Amanita sp. from Subtropical Region of Saudi Arabia as a Source of Chitinase Enzyme and its Antifungal Activity

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Mushroom products have been used as a biotechnological tool for many applications. Particularly, thermostable chitinase plays a vital role in biowaste management and biological control. In the present investigation, *Amanita sp.* was recorded in the subtropical region of Saudi Arabia, therefore, it was utilized for chitinase production using substrates chitin and dead fungal mycelia (DFM). Compared with the DFM, chitin was more suitable for chitinase activity at different temperatures and pH. *Amanita sp.* produced chitinase up to 70 °C, but the optimum was 50 °C. The chitinase activity was 4.98, 3.5, and 0.9 $U.mg^{-1}$ with the use of chitin, while it was 4.6, 3.1, and 0.6 $U.mg^{-1}$ with the use of DFM at 50, 60, and 70 °C, respectively. Chitinase activity was stable up to 60 °C, then it began to decrease at 70 °C. The chitinase activity was better at pH 4 and 5 than pH 8 and 9. The antifungal effect of the produced chitinase at 50 °C was more effective than at 60 °C. For instance, the *Alternaria alternata* colony radius was 3.50 cm and 2.26 cm at 50 °C while it was 4.35 cm and 4.13 cm at 60 °C when using DFM and chitin, respectively.

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INTRODUCTION

The genus *Amanita* is one of the biggest genera of basidiomycetous with around 500 taxa identified globally. Some of its most toxic species include *Amanita phalloides* (Fr.) Link, *A. verna* (Bull.: Fr.) Lam., *A. virosa* (Fr.) Bertillon, and *A. fuliginea*. One of the dangerous *Amanita* species has been linked to more than 90% of the fatal occurrences of mushroom poisoning in humans. The death cap, *A. phalloides*, is the most notorious member of this genus and has attracted a lot of media attention (Enjalbert *et al.* 1996). Bioremediation of heavy metals was successful using *Amanita spp.* According to Podlasińska *et al.* (2015), *A. citrina* collected Pb at the greatest amount. According to Yang *et al.* (2018), the Amanitaceae family has 1000 species globally, including roughly 50 species in tropical Africa. According to descriptions, the genus *Amanita* (Amanitaceae) is an ECM genus that forms symbiotic relationships with its neighboring plants (Onguene and Kuyper 2012). In Saudi Arabia, Manzelat (2019) reported the occurrence of numerous mushrooms in the Jizan and Abha regions, including *Amanita spp.*, *Morchella spp.*, *Agrocybe spp.*, *Boletus spp.*, *Coprinus spp.*, *Podaxis spp.*, *Lepiota spp.*, *Pleurotus spp.*, and *Agaricus spp.*

The capacity of numerous ECM fungi to enzymatically hydrolyze different components of plant and fungal cell walls is becoming increasingly clear. This suggests that certain nutrients may be obtained from refractory materials as well as simple organic substrates (Leake *et al.* 2002). Because the majority of the *Amanita* species in this genus participate in ECM connections with higher plants, cultivating an *Amanita* species is often a challenging process. Despite this, more than 10 different *Amanita* species have been successfully cultivated (Pringle *et al*. 2009).

The second most prevalent biopolymer on this planet next cellulose is chitin, a linear polymer formed of 1,4-N-acetylglucosamine. In addition to the interior structures of various invertebrates, chitin may be found in the external skeletons of crabs, algae, shrimp, yeasts, insects, lobsters, and fungi (Shahidi and Abozaytoun 2005). Sizes of chitinases, which are glycosyl hydrolases, range from 20 kDa to approximately 90 kDa (Bhattacharya and Gupta 2007). Both live and dead fungi have been found to have their cell walls lysed by chitinase. Numerous biotechnological uses for chitinases include the production of pharmaceutically remarkable chitooligosaccarides and acetyl-D-glucosamine (Sørbotten *et al.* 2005), controlling pathogenic fungus (Bakri *et al.* 2022; Ekundayo *et al.* 2022), treating chitinous waste (Dahiya *et al.* 2005), isolating protoplasts from fungi and yeasts (Vyas and Deshpande 1991), making single-cell proteins (Krishnaveni and Ragunathan 2014), and dye removal (Abdel Ghany *et al.* 2019). Chitinases have crucial physiological and ecological roles in a variety of species, including fungi, bacteria, insects, higher plants, and mammals (Mathew *et al.* 2021; Al-Rajhi *et al.* 2022a,b). Previously, Mucha *et al.* (2006) studied the creation of chitinase by *Amanita muscaria*, *Laccaria laccata*, and *Suillus bovinus* with the use of various substrates. They found that monitored that *Amanita muscaria* was the highest producer of chitinase. The advantage of thermostable chitinases have an advantage over mesophilic chitinases is that Thermostable chitinases can break down the substrate at relatively high temperatures and display decreased viscosity, significantly lower risk of contamination, thermal and chemical stability and better solubility (Akram *et al.* 2021). Thermostable as well as thermophilic chitinases are increasing popularity in current years, as they can tolerate high temperatures and preserve the stability of chitinases for longer periods.

The Jazan region in Saudi Arabia is characterized with hot climate conditions; thus, these environments were thriving with thermophilic microorganisms and its thermostable products such as enzymes. Most previous investigations are focused on a newly discovered species of mushrooms in Saudi Arabia but have not considered their biotechnological. Therefore, the production of chitinase under stress conditions of temperatures and its antifungal activities were the target of the present investigation.

