Halostability and Thermostability of Chitinase Produced by Fungi Isolated from Salt Marsh Soil in Subtropical Region of Saudi Arabia

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Strategies based on halo- and thermostable enzymes are promising and attractive for biotechnological applications. Three fungal isolates, namely *Aspergillus flavus*, *Cladosporium cladosporioides*, and *Alternaria alternata*, and were subjected to chitinase production using a medium with different concentrations of NaCl up to 10%. *C. cladosporioides* was found to be the main chitinase producer at high concentration of NaCl; therefore, its identification was confirmed using 18S rDNA. The highest chitinase production (88.67 U/mL) was obtained by *C. cladosporioides*, followed by *A. flavus* (76.17 U/mL), and *A. alternata* (70.67 U/mL) at 5% NaCl, while their production without NaCl was 35.07 U/mL, 22.83 U/mL, and 21.33 U/mL, respectively. Thermal stability of chitinase was recorded at 50 °C at 20 min. Chitinase halostability at 20 min indicated that 10% NaCl was the optimum level, with activity 88.3 U/mL. Safranin dye decolorization by *C. cladosporioides* was enhanced to 88.25% *via* the addition of 5% NaCl to growth medium containing chitin. The inhibitory activity of chitinase was detected against *C. lunata* and *F. oxysporium* with or without NaCl. *Culex pipiens* larvae were more affected by *C. cladosporioides* chitinase produced at 5% than 10% NaCl. Energy scores of the molecular docking investigations confirmed the insecticidal activity of chitinase against *C. pipiens* larvae.

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INTRODUCTION

In recent decades, extreme environments have gained great attention from researchers for numerous reasons. Natural inhabitants from such sources have been adapted to produce several industrial, medicinal, and agricultural valuable compounds, such as hydrolytic enzymes. According to Liu *et al.* (2019), salt-tolerant enzymes secreted by microorganisms, particularly halophilic/halotolerant, are highly effective compared to mesophilic enzymes for carrying out various industrial reactions at high salinity stress.

Recent literature reviews (Amoozegar *et al.* 2019; Ruginescu *et al.* 2022) have discussed the breakdown of diverse types of biomasses, bioremediation of hyper-saline locations from pollutants, biocleaning of saline waters from dyes, and biofuels production performed using halophilic enzymes such as amylase, lipase, cellulase, and xylanase. Despite all the eco- and bio-beneficial advantages of halophilic enzymes, there still have been only limited studies of the exploration and exploitation of marine habitats for novel enzyme creators if compared to other habitats (Pasqualetti *et al.* 2019). There are only a scarce number of scientific papers discussing the discovery of marine fungi to produce enzymes. In nature, chitin is considered the second most abundant polysaccharide next to cellulose. It consists of β-1-4 linked N-acetylglucosamine as a homopolymer (Duo-Chuan 2006). Chitinase is a natural extracellular enzyme and belongs to the group of hydrolytic enzymes applied to fight insects and their larvae *via* breaking down chitin in the peritrophic membrane (PM), permitting the passage of most molecules that are present in the midgut of larvae across PM. Chitinases are found in fungi, bacteria, plants, animals, and insects. They play an essential role in growth and development of each organism. For example, chitinases of insects and crustaceans are associated with degrading old layers of cuticle (Veliz *et al.* 2017). Several reports mentioned the application of enzymes, particularly chitinases, in biocontrol of pests, such as fungi, bacteria, nematodes, and insects. Chitinases have been regarded as attractive and ecologically safe, particularly in management of these pests as well as other industrial applications (Bahar *et al.* 2012; Abdelghany *et al.* 2018; Abdelghany and Bakri 2019; Abdelghany *et al.* 2020; Nofal *et al.* 2021a,b,c; Al-Rajhi *et al.* 2022a,b).

According to previous literature, chitinolytic microorganisms have displayed insecticidal activity (Khmel *et al.* 1998; da Silva *et al.* 2005; Yanhua *et al.* 2007). Sánchez-Pérez *et al.* (2014) and St. Leger *et al.* (1991) reported the synergic action of proteases with chitinases to degrade the cuticle of insects. Efficacy of the proteases with chitinase against cotton aphid were reported in Kim *et al.* (2010), but the main insecticidal compound/mode or action is potentially due to chitinase activity. In another study (Ali *et al.* 2010), chitinase application suppressed the growth of *Plutella xylostella* larvae and caused metamorphosis. Chitinase from *Pseudomonas fluorescens* exhibited complete mortality of a tea mosquito bug (Suganthi *et al.* 2017). Previously, the killing effect of chitinase from *Beauveria bassiana* was evaluated against aphids (Fang *et al.* 2005). Chitinase activity of halophilic fungus *Aspergillus flavus* was reported at 0.8 M NaCl with thermal stability at 50 °C for 15 min (Beltagy *et al.* 2018). Several benefits of chitin derivative and chitinase enzyme were reported in various applications. Thus, continuous investigation to find a great action, low-cost, and stable chitinase is critical. This study evaluated the fungal chitinolytic activity and halostable and thermostable chitinase produced from fungal isolates from a subtropical region in Saudi Arabia. Additionally, its biological and chemical applications were investigated.

EXPERIMENTAL

Materials

The utilized chemicals, including media growth contents, solvents, colloidal chitin (crab shell chitin), and buffers, were delivered from Sigma–Aldrich, St. Louis, MO, USA. The insecticide used was Reldan (AGROCHEM, Alexandria, Egypt) containing 5 g/100

mL of active ingredient (chlorpyrifos-methyl), [O, O-dimethyl-O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate].

