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INHIBITORY EFFECT OF YEAST LYTIC ENZYMES ON FUNGAL PATHOGENS AND SHELF-LIFE EXTENSION OF TOMATO FRUIT (*LYCOPERSICON ESCULENTUM*) AT ROOM TEMPERATURE

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Abstract

Extracellular lytic enzymes are essential for combating phytopathogens as biological control agents. Consequently, an assessment was conducted to examine the lytic enzyme-producing capabilities of epiphytic yeast isolates. This assessment also included studying the *In vitro* antifungal activity and the biocontrol efficacies of yeast on the compositional attributes of stored tomato fruit. Out of the 25 yeast isolates evaluated for their potential to produce lytic enzymes, all exhibited positive β-1,3-glucanase activity. Additionally, isolates 2, 5, 15, 18, 21, and 25 showed positive chitinase activities. The yeast isolates successfully inhibited the *In vitro* growth of *Fusarium solani* and *Alternaria alternata*. Epiphytic yeast isolates (1, 5, 14, 15, 17, 18, 21, and 23) displayed strong *In-vitro* antifungal activities were further cultured in broths (10^7 CFU/mL) were applied to partially ripened tomato fruits and were stored at 21±2°C with 20-75% relative humidity. Fruits treated with potassium sorbate (1% K-sorbate) served as the positive control set. The epiphytic yeast-treated fruits showed the least percent loss of weight (PLW), Total Soluble Solids (TSS), and decay percentage. The firmness of yeast-treated tomato fruit was higher compared to the control set. Although the lycopene content was higher in the control, a gradual upsurge in the amount of lycopene was noted in fruit treated with isolate 1, followed by isolate 21 on day 15. Maximum decay of the tomato fruit was observed in the control and positive control sets as compared to the yeast-treated fruit. This study suggests that the postharvest storage life and quality of tomato fruit can be maintained by utilizing epiphytic yeast, which is also known as the most suitable and potent biocontrol agent for controlling postharvest losses.

Key words: Yeast, *Alternaria alternata,* Glucanase, Chitinase, *Fusarium solani,* Tomato.

Introduction

Originally imported from the Andes to Europe in the 16th century, tomatoes (*Lycopersicon esculentum* Mill.) are now a worldwide important vegetable crop (Gatahi, 2020). The tomato, widely recognized as a common home garden plant, is the most popular vegetable after the potato (*Solanum tuberosum* L.) (Frenkel & Jen, 2021). Tomatoes are known sources of vitamins (A and C), mineral content, and antioxidants (Ali *et al*., 2020). It is characterized to have a life span of around 2-3 weeks at a temperature of 20-25°C and having a relative humidity of 70-75% (Tolasa *et al*., 2021).

Tomatoes are prone to postharvest degradation, and the postharvest losses escalate over the extended storage. The postharvest fungal pathogens of tomato include., *Alternaria alternata, Fusarium solani, Fusarium oxysporum, Geotrichum candidum, Rhizopus stolonifera,* and *Rhizoctonia solani* (Ramudingana *et al*., 2024). Several methods have been instigated to curb post-harvest diseases and improve the storage life of fruits without causing an undesirable effect on their quality, such as storage at low temperatures (Sivankalyani *et al*., 2016), treatment with fungicides (Habiba *et al*., 2021), proper sanitation of fruits and treatment with hot-water (Tolesa & Workneh, 2017), application of edible coatings such as wax (Kore *et al*., 2017; Salehi, 2020) modified and controlled atmosphere (CA) and application of biocontrol agents (Habiba *et al*., 2017; Habiba *et al*., 2019). Even though synthetic fungicide is the conventional means to resist postharvest diseases but development of resistance in pathogens as well as growing community concerns about the remnants of fungicides on the atmosphere and human health due to fungicide application creates the need for alternative methods (Wisniewski *et al*., 2016; Qadri *et al*., 2020). In recent years, biological control agents, such as epiphytic yeast, have emerged as a sustainable and eco-friendly approach to managing postharvest diseases in tomatoes (Palmieri *et al*., 2022).

β-1, 3-glucanase, and chitinase are important enzymes which are involved in the breakdown and deterioration of fungal cells and walls (Roncero & Vázquez, 2020). These enzymes are synthesized by various fungi and bacteria and could play a significant role in biological control (Khan & Umar, 2021). The yeast isolates can control the growth of postharvest pathogens viz., *Alternaria alternate*, *Penicillium expansum*, and *Botrytis cinerea*, etc., (Li *et al*., 2024; Gramisci *et al*., 2018). Various underlying mechanisms behind the antagonistic activity of yeast include nutrient and space competition, antimicrobial compound production, biofilm formation, and the production of lytic enzymes. Yeast grows intensively, and rapidly, and forms biofilm on the plant surface (single microbe or consortium that are interconnected and form membrane) which inhibits the growth of fungal mycelium and spore production (Costa-Orlandi *et al*., 2017). Moreover, yeast colonizes the plant surface, mainly the injured area where the chance of fungal infection is maximum that accesses released nutrient substrates (Klein & Kupper, 2018). Yeasts reduce the nutrient availability to build their biomass and thus limits the proliferation of pathogens.