EXPERIMENTAL

Materials and Methods

Isolation and Culturing of Pure Fungus

The chitinase producer was isolated from Subtropical Region of Saudi Arabia known Abu Arish, Jazan governorate (16°96′26.5″N, 42°83′55.6″E). In a rich home garden with grasses and wood residues, mature basidiocarps of basidiomycetes were observed. Under appropriate conditions they were collected for identification according to Yang (2015), Kounbo *et al.* (2019), and Vizzini *et al.* (2020). Macroscopic examination was

recorded utilizing standard manuals and keys for identification as well as structure of gills and spores. Additionally, stipe width and length, spacing of the lamellae, and existence of ring and volva were remarked. Structure of the collected fruiting bodies was documented *via* different sections of the sample of collected mushroom. Moreover, a spore print illustrating the internal construction of the collected mushroom gills was taken. Pure fungus culture was obtained from young of fruiting body, a primary culture was prepared by eliminating a small piece from gill , then placing it in tube containing appropriate volume medium containing the following ingredients per 100 mL distilled water (Dextrose 1.5 g, peeled potato 20 g, $MgSO_4$ 0.1 g, KH_2PO_4 0.1 g, KNO_3 0.1 g, $(NH_4)_2HPO_4$ 0.05 g, $CaCl_2$ 0.025 g, thiamin–HCl 0.02 mg, malt extract 12 mL and 2 g agar) adjusted at pH 5.6 .Fungus culture was incubated at 30 °C for 15 days. The appeared mycelial masses were purified several times using the same growth medium to obtain pure culture.

Chitinase Production and its Thermal Stability at Different Temperatures

Chitinase production was estimated at a range of incubation temperatures from10 to 70 °C. The flasks containing growth media with colloidal chitin or dead fungal biomass (*Aspergillus niger via* autoclaving) were inoculated with the tested fungus, followed by incubation for 12 days**.** Once the period of incubation finished, the mycelia that had appeared were detached from the metabolized growth medium, then *via* 0.5 µM bacteria-proof filter the broth was filtrated, which was followed by centrifugation for 5 min at 8,000 rpm. The obtained supernatant was precipitated using ammonium sulphate at 0-100% ranges at 4 °C and continuously stirred up to 12 h. To obtain the precipitate, the supernatant was re-centrifuged at 9,000 rpm at 4 °C for a period of 30 min. The obtained precipitate was dissolved within 0.2 M of phosphate buffer (pH 9), then dialyzed against the same buffer to obtain crude chitinase, then the activity of produced chitinase at each incubation temperature was estimated. The thermal stability of chitinase was assessed, where the obtained enzyme was incubated at separate temperature from 10 °C up to 70 °C for 60 min, and then utilized a water bath (WBL-10LC-SSD1**,** LW Scientific, Lawrenceville, GA, USA). Activity of enzyme was detected as mentioned previously (Al Abboud *et al.* 2022).

Chitinase Production at Different pH

Chitinase production was estimated within a range of pH from 3 to 9. The flasks containing growth media with colloidal chitin or dead fungal biomass were adjusted at different pH, then inoculated with the tested fungus, followed by incubation for 12 days**.** Once the incubation period finished, the activity of chitinase was estimated.

Chitinase Activity Assessment

The filtrate metabolized medium of fungus growth (100 µL) was fused with colloidal chitin (200 µL) and mixed with sodium acetate buffer (10 mM) that adapted at pH 5.2. For 1 h at 50 °C, the reaction mixture was reserved, then using dimethylamino benzaldehyde (DMAB) reagent (Sigma-Aldrich Chemical Co., Ltd., St. Louis, MO, USA), the liberated N-acetylglucosamine was quantified. A certain. Certain volume of the mixture reaction (0.5 mL) was mixed in the test tube with the prepared 120 mM potassium borate buffer (0.5 mL) that equilibrated at pH 8.9 followed by warming for 3 min in boiling water *via* water bath. After that, it was cooled, and 3 mL DMAB were added and subsequently kept at 38 °C for 20 min, re-cooled at 20 °C, then at 544 nm the absorbance was recorded spectrophotometry (Jasco, Japan). The liberated N-acetylglucosamine was estimated *via* calibration of stock curve of N-acetylglucosamine

in borate buffer (Reissig *et al.* 1955), and the quantity of liberated protein was evaluated according to Bradford (1976) using bovine serum albumin.

Fungal Inactivation by Chitinase

Under sterilized conditions,100 U/mL of the produced chitinase using the two substrates (Chitin and dead fungal biomass) were added to the autoclaved Czapek Dox Agar medium (Sigma-Aldrich Chemical Co., Ltd ,St. Louis, MO, USA). Then, the growth medium was poured into sterilized Petri plates, followed by inoculation with 0.6 mm of active colonies of tested fungi (*Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus terreus*, *Curvularia lunata*, *Cladosporium cladosporioides*, and *Alternaria alternata*) in the center of plates. They were incubated at 30 °C for 7 days. Once the incubation time finished, the inhibitory potential of chitinase was measured by measuring the colony radius.

Statistical Analysis

Standard deviation of all results were recorded from calculation of the mean three obtained results.

RESULTS AND DISCUSSION

Typical mature ascocarps (Fig. 1) were observed during a dry period after 14 days of rains in Abu Arish rejoin. The collected ascocarps (