Isolation and Identification of Chitinase-producing Fungi at Halophilic Conditions

Salt marsh soil samples were collected from the Jazan rejoin, Saudi Arabia (16°57′22.2″N, 42°32′20.3″E), which is characterized by the salt precipitate on the soil surface. It situated in the subtropical zone and stretches 300 km (190 mi) along the southern Red Sea coast. Three samples of salt marsh soil were taken at 5 to 20 cm depth, then placed in sterilized polyethylene bags. Though the serial dilution method, the soil suspension was inoculated on growth medium (Czapek Dox agar) supplemented with different NaCl concentrations (2, 5, and 10%), incubated for 8 d at 30 ºC to isolate halophilic fungi (Abdelghany *et al.* 2019a), and followed by incubation at 28 ºC for 6 days. The developed fungi were purified, identified using different growth media (Malt extract, Czapek Dox agar, and potato dextrose agar), and investigated according to morphological and microscopic examination based on growth rate, colony color and texture, conidiophores, conidia, and hyphae features (Ellis 1971; Raper and Fennell 1973; Domsch *et al.* 1980; Rotem 1994). The identified fungi isolates were tested for chitinase production. The most potent producer of chitinase was further identified depending on molecular characterization as follows: The collected fungal mycelia were subjected to DNA extraction and polymerase chain reaction (PCR) amplification of 18S rDNA region *via* Quick-DNA Fungal Microprep Kit (Zymo research; D6007; Irvine, CA, USA) in Sigma Scientific Services Company, Cairo, Egypt. Maxima Hot Start PCR Master Mix (Thermo; K1051) was applied for PCR. The following primers were used, ITS1-forward (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4-reverse (5′-TCCTCCGCT TATTGA-TATGC-3′). The following contents (10 µL, 1 µL, 1 µL, 13 µL, and 25 µL) of genomic DNA, forward primer 18S rRNA, reverse primer 18S rRNA, nuclease, and Maxima® 2X Hot Start PCR Master Mix, respectively, were mixed to perform the PCR reaction. The PCR was performed at the following conditions: Taq polymerase activation for 10 min at 95 °C, 35 cycles at 95 °C for 10 min, followed by 30 s required for initial denaturation, 1 min annealing at 55 °C, 1 min initial extension at 72 °C, and for 15 min final extension at 72 °C. The PCR reaction products were subjected to agarose gels (1%) electrophoreses to detect the separated bands with ethidium bromide at specific dose (10 mg/mL) (White *et al.* 1990; Abdelghany *et al.* 2019b).

Assessment of Chitinase Production at Different NaCl Concentrations

Czapek Dox broth medium containing chitin (2%) and different concentrations of NaCl up to 10% was inoculated with fungal isolates (5-mm diameter disk of active margin colony, 10^6 spores/mL), then incubated for 8 days at 30 °C. The fungal mycelia after ending the incubation period were removed. Then the medium broth was filtrated through a 0.5 µM bacteria-proof filter (A filter fine enough to prevent the passage of mycelia or spores at size of bacteria) to obtain clear filtrate. Culture fluid (filtrate) was centrifuged (8,000 rpm at 4 °C for 10 min). The supernatant was subjected to precipitation *via* ammonium sulphate to 80% saturation at 4 °C with continuous stirring for 12 h. Then, the precipitate was collected *via* centrifugation (9,000 rpm for 30 min at 4 °C), followed by dissolving in an appropriate amount of phosphate buffer (0.2 M, pH 9), and extensively dialyzed in contradiction of the used buffer. The resultant dialysate was applied as crude chitinase.

Preparation of Colloidal Chitin and Chitinase Assay

To obtain colloidal chitin, 1% chitin powder was suspended in citrate phosphate buffer with pH 6.6. The reaction mixture for assaying chitinase activity contained 1 mL of prepared colloidal chitin with 1 mL of supernatant containing chitinase. The mixture was incubated at 37 °C in a water bath for 40 min. The reaction mixture was cooled and centrifuged (10,000 rpm up to 10 min) to assay enzyme (Reid and Ogryd-Ziak 1981). The obtained supernatant was also used to detect the reducing sugars *via* 3,5-dinitrosalysilic acid (DNS) method. The quantity of enzyme (one unit) was defined as the quantity of enzyme required to reduce the colloidal chitin.

Chitinase Thermostability and Halostability

Chitinase was incubated in a water bath at different temperatures from 10 °C to 70 °C, and times of 20, 40, and 60 min to study the thermal stability of chitinase (Bakri *et al.* 2022). Enzyme activity was detected as previously mentioned. To measure halostability of chitinase, the enzyme was pre-incubated without chitin in 50 mM of sodium phosphate buffer (pH 8.0) appended with 0 to 15% NaCl at 5 °C for 20, 40, and 60 min.

Decolorizing Assay of Used Dye by Chitinase

Safranin O (chemical formula $C_{20}H_{19}N_4Cl$) dye at 500 µg/100 mL was used to assess the ability of *Cladosporium cladosporioides* chitinase to decolorize the dye in the presence of chitin (Sadhasivam *et al.* 2005) in Czapek Dox broth as growth medium. The following treatments were used: 1- Growth medium (Czapek Dox broth medium), Growth medium containing dye (GMD) without fungus (F) inoculation, 2- GMD with F, 3- GMD and 5% NaCl with F, 4- GMD and chitin (2%) with F, 5- GMD, chitin (2%), and 5% NaCl with F, and 6- GMD, chitin (2%), and 10% NaCl with F. After incubation period (8 d) at 30 °C, the fungus mycelia were removed and culture media were filtrated. The percentage of decolorized dye was measured *via* a UV-spectrophotometer (Model 6300, EU, JENWAY, Stone, UK) at 521 nm wavelength using the following Eq. 1; medium containing dye was applied as blank control:

$$\text{Decolorization \%} = (\text{Initial wavelength} - \text{Final wavelength (nm)} / \text{Initial wavelength (nm)}) \times 100 \quad (1)$$

Antifungal Activity of Chitinase

Different concentrations of chitinase (0, 25, 50, and 100 U/mL) were added to sterilized growth medium. Czapek Dox Agar supplemented with different concentrations 0, 5, and 10% of NaCl under aseptic conditions. Then, the growth medium was inoculated with 0.6 mm of actively growing colonies of different fungi, including *Curvularia lunata*, *Fusarium oxysporium*, *Aspergillus niger*, and *A. terres*. The inoculated growth medium was incubated at 30 °C for 8 days, and the colony radius was measured to assess the activity of chitinase against tested fungi.