The lytic enzyme production by yeast upon direct contact with pathogens has been studied well. Yeasts secrete enzymes such as chitinases, glucanases, lipases, or proteases (Zhang *et al*., 2020). Chitinases degrade the components of the cell wall of phytopathogens, and their production is beneficial for the action mechanism of biocontrol. Moreover, chitinase stimulates the immune system by means of breaking down the chitin (Di Francesco *et al*., 2023). β-glucan is an essential component of the cell wall of the fungi, it gives resistance to toxins and helps in the adhesion of cells. Several yeast isolates can produce glucanase namely *Candida famata, Rhodotorula mucilaginosa*, *W. anomalus* (Zhang *et al*., 2019), effectively retards the pathogen growth. For plant protection, mycoparasitism (where another organism consumes the fungus) is a crucial mechanism. This mechanism involves the adherence of yeast cells to the fungal cell wall, which is then perforated, stops the cell dividion, disrupts its structure, and lowers its turgor (Kowalska *et al*., 2022).

Many biological control agents have been tested in recent years, and some are commercially available. However, there is still significant interest in finding more efficient biocontrol agents, which vary considerably in their biocontrol effectiveness (Ayaz *et al*., 2023).In our earlier studies, investigations have been made to study the impact of fluorescent *Pseudomonas* and yeast in controlling postharvest disease and the quality of tomato fruit (Habiba *et al*., 2017, 2019). However, the current study aims to investigate the lytic activities of epiphytic yeast isolates along with the analysis of their impact on the compositional characteristics and decay percent of stored tomato fruit.

Material and Methods

Isolation of biocontrol agent, postharvest fungal pathogens, and antagonistic activity: Isolation of epiphytic yeast from un-bruised fresh vegetables and fruits such as lemon, green chili, orange, grapefruit, mango, and tomato along with their molecular identification has been reported in our earlier study (Habiba *et al*., 2019). Diseased tomato fruit was surface sterilized and then was transferred on the potato dextrose agar (PDA) plates. The inoculated plates were incubated at room temperature for 7 days. Selected isolates of epiphytic yeast were maintained at 4°C on the Yeast peptone dextrose agar (YPD) medium containing yeast extract, 10 g; peptone, 20 g; and dextrose, 20 g per liter, before use. The antagonistic activity of isolates was determined against postharvest fungi by dual culture plate technique (Ramirez-Carino *et al*., 2023).

Enzymatic activity of epiphytic yeast

Chitinase activity: Chitinase activity was determined using a minimal medium, supplemented with 1.2% colloidal chitin. Briefly, fresh yeast was inoculated in the middle of the medium plate and kept at room temperature for five days. The chitinase activity was revealed by the clear zone formed around the yeast (Kuddus & Ahmad, 2013).

Glucanase activity: The method given by Lutz *et al*., (2013) was used with some changes. Yeast isolates were cultured on NYDA containing 5 g per liter laminarin. The plates were kept for 72 h at room temperature and after incubation, the plates were stained with Congo red (0.6g/L), again plates were incubated for 90 min at room temperature. The extra stain was removed and the formation of colored zone around the isolate was observed.

Sample collection and surface sterilization: Tomato fruit (*Lycopersicum esculentum*) at the partially ripened stage were brought to the laboratory from supermarkets and fields in Karachi, Pakistan. The fruit surface was sterilized using sodium hypochlorite (2%) for two min and later washed with water thoroughly (Wardana *et al*., 2022).

***In vivo* activity of epiphytic yeast isolates against postharvest quantitative and qualitative losses in tomato fruit:** To check the *In vivo* efficacy of yeast isolates method suggested by Habiba *et al*., (2019) was followed. Pure culture broths of isolates 1, 5, 14, 15, 17, 18, 21, and 23 were prepared and incubated for 48 hours. A concentration of 10^7 CFU/mL of each suspension was applied to fresh, diseased-free surface disinfected tomato fruit. The tomatoes were soaked in solutions for five minutes. The tomatoes were air-dried again and were placed in perforated baskets. For positive control, an aqueous suspension of K-sorbate (1%) was used while sterile water acted as a negative control. Relative humidity of 20-75% and room temperature (21±2ºC) was recorded during the period of study. After every 5 d, physiochemical parameters were recorded for 15 d of experimentation.

