eight	Nine
AUN-AH14	7.3×10^8	8.3×10^7	2.3×10^7	5.2×10^7	1.5×10^7	1.7×10^6	2.5×10^6	2.1×10^6	7.6×10^5	2.6×10^5
AUN-AH23	4.6×10^8	3.6×10^7	2.6×10^7	2.1×10^7	1.3×10^7	1.0×10^6	0.4×10^6	0.1×10^6	5.4×10^5	1.8×10^5

JQ713185.1, DQ849321.1, and HM754448.1. Interestingly, the genus, *Galactomyces* has previously been considered as a biological control agent (Freimoser et al. 2019). A previous study demonstrated the potential of *Galactomyces* spp. in controlling pre- or post-harvest diseases (Cai et al. 2021).

The in vivo treatment of onion bulbs with formulated yeast isolates or their CFs significantly reduced the severity of black mold disease at different degrees relative to control treatments. Generally, formulations of AUN-AH14 and AUN-AH23 showed the highest reduction of DS%, followed by filtrate formulations of CF-AUN-AH14 and CF-AUN-AH23. Yeasts exhibit a wide range of uses, and they have been considered as effective biocontrol agents against pathogenic microbes (Costa-Orlandi et al. 2017). The ability of biocontrol yeasts to mainly form adhesive biofilm in the phyllosphere and fruit carposphere (wounds) is currently considered as their key mode of action (Freimoser et al. 2019). Formation of yeast biofilm is initiated by the attachment of cells to the surface, followed by cell wall modifications, extracellular matrix release, and development of pseudohyphae or hyphae (Cavalheiro and Teixeira 2018). Developing a commercial biocontrol formulation with prolonged efficiency and shelf life remains necessary.

The influence of storage period on the bioactivity of formulated yeast strains revealed a steady decrease in survivability, as storage time at 4 °C increased. The reduction in propagules density among fungal formulations developed in this study beginning from the fifth month, and the gradual decline continued until the ninth month of storage. This observation was consistent with that made by Gupta and Dohroo (2014) who reported an initially high microbial population which gradually decreased with the increase in storage time. Similarly, propagules density of *Trichoderma* spp. formulations exhibited a 50% decrease, following a six-month storage period at 24 °C (Jayaraj et al. 2006). Generally, dried yeast formulations showed prolonged shelf life without refrigeration, low contamination, and are more flexible during transportation (Melin et al. 2007). Talc is considered as suitable carrier for formulation development because of its stable characteristics and its availability. In addition, talc has been shown to be a strong carrier capable of retaining most active propagules after six months of storage (Gupta and Dohroo 2014).

Conclusion

The onion bulb treated with formulated yeast of AUN-AH14 with AUN-AH23 followed by their filtrations showed the highest reduction of black mold disease severity. The shelf life of formulated yeast isolates began to gradually deteriorate after 5 months of storage at 4 °C.

Abbreviations

CF	Cultural filtrate
PDA	Potato dextrose agar
PCR	Polymerase chain reaction
ITS	Internal transcribed spacer
ANOVA	Analysis of variance

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Not applicable.

Author contributions

All authors contributed equally in the manuscript, NMAS suggested the idea of the work and contributed to data curation and their validation. HMMKB performed the experiments and prepared the draft and contributed to the formal analysis of the data. KAMA, AH, NMAS and HMKMB contributed to the reviewing and editing the manuscript. All authors reviewed and approved the final version of the manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

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Consent for publication

Not applicable.

Competing interests

No potential conflict of interest was reported by the authors.

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