Insecticidal Potential of Chitinase

The insecticidal activity of chitinase was measured against larvae of *Culex pipiens* (30 larvae) that was kept at 28 ± 2 °C and 12:12 h light:dark photoperiod in wood chamber insectary. Plastic enamel dishes containing 1 L of distilled water amended with fish food were used for rearing the larvae. Supernatant containing chitinase was added to distilled water in dishes containing larvae (1/10 v/v). The chemical insecticide as a positive

control was added at concentration 20 ppm, while the larvae medium lacking any treatment was used as a negative control. Any developed larvae were transferred to wooden cages amended with solution of sucrose (10%) and a pigeon required for feeding.

All ethical review committees approved the experimental process, and international and national guidelines were followed in this experiment.

Molecular Docking

A molecular modeling investigation using the Molecular Operating Environment (MOE) (Murad *et al.* 2022; Al-Rajh *et al.* 2022c) module was conducted to explain the observed insecticidal activity of chitinase. The structural model was built utilizing the 'BUILDER' module of MOE. Conformational analyses of the built molecules were performed using a two-step route. First, chitinase was submitted to an energy minimization tool utilizing the included MOPAC 7.0 software (Chemical Computing Group Inc., Montreal, QC, Canada, v.2008). The geometry of the compounds was optimized utilizing the semiempirical PM3 Hamiltonian with Restricted Hartree-Fock (RHF) and root mean square (RMS) gradient of 0.05 Kcal/mol. The obtained model implemented the 'Systematic Conformational Search' of the MOE. To rank the binding affinity of the chitinase to (5V13) *Culex* sp. hormone-binding protein, the binding free energy and hydrogen bonds among the chitinase and amino acid into (5V13) were used. The hydrogen bonds were evaluated by measuring the hydrogen bond length. Additionally, root mean square deviation (RMSD) of the co-crystal ligand position compared to the docking pose was used in ranking. Both RMSD and the mode of interaction of the native compound within the *Culex* sp. hormone-binding protein (5V13) receptor were utilized as a standard docked model.

Statistical Analysis

The investigation findings were confirmed in triplicate, and the standard deviation (SD) was recorded. The SPSS ver. 22.0 (SPSS Inc., Chicago, IL, USA) software was utilized for statistical analyses.

RESULTS AND DISCUSSION

Different samples of salt marsh soil from the Jazan rejoin of Saudi Arabia (Fig. 1) were used as the source of chitinase-producing halophilic fungi. Through serial dilution, the soil suspension was inoculated on growth medium supplemented with different NaCl (2, 5, and 10%) concentrations. With increasing NaCl concentration, the number of fungal colonies decreased, particularly at 5% and 10% NaCl (Fig. 2A and 2B). Three fungi appeared on the growth medium containing 10% NaCl. The three fungi were purified and identified according to previous keys as *Aspergillus flavus*, *Alternaria alternata*, and *Cladosporium cladosporioides* (Fig. 3). The three fungi were tested for chitinase production, the potent producer was subjected to approve the identification *via* molecular characterization. Molecular identification was performed using ITS rRNA gene. Using the alignment search tool (https://blast.ncbi.nlm.nih.gov), the ITS rRNA homology value of the strain showed similarity (99.61%) with *C. cladosporioides* (OM836434.1) as presented in the phylogenetic tree (Fig. 4), the sequence was deposited in GenBank under accession number OM836434.1.

Diverse genera, comprising *Cladosporium* sp., *Aspergillus* sp., *Alternaria* sp., and *Acremonium* sp. are reported to exist under saline stress (Grum-Grzhimaylo *et al.* 2013).

Aspergillus sp. and *Cladosporium* sp. were the main isolates grown at different NaCl concentrations up to 15% (Orwa *et al.* 2020).

Fig. 1. Salt marsh soil samples (A, B, and C) black arrows point to salt precipitate in Jazan rejoin of Saudi Arabia (D) as a source of halophilic fungi

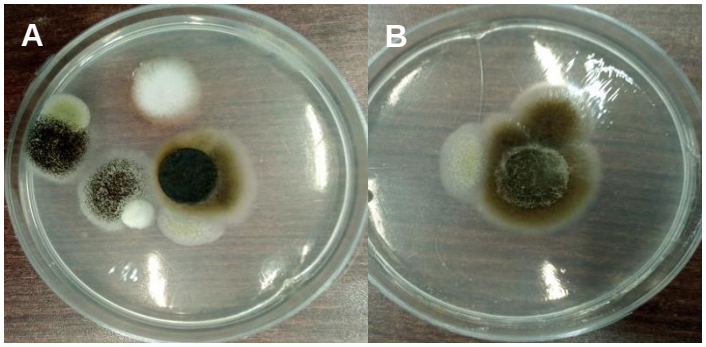


Fig. 2. Fungal isolates at 5% (A) and 10% NaCl (B)