Physiochemical parameters of tomato fruit

Percent loss of weight: The weight loss of tomato fruit was determined by following the procedure presented by Anon., 2019.

Weight loss = A-B/A × 100

Where:
A = Tomato fruit weight on the day of experimentation
B = tomato fruit weight on succeeding days

Fruit firmness: The Firmness of the fruit from two points on its cheeks was measured through a hand-held penetrometer (PCE-PTR 200) having a cross head of 8 mm.

Total soluble solids (TSS): To determine the total soluble solid of tomato fruit juice handheld refractometer (ATC) was used (Anon., 2019).

pH: The method of Anon., (2019) was used to determine the pH of tomato fruit.

Titratable acidity (TA): The method provided by Anon., (2019) was used to determine the % citric acid of tomato fruit, in this procedure where titration of 5 mL tomato juice against 0.1 N NaOH in the presence of phenolphthalein indicator was performed. Titratable acidity was calculated according to Habiba *et al*., (2021).

Estimation of total phenol content in tomato fruit juice (TPC): The Folin-Ciocalteu method presented by Chandini *et al*., (2008) was followed to measure the total phenolic content (TPC) of tomato fruit. Tomato juices were centrifuged for 15 min at 1007×g, 10µL supernatant was used and made up to 100µL with distilled H_2O. 2ml of 2% Na_2CO_3 was poured and incubated for 2min. After the period of incubation, 50% Folin-Ciocalteu phenol reagent (100µl) was added and kept at room temperature in darkness (30 min). Samples' absorbance was recorded using a spectrophotometer at 720nm against the blank and was presented in mg/g GAE.

Lycopene content (mg/g of tissue): For the determination of lycopene content in tomato, a 60 mg sample was added with five mL of ethanol (95%), butylated hydroxytoluene (0.05% w/v) prepared in acetone, ten mL hexane, and centrifugation was carried out at 180 rounds per minute for ten minutes at 0°C. 3 mL of sterilized H_2O was added and again centrifugation was carried out at 0°C for five minutes. For the separation of the phases, the test tubes were placed for 15-20 min at room temperature. The lighter layer (upper layer) was used for lycopene estimation after separation. The optical density was measured at 503 nm. Hexane was used as blank, while the lycopene content was expressed in mg/g of tissue (Fish *et al*., 2002).

Decay/rotting percent: Visual observations determined the decay %. The calculation was done according to Habiba *et al*., (2019).

Data analysis: Statistical analysis was run by the software package SPSS-26. In this experiment, 20 fruits per treatment divided into four replicates were used and means and standard deviations were calculated for weight loss (%), firmness (N), TSS (% sucrose), pH, TTA (% citric acid), TPC (% mg GA), lycopene content (mg/g of tissue) and decay (%) to determine the effect of epiphytic yeast on tomato fruit. To determine the differences between means, one-way ANOVA and LSD (least significant difference) measures were used. To measure the effect of yeast on the % weight loss, TSS, firmness, pH, TTA, TPC, and % decay of stored tomato fruit, data was subjected to the General Linear Model (GLM).

Results and Discussion

The perishability of vegetables and fruits prone them to deteriorate biologically, chemically, and physiologically which causes alteration in their nutritive values and quality parameters (Ufitinema *et al*., 2024). Antagonistic microorganisms, considered as an alternative to chemical fungicides, can reduce the postharvest disease incidence of fruit and vegetables. In this study, the lytic enzyme-producing capabilities of epiphytic yeast were evaluated along with the study of the biocontrol efficacies of yeast on the compositional attributes of stored tomato fruit.

In this study, the outer surface of fresh vegetables and fruits such as lemon, green chili, mango, grapefruit, orange, and tomato were used to isolate epiphytic yeast. In this study, *Fusarium solani* and *Alternaria alternate* were observed as common postharvest fungal pathogens of tomato fruit. Our observation was similar to the study conducted by Coelho. (2023) where *F. solani* and *A. alternate* cause spoilage of tomato fruit in post-harvest conditions. Several reports showed that several *Fusarium* spp. are responsible for causing postharvest deterioration of fruits and vegetables (Dayok *et al*., 2024). However, in tomatoes, *F. solani* and *A. alternata* are the most common postharvest pathogens which cause substantial fungal losses in storage (Amadioha & Uchendu, 2003). In addition to tomatoes, *F. solani* also attacks tropical crops (Qi *et al*., 2024). *A. alternata* produces the enzyme endo-1,4-β-glucanase, which aids in colonizing infected plant tissues (Sethi *et al*., 2023).