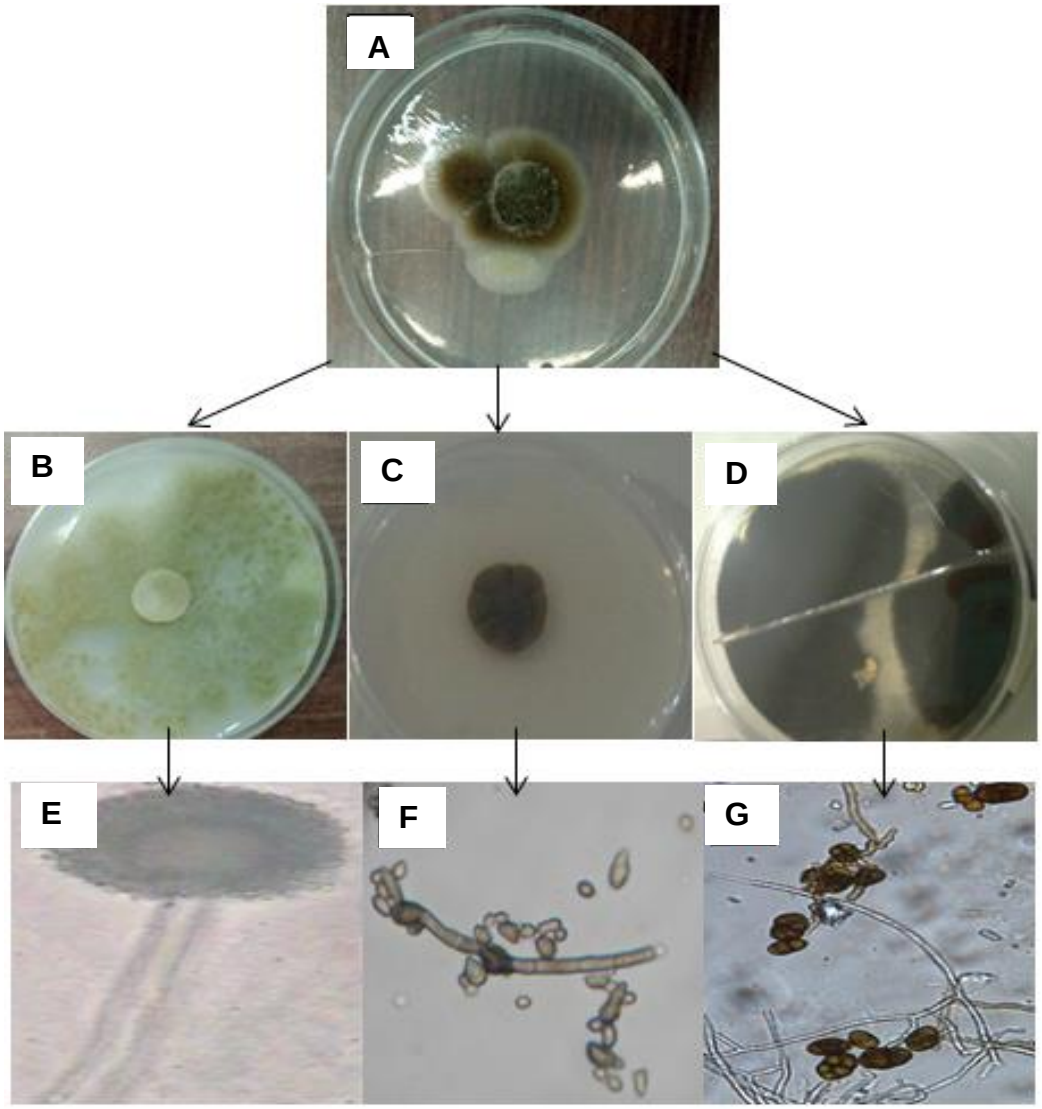


Fig. 3. Fungal isolates from rhizospheric soil: A: unpurified fungal isolates at 10% NaCl; B: pure colony of *A. flavus*; C: pure colony of *C. cladosporioides*; D: pure colony of *A. alternata*; E: microscopic feature of *A. flavus*; F: microscopic feature of *C. cladosporioides*, and G: microscopic feature of *A. alternata* (400X)

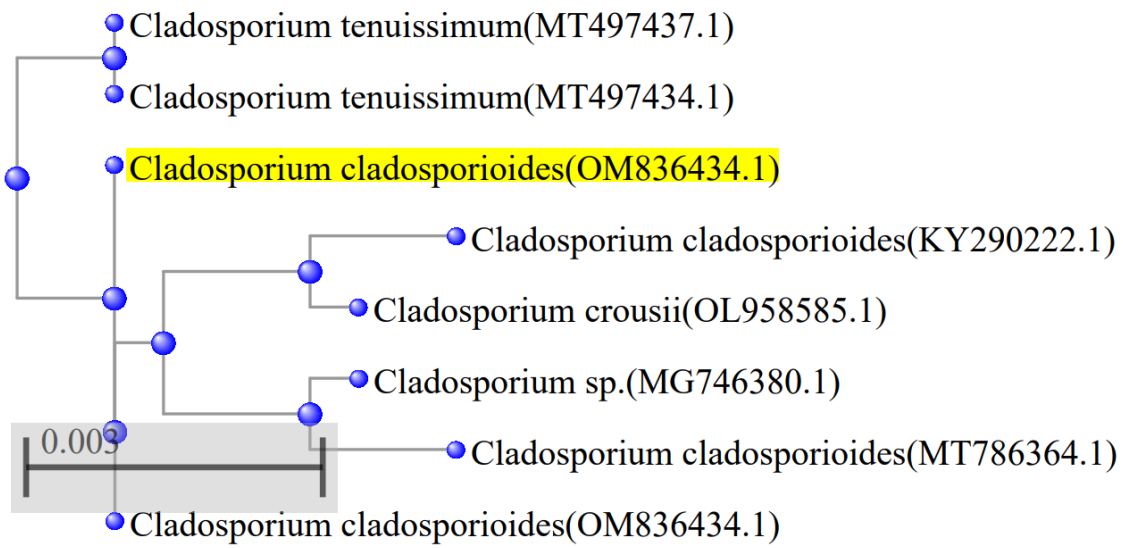


Fig. 4. Identification of *Cladosporium cladosporioides* (OM836434.1) with neighbor joining phylogenetic tree (Yellow highlight)

The results showed that the three fungal isolates were able to produce chitinase enzyme but the productivity depended on fungal species and NaCl concentration (Fig. 4). *C. cladosporioides* was the most potent producer at 88.67 ± 0.58 U/mL, followed by *A. flavus* (76.17 ± 0.29 U/mL) and by *A. alternata* (70.67 ± 0.15 U/mL) on medium containing chitin with 5% NaCl, while their productivity was 86.83 ± 0.29 U/mL, 71.67 ± 0.58 U/mL, and 86.83 ± 0.29 U/mL, respectively, using chitin with 10% NaCl. In contrast, the presence of chitin without NaCl resulted in weak productivity of chitinase by all fungal isolates (Fig. 5).