Among the 25 isolates, six isolates showed positive chitinase activity, while all 25 isolates exhibited positive β-1,3 glucanase activity (Table 2; Fig. 3). The lytic enzyme-producing ability of epiphytic yeast demonstrates its inhibitory activity against fungal pathogens (Oztekin & Karbancioglu-Guler, 2021). All 25 isolates inhibited the growth of *F. solani* and *A. alternata* in the *In vitro* test. However, yeast isolate. 1 (HAB-31) showed the highest inhibition zone of 27.33 mm against *F. solani* and 26.33 mm against *A. alternata* (Table 1; Figs. 1, 2). The cell wall degrading enzymes produced by yeast cells could diffuse into the external environment which restricts or inactivates the nearby pathogen (Freimoser *et al*., 2019). The main component of the cell wall of pathogens includes chitin, the enzyme chitinase cleaves the β-1,4 glycosidic linkages of chitin (Vaghela *et al*., 2022). The enzyme glucanase catalyzes the breakdown of glucan, another component of the cell wall, by breaking the β-glycosidic bonds (Theis *et al*., 2019).

Partially ripened tomatoes were used for the *In vivo* study and stored at 21±2°C with relative humidity ranging from 20 to 75% for fifteen days of storage. During the storage time, an increasing trend in weight loss of tomato fruit was observed, while the percentage was higher in control sets in comparison to treatments. On the 15th day, maximum loss of weight was observed in the positive control set (21.45%) while the minimum weight loss percentage amongst treatments was presented by isolate 1/HAB-31 (15.15%) tailed by isolate 23 /HAB-53 (17.9%) as displayed in (Fig. 4a). The parameter responsible for decreasing acceptance of commodities amongst consumers is weight loss, it may be caused by infection of fungi and low relative humidity during the study period (Grande *et al*., 2019). Our findings agree with the earlier findings presented by Peralta-Ruiz *et al*., (2020) and Gürdal & Çetinkaya, (2024) who demonstrated that the use of an external barrier as coating or yeast surface application helps in decreasing weight loss by providing the barrier for the moisture loss. Typically, weight changes in fruit occur primarily because of water loss (Pott *et al*., 2020).

The decreasing trend of tomato fruit firmness was observed in control and treated fruit sets at different time intervals. The positive and negative control (1.1N) showed the least fruit firmness, while yeast suspension-treated fruits showed high firmness of fruits on day 15. However, the greatest firmness was seen in fruit preserved with isolate 23/HAB-53 (Fig. 4b). During the process of ripening of fruit, softening occurs which causes a loss in fruit firmness (Kaewklin *et al*., 2018). While a significant increase in the TSS in tomato fruit juice was observed for 15 days. However, treatments showed a gradual increment in the amount of TSS in comparison to the control set (Fig. 4c), maximum TSS was observed in isolate 21/HAB-51 treated fruit, which is probably caused by the conversion of starch into simpler sugar by hydrolytic enzymes during the period of storage (Souza, 2010).

On one hand, pH of tomato fruit showed an increasing trend concerning the storage period (Fig. 4d). The pH was maximum in isolate 15/HAB-45 followed by isolates 1/HAB-31 and 23/HAB-53. On the other hand, the TA of tomato fruit showed a decreasing pattern from 0-15d notwithstanding the treatments; this increasing form was steady and gradual in isolate 23/HAB-53 and isolate 14/HAB-44 treated sets (Fig. 5a). The changes in pH and TA occur due to the increasing rate of metabolism, respiration as well as ethylene production (Habiba *et al*., 2019). Our studies agree with the previous findings of Morales *et al*., (2024); Umeohia & Olapade. (2024); and Fernandes *et al*., (2024) on commodities such as citrus, tomato, and mango with a decreasing trend in titratable acidity during the period of storage under ambient temperature.

The carotenoid responsible for causing the red color in tomato fruit is lycopene, it is an important phytonutrient and antioxidant (Laayouni *et al*., 2023) and is known as the main maturity index. During the study period, the content of lycopene also demonstrated an increasing pattern in treatments and the control set. Fruit treated with yeast by isolate 23/HAB-53 showed higher lycopene content followed by isolate 18/HAB-48 and isolate 5/HAB-35 after 15 days of incubation (Fig. 5b). Nevertheless, the gradual increment of lycopene content was caused by the conversion of chlorophyll to chromoplast at a slower rate.

Tomato fruit treated with biocontrol isolates of yeast such as isolate 1/HAB-31, isolate 15/HAB-45, and isolate 14/HAB-44 showed a minimum decaying percentage which was >10% (Fig. 5c), which might have occurred due to higher phenolic content in fruit and the enzyme activity by yeast which retards the proliferation of fungi present in the vicinity. Comparative analysis of tomato fruit on zero-day (Fig. 6) and fifteenth day (Fig. 7) has been shown to prove the significant difference. Assimilation of secondary metabolites such as phenols helps scavenge free radicals (ROS) and limit oxidative stress during storage (Muscolo *et al*., 2024).

Table 1. *In vitro* biocontrol activity of epiphytic yeast against postharvest pathogenic fungi (*Alternaria alternata* and *Fusarium solani*) of fruits and vegetables and enzymatic activity.

			Zone of inhibition (mm)	
Isolate No.	**Culture No.**	**Source**	***F. solani***	***A. alternata***
1.	HAB-31	Mango	27.33*	26.33*
2.	HAB-32	"	19.66	15.53
3.	HAB-33	"	19.66	17
4.	HAB-34	"	19.33	10.03
5.	HAB-35	"	22.66*	17
6.	HAB-36	Lemon	18	13.66
7.	HAB-37	"	20	10.66
8.	HAB-38	"	20	12.33
9.	HAB-39	Orange	19	11.66
10.	HAB-40	"	18.66	18
11.	HAB-41	"	17.33	15.66
12.	HAB-42	"	20	15.66
13.	HAB-43	Tomato	20	12.33
14.	HAB-44	"	23*	21
15.	HAB-45	"	18.33	19
16.	HAB-46	"	20	11.66
17.	HAB-47	"	21	17.66
18.	HAB-48	"	20	19.33
19.	HAB-49	Grapefruit	20	14.66
20.	HAB-50	Orange	20.16	11
21.	HAB-51	"	20.66	17.66
22.	HAB-52	"	19	18.66
23.	HAB-53	"	21.33*	22
24.	HAB-54	Green chilli	22	21
25.	HAB-55	"	15	13.33

* = Lysis of mycelium

Table 2. *In vitro* enzymatic activity (Chitinase and Glucanase) of epiphytic yeast.

Culture No.	Chitinase	Glucanase
HAB-31	-	+
HAB-32	+	+
HAB-33	-	+
HAB-34	-	+
HAB-35	+	+
HAB-36	-	+
HAB-37	-	+
HAB-38	-	+
HAB-39	-	+
HAB-40	-	+
HAB-41	-	+
HAB-42	-	+
HAB-43	-	+
HAB-44	-	+
HAB-45	+	+
HAB-46	-	+
HAB-47	-	+
HAB-48	+	+
HAB-49	-	+
HAB-50	-	+
HAB-51	+	+
HAB-52	-	+
HAB-53	-	+
HAB-54	+	+
HAB-55	-	+

+ Positive enzyme activity

– Negative enzyme activity

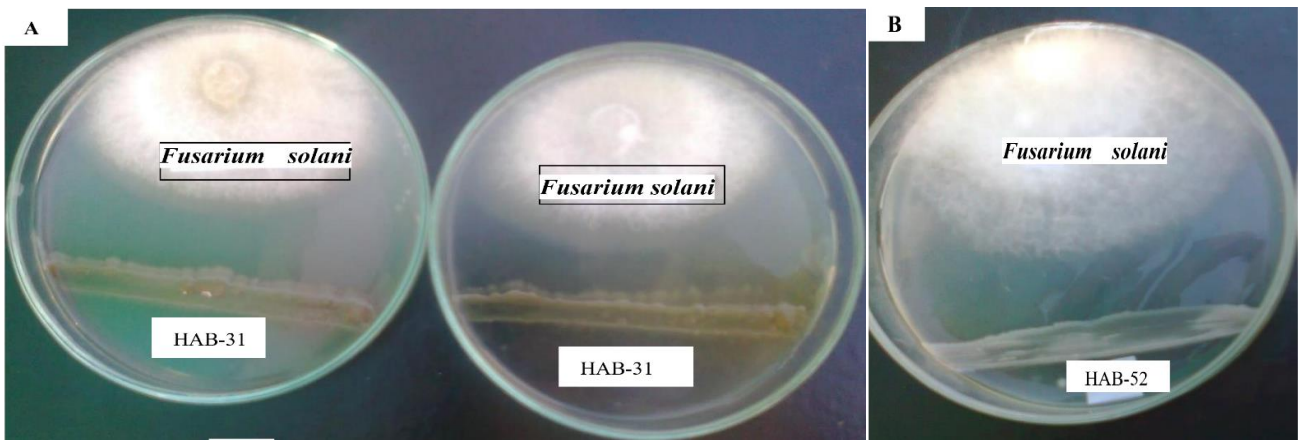


Fig. 1. Inhibition of radial growth of *Fusarium solani* (Top of the plates) by epiphytic yeast (HAB) (Bottom of the plates) isolates in dual culture plate assay showing zone of inhibition.
A=HAB-31, **B** =HAB-52