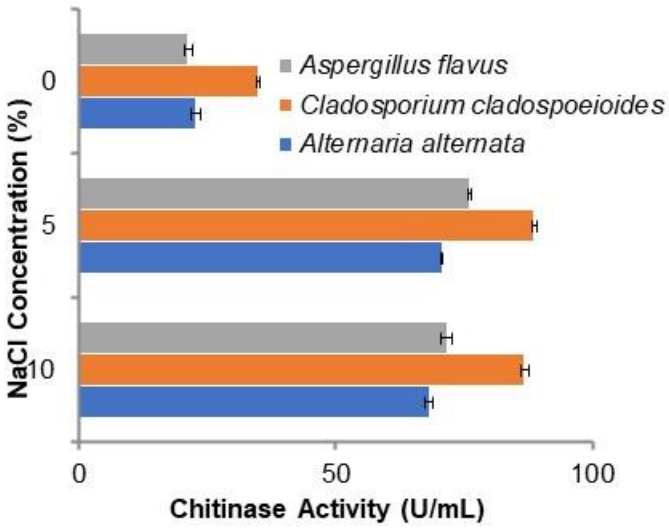


Fig. 5. Chitinase activity of fungal isolates using chitin with different concentrations of NaCl

Previous studies reported that many filamentous fungi were able to degrade chitin-containing compounds *via* chitinase but with variable levels (Alves *et al*. 2018; Atalla *et al*. 2020). One isolate of *A. flavus* and two isolates of *Cladosporium* sp. were isolated from

marine habitats and tested for chitinase production. The highest activity was recorded for *A. flavus* but not for *Cladosporium* sp. (Pasqualetti *et al.* 2019). *Aspergillus niger* and *A. niveus* were recognized for chitinase production using chitin substrate (Brzezinska and Jankiewicz 2012; Alves *et al.* 2018). Varied amounts of chitinolytic activity (0.0261 U mL^{-1} to 0.1340 U mL^{-1}) were detected from different isolates of *Metarhizium anisopliae* cultivated in diverse culture media (Braga *et al.* 1998). *A. flavus* was isolated as halophile fungus with chitinolytic activity at 0.8 M of NaCl (Beltagy *et al.* 2018).

The thermal stability of chitinase produced by *C. cladosporioides* at different temperature and times revealed that chitinase activity was approximately stable up to 50 ºC, 40 ºC, and 30 ºC at 20, 40, and 60 min, respectively, and with increased temperature the activity decreased (Fig. 6). The activity of chitinase sharply decreased at 70 ºC particularly after 60 min from 86.67 ± 0.29 U/mL at 40 ºC to 80.83 ± 0.29 U/mL at 70 ºC. The present findings partially agreed with previous reports that the activity of chitinase of *Trichoderma viride* culture was stable at 40 to 50 °C (Ekundayo *et al.* 2016). Recently, chitinase activity reached stability for *Penicillium chrysogenum* after 60 min at 50 °C, then declined with increased temperature (Atalla *et al.* 2020). In another study, thermal stability of halophile *A. flavus* was recorded at 50 °C for 15 min (Beltagy *et al.* 2018). A loss of chitinase activity for *A. niger* was observed after 3 h at 60 °C (Brzezinska and Jankiewicz 2012).

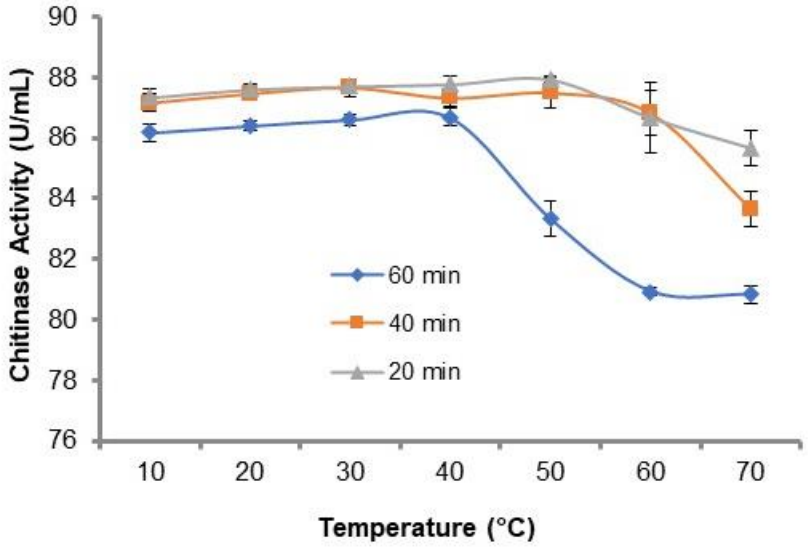


Fig. 6. Thermal stability of chitinase produced by *C. cladosporioides* at different temperature and time

Many industries depend on waste with a high level of salinity. Therefore, the halotolerant nature of enzymes plays a critical role in salinity dependent industries, *i.e.* the halotolerant enzymes convert or degrade high salinity wastes to industrial products. Moreover, halotolerant chitinase restricted the proliferation of microbial pathogens of halophilic plants (Essghaier *et al.* 2012). In the current study, halostability of chitinase was observed at different concentrations of NaCl from 0 up to 15% (Fig. 7). Exposure of chitinase to NaCl for 20 and 40 min revealed that 10% NaCl was the optimum concentration with activity of 88.3 and 86.2 U/mL, respectively. While exposure for 60 min revealed that 5% NaCl was the optimum concentration with activity of 84.46 U/mL.

At 15% NaCl, slight gradually decline in halostability of chitinase was recorded. Previously, Makhdoumi *et al.* (2015) noticed that 10% NaCl was the optimum concentration for bacterial chitinase production, while its stability was recorded up to 15% (w/v). An early study reported that some enzymes produced by *Cladosporium sphaerospermum*, namely fructose 1,6 diphosphate, glyceraldehyde phosphate dehydrogenase, and aldolase, were more active under stress of NaCl compared to its activity in a NaCl-free medium (Karlekar *et al.* 1985).

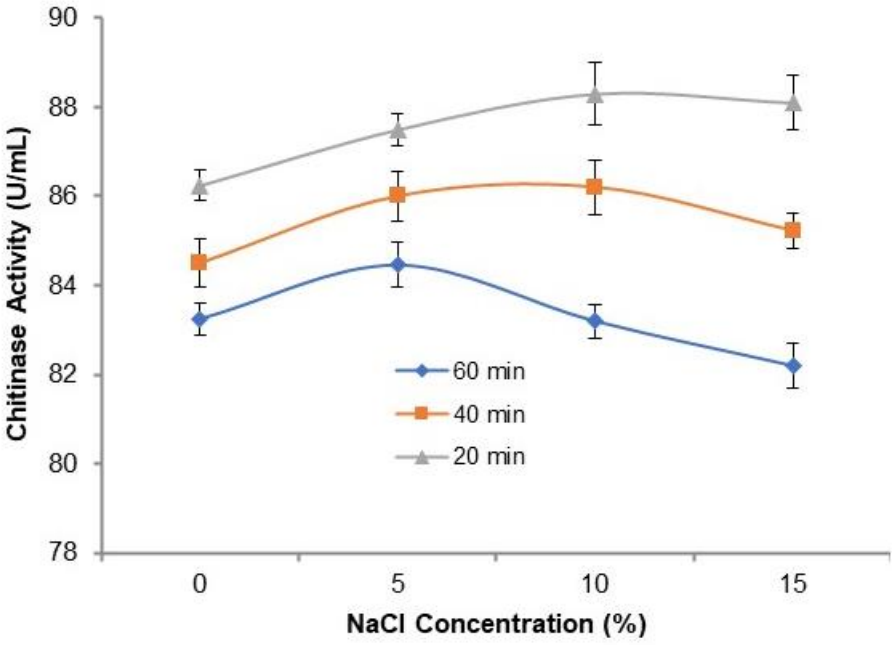


Fig. 7. Halostability of chitinase produced by *C. cladosporioides* at different NaCl concentration and time

Table 1 shows the various decolorization percentage of safranin and color change depending on culture conditions. The ability of *C. cladosporioides* to decolorize safranin was recorded (18.68%), but this capability was enhanced (50.04%) with the presence of chitin in the growth medium. A slight increment of decolorization (39.76%) was observed at 5% NaCl in growth medium without chitin. Moreover, the presence of NaCl with the chitin increased the decolorization of safranin to 88.25% particularly at 5% NaCl. The results indicated that decolorization of safranin may have been because of chitinolytic activity, at the same time, salinity encourages the chitinase production leading to increased decolorization. The ability of fungus to decolorize safranin without the presence of chitin may be associated with secretion of organic acids or dye adsorption on fungus mycelia as mentioned previously by Nakajima-Kambe *et al.* (1999). In another studies, the decolorization of dyes is performed either with microbial enzymes or adsorption on the mycelia (Abdel Ghany and Al Abboud 2014; Abdel-Ghany *et al.* 2019).

Table 1. Effect of Chitinolytic Activity of *C. cladosporioides* on the Decolorization of Safranin at Salt Stress

Treatment	Decolorization (%)	Rest Quantity (µg/100 mL)	Rest Quantity (%)	Color Shown
GMD without F	0.0 ± 0.0	500	100	
GMD with F	18.68 ± 0.52	406.60	81.32	
GMD and 5% NaCl with F	39.76 ± 0.33	301.20	60.24	
GMD and chitin (2%) with F	50.04 ± 1.20	249.80	49.96	
GMD, chitin (2%), and 10% NaCl with F	62.70 ± 1.33	186.5	37.30	
GMD, chitin (2%), and 5% NaCl with F	88.25 ± 1.5	58.75	11.75	
where GMD refers to the growth medium containing dye, and F refers to the fungus				

The inhibitory activity of *C. cladosporioides* chitinase against various fungi at different concentrations of NaCl is visualized in Table 1. Activity of chitinase was observed against *C. lunata* and *F. oxysporium* with or without NaCl, where the colony radius decreased with increased enzyme up to 100 U/mL. The colony radius of *C. lunata* was 3.25, 2.96, and 2.08 cm using 100 U/mL of chitinase, compared to a colony radius of 6.05, 5.25, and 4.05 cm without chitinase at 0, 5, and 10% NaCl, respectively. The same effect of chitinase on *C. lunata* was observed on *F. oxysporium*, while negligible inhibitory activity of chitinase was noticed on *A. niger* and *A. terres*. For example, the colony radius of *A. niger* and *A. terres* was 6.54 cm and 4.72 cm, respectively, without chitinase while it decreased to 6.15 cm and 4.40 cm at high concentration of 100 U/mL of chitin, respectively (Table 2).