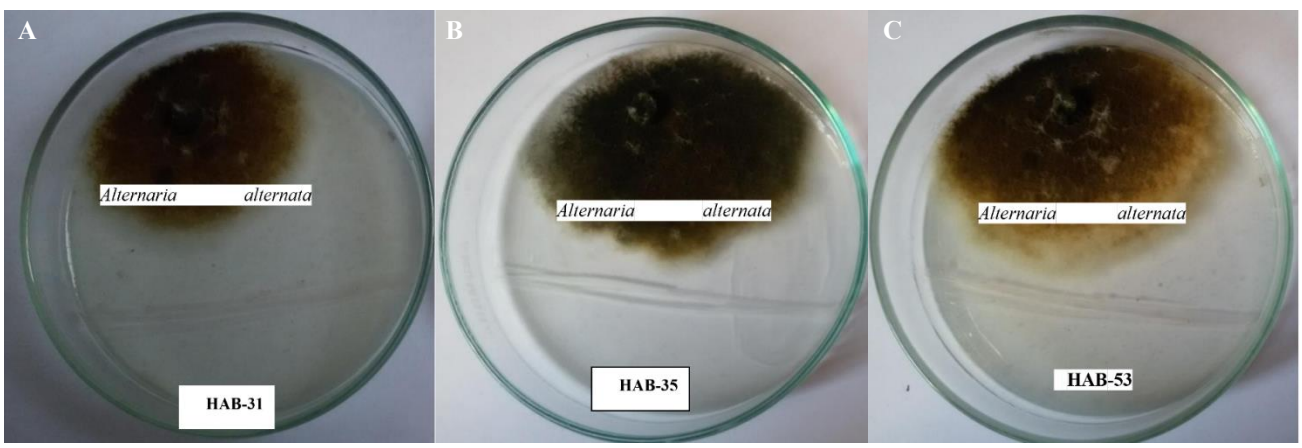


Fig. 2. Inhibition of radial growth of *Alternaria alternata* (Top of the plates) by epiphytic yeast (HAB) (Bottom of the plates) isolates in dual culture plate assay showing zone of inhibition.
A=HAB-31, **B**=HAB-35, **C**=HAB-53

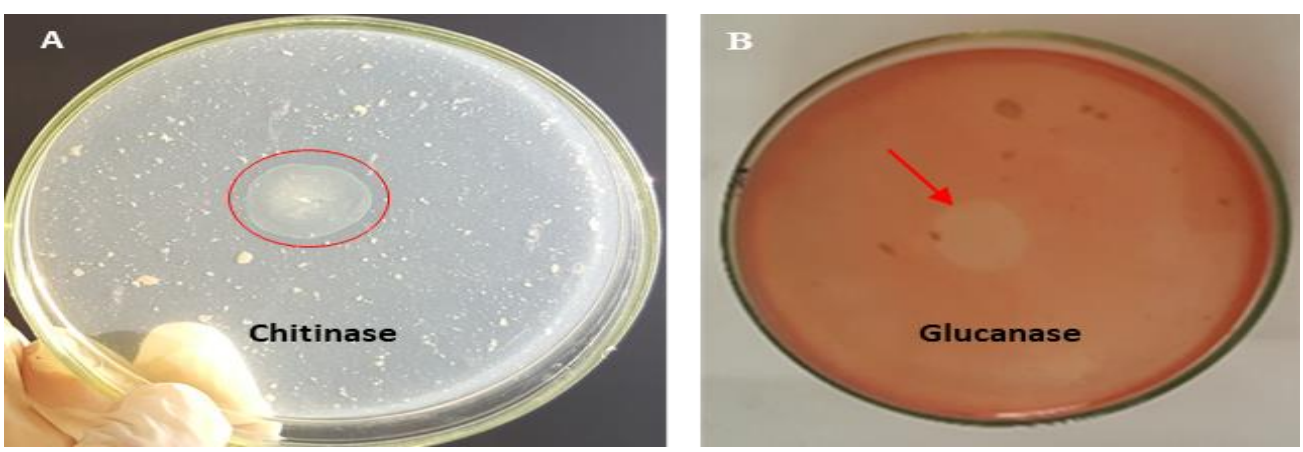
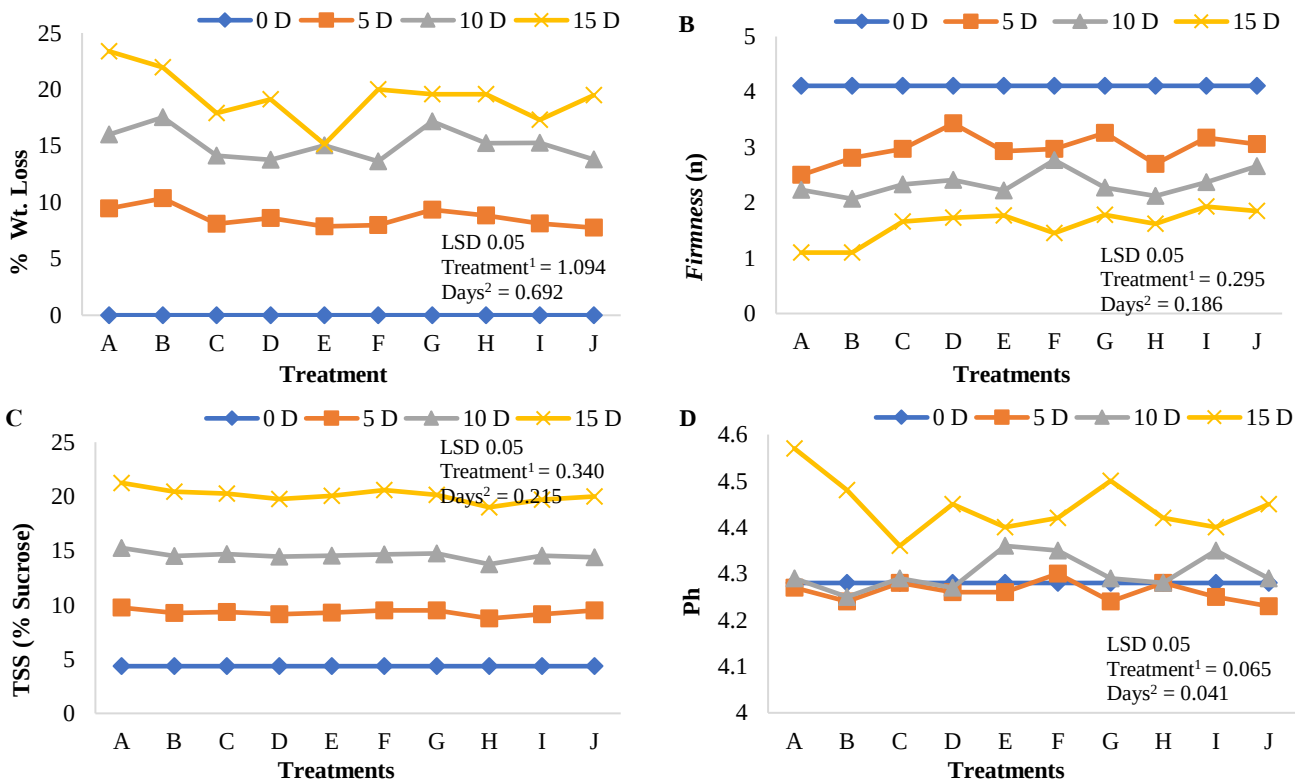


Fig. 3. Enzymatic activity A= Chitinase (HAB-48) and B= Glucanase (HAB-51).

Conclusions

The inhibitory effect of lytic enzymes and biocontrol potential of yeast isolates against common postharvest pathogens of tomato fruit in *In vitro* as well as *In vivo* conditions addresses the biocontrol activity of epiphytic yeast against fungal pathogens. Additionally, *In vivo* studies revealed that yeast isolates significantly control the percent decay, weight loss, and fruit firmness. The gradual increasing trend of TSS, pH, and lycopene content and decreasing trend of TA addresses epiphytic yeast as an efficient candidate for the quality maintenance of tomato fruit during storage. However, further studies on the mechanism of fungal lysis are required.