Table 2. Inhibitory Activity of *C. cladosporioides* Chitinase against Various Fungi at Different Concentrations of NaCl

Concentration of Chitinase (U/mL)	Colony Radius (cm) at Different NaCl Concentrations (%)					
	C. lunata			*F. oxysporium*		
	0	**5**	**10**	**0**	**5**	**10**
0	6.05 ± 0.10	5.25 ± 0.10	4.05 ± 0.10	7.08 ± 0.06	6.08 ± 0.06	4.25 ± 0.06
25	5.50 ± 0.07	4.55 ± 0.09	3.48 ± 0.08	6.70 ± 0.12	6.05 ± 0.05	4.12 ± 0.04
50	5.05 ± 0.05	4.40 ± 0.04	2.68 ± 0.10	5.43 ± 0.05	4.75 ± 0.04	3.08 ± 0.05
100	3.25 ± 0.03	2.96 ± 0.09	2.08 ± 0.05	4.00 ± 0.04	3.72 ± 0.06	2.20 ± 0.03
Concentration of Chitinase (U/mL)	Colony Radius (cm) at Different NaCl Concentrations (%)					
	A. niger			*A. terres*		
	0	**5**	**10**	**0**	**5**	**10**
0	6.54 ± 0.03	5.50 ± 0.03	4.44 ± 0.04	4.72 ± 0.05	3.85 ± 0.14	2.88 ± 0.14
25	6.50 ± 0.04	5.48 ± 0.09	4.80 ± 0.03	4.70 ± 0.04	3.65 ± 0.04	2.08 ± 0.03
50	6.48 ± 0.05	5.43 ± 0.06	4.48 ± 0.03	4.55 ± 0.05	3.60 ± 0.03	2.02 ± 0.05
100	6.15 ± 0.08	5.38 ± 0.03	4.33 ± 0.06	4.40 ± 0.03	3.50 ± 0.06	1.75 ± 0.03

The inhibitory activity of *A. niger* and *A. terres* is possibly because of the effect of NaCl but not to chitinase. Several studies applied the chitinase to repress numerous fungi including phytopathogens and mycotoxigenic fungi. Two fungal species *Colletotrichum* sp. and *Sclerotium rolfsii* were sharply inhibited more than 95% using 60

U/mL of chitinase (Nguyen 2020). The authors' results are in line with a previous study (Brzezinska and Jankiewicz 2012), where *Fusarium culmorum*, *F. solani*, and *Rhizoctonia solani* were inhibited by chitinase secreted by *A. niger* whereas *F. oxysporum*, *Alternaria alternata*, and *Botrytis cinerea* were not affected. In another study (Aoki *et al.* 2020), chitinase produced by *Trichoderma* sp. played a vital role in the control of *B. cinerea*. The inhibitory activity against fungi may been because of lysis and the destroyed fungal cell walls by chitinolytic activities.

Insecticidal Activity

The chitinase activity grown on medium amended with different NaCl concentrations was compared with chemical insecticide tested against larvae of *Culex pipiens* (Table 3). Chitinase activity exhibited the highest insecticidal activity using 5% NaCl. The mean number of dead larvae was 19.33 represented by 64.4%, followed by addition of 10% NaCl as inducer of chitinase production. The mean number of dead larvae was 19 represented by 63.3%. Weak insecticidal activity was observed using medium with chitin but without NaCl the mean number of dead larvae was 5 represented by 16.7%. Conversion of living larvae to pupa or adult was more affected by chitinase produced using chitin with 10% NaCl followed by chitin with 5% NaCl. The mean number was 1.33 ± 0.58 (represented by 4.43%) and 1.67 ± 0.58 (represented by 5.56%) adults, respectively (Table 3). As mentioned in literature, the mechanical insecticidal activity of fungi may due to lysis of whole body tissues as well as cuticle *via* chitinase, protease, and lipase (Hamama *et al.* 2022). High mortality rates of *C. pipiens* larvae were recorded as a result of exposure to *Penicillium chrysogenum* chitinase (Mansour *et al.* 2019). Additionally, *Pseudomonas fluorescens* chitinase exhibited toxicity effect against tea mosquito bug with complete mortality (Suganthi *et al.* 2017). Previously, fungal chitinase was applied against *Galleria mellonella*, *Trichoplusia ni*, *Aedes aegypti* (Deshpande 1999) and aphids (Fang *et al.* 2005).

Table 3. Insecticidal Activity of Chitinase Produced at Different NaCl Concentrations against Larvae of *Culex pipiens*

NaCl Concentration (%)	No. of		No. of Transformed Larvae to	
	Dead Larvae	Living Larvae	Pupa	Adult
0.0	5.0 ± 1.0	21.33 ± 1.15	1.33 ± 0.58	2.33 ± 1.15
5	19.33 ± 1.15	6.67 ± 0.58	3.0 ± 1.0	1.67 ± 0.58
10	19.00 ± 1.73	8.33 ± 0.58	1.33 ± 0.58	1.33 ± 0.58
*Control 1	27.33 ± 0.58	2.67 ± 0.58	0.00	0.00
**Control 2	3.33 ± 0.58	16.0 ± 1.0	5.33 ± 0.58	5.33 ± 0.58

*Control 1, Chemical insecticide, Reldan (20 ppm); **Control 2, insect medium without treatments

Molecular Modeling: Docking Study

Molecular docking is a method of examination providing important information on the reasoning of designing ligands for an especially active site of chitinase. This is an economical and present-day drug disclosure trend where a technology base ligand-protein interaction uncovers the pre-synthesizing prospects (Barik *et al.* 2020). The docking process was completed by simulating the interaction of chitinase with *Culex* sp. protein (PDB = 5V13) (Fig. 8) that was selected from the literature to look into the binding mode and conformation structure that contributes to the interaction between protein and compound.

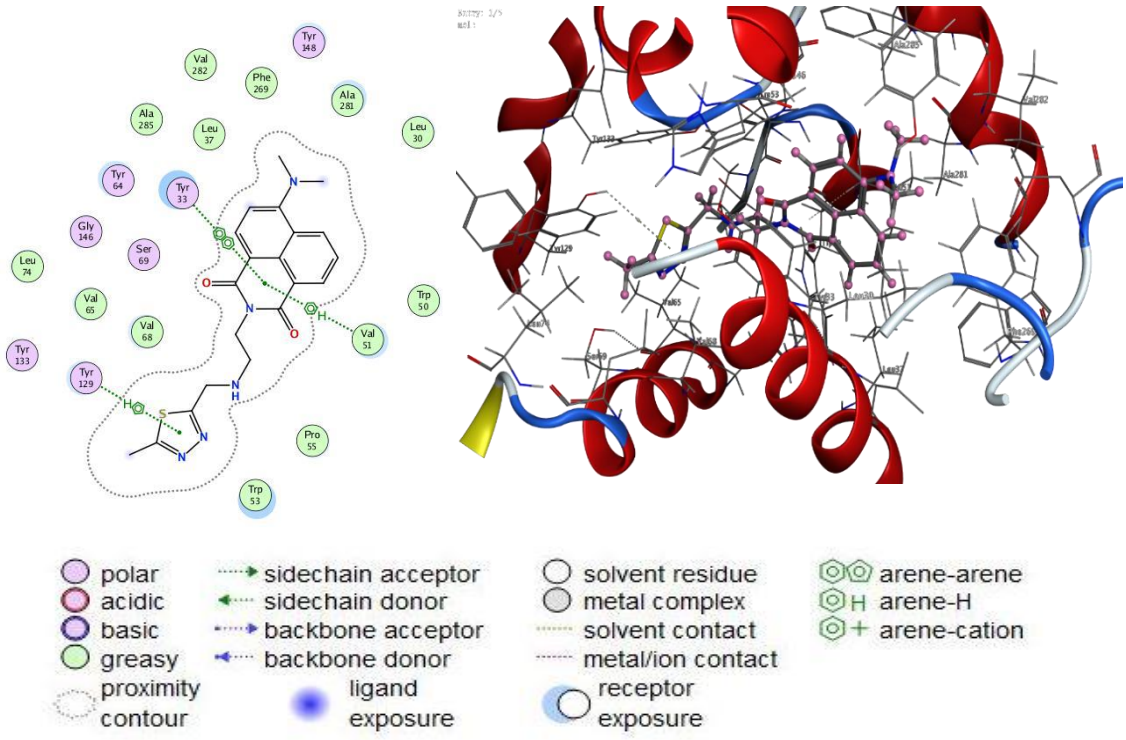
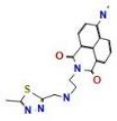
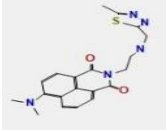
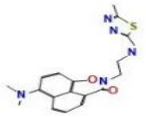
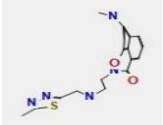
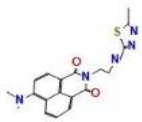


Fig. 8. 2D and 3D diagram showing the interaction between chitinase and active sites of 5V13 protein, with the representative key for the types of interaction between chitinase and protein

Table 4. Docking Score and Energies of Chitinase Acid with Protein (PDB = 5V13)

Molecule					
S	-7.6646	7.6372	-7.5996	-7.5309	-7.3158
RMSD refine	0.9821	1.4207	1.0038	1.3566	1.3440
E_conf	57.6989	60.4202	74.4378	70.3096	54.7209
E_place	-106.7121	-87.7448	-87.4209	-100.5180	-82.0538
E_score1	-11.6418	-12.8521	-11.8364	-10.8511	-11.7138
E_refine	-27.7740	-28.4611	-26.2961	-26.8656	-23.0188
E-score2	-7.6646	-7.6372	-7.5996	-7.5309	-7.3158

where S = Final score, which is the score of the last stage that was not set to none; RMSD = Root mean square deviation of the pose, in Å, from the original ligand. RMSD_refin e = RMSD between the pose before refinement and the pose after refinement. E_conf = Energy of the conformer; E_place = Score from the placement stage. E_score 1 and E_score 2 = Score from rescoring stages 1 and 2, respectively. E_refine = Score from the refinement stage, calculated as the sum of the Van der Waals electrostatics and solvation energies, under the Generalized Born solvation model (GB/VI).

From the docking investigations, the results predicted that the inhibitor chitinase has strong interaction with the active site bound to chain (B) of (5V13) and its energy value (-7.6646 kcal mol^{-1}) (Table 4). The obtained finding indicated that the energy scores of the molecular docking investigations are in good agreement with the experimental results. *Culex* sp. protein (PDB = 5V13) interacted *via* amino acid pocket molecules with chitinase through 6-ring, and 5-ring, with VAL 51 and TYR 129, respectively, by their H atoms. The distance was 4.26 Å and 4.58 Å, as illustrated in Table 5.

Table 5. Chitinase Interaction with 5V13 Protein

Chitinase	Receptor	Interaction	Distance (Å)	E (kcal/mol)
6-ring	CA VAL 51 (B)	Pi-H	4.26	-0.5
5-ring	OH TYR 129 (B)	Pi-H	4.58	-0.8
6-ring	6-ring TYR 33 (B)	Pi-Pi	3.86	-0.0

CONCLUSIONS

1. NaCl at 5% enhanced the productivity of chitinase under most conditions as well as its applications. Halostability of chitinase was better at 10% NaCl than 5% NaCl, particularly for 20 and 40 min.
2. From the obtained results, chitinase may applied to fight phytopathogenic as well as mycotoxigenic fungi, namely *C. lunata*, *F. oxysporium*, *A. niger*, and *A. terreus* to avoid its harmful effect on plants and grains.
3. Inhibitory activity of chitinase against *C. pipiens* was confirmed *via* a molecular docking study.
4. Halotolerant nature of the chitinase has great biotechnological potential, prompting a more detailed investigation in a future study.

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