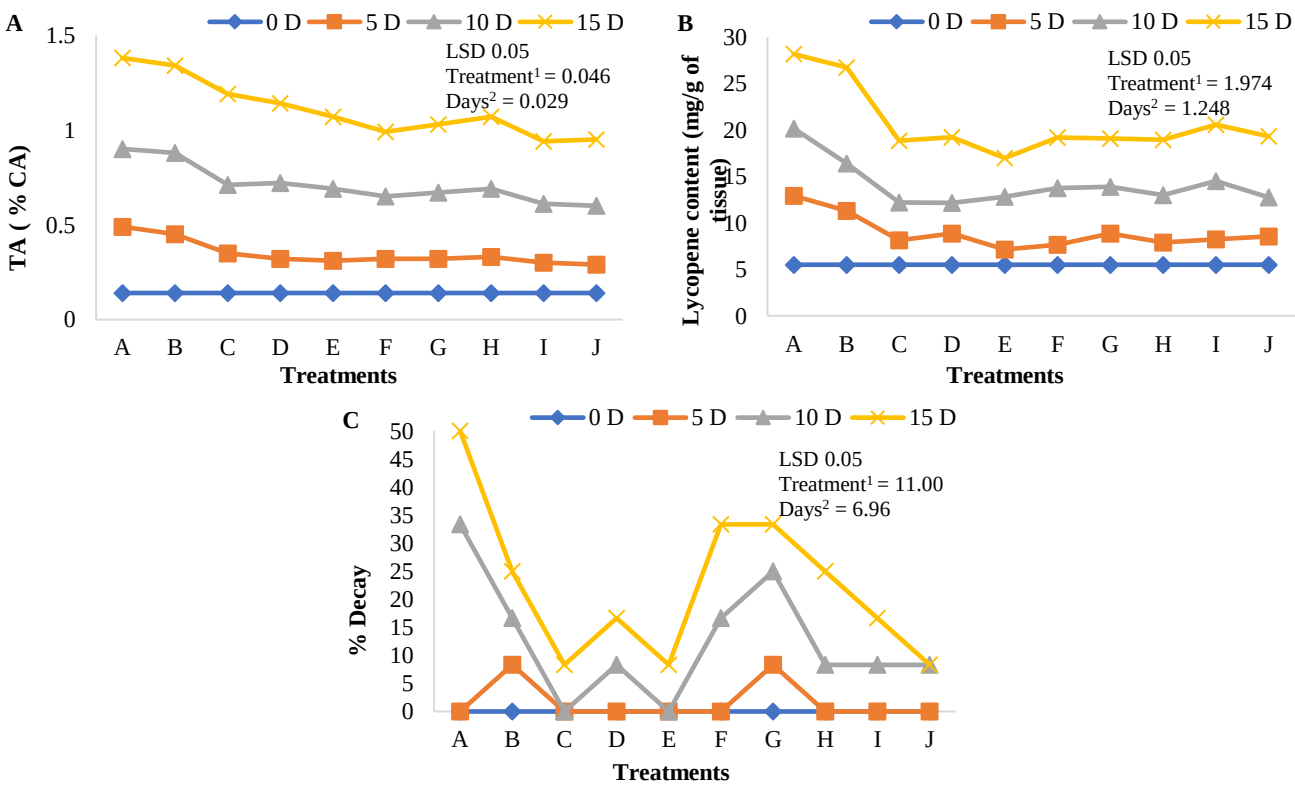


Whereas, A= Control (distilled water); **B**= Positive control (1% K-sorbate); **C**= HAB-45; **D**=HAB-48; **E**=HAB-31; **F**=HAB-35; **G**=HAB-47; **H**=HAB-51; **I**=HAB-53; **J**=HAB-44

[1]Mean values in graph for treatment showing differences greater than LSD values are significantly different at p<0.05.

[2]Mean values in graph at different days showing differences greater than LSD values are significantly different at p<0.05.

Fig. 4. Effect of yeast on % weight loss (A), fruit Firmness N (B), TSS% (C), and pH (D) in tomato fruit kept at 21±2°C with 20%-75% of relative humidity for 15d.



Whereas, A= Control (distilled water); **B**= Positive control (1% K sorbate); **C**= HAB-45; **D**=HAB-48; **E**=HAB-31; **F**=HAB-35; **G**=HAB-47; **H**=HAB-51; **I**=HAB-53; **J**=HAB-44

[1]Mean values in graph for treatment showing differences greater than LSD values are significantly different at p<0.05.

[2]Mean values in graph at different days showing differences greater than LSD values are significantly different at p<0.05.

Fig. 5. Effect of yeast on TA (A), lycopene content (B), and decay % (C) in tomato fruit kept at 21±2°C with 20%-75% of relative humidity for 15d.

Fig. 6. Tomato fruit on the day of experimentation stored at 21±2°C with 20%-75% relative humidity for 15d.

Whereas, A = Control; B = 1% K-sorbate; C = HAB-31; D = HAB-47; E= HAB-45
Fig. 7. Biocontrol effect of epiphytic yeast compared with control and positive control (1% K-sorbate) on stored tomato fruit at 21±2°C with 20%-75% relative humidity on the 15d of storage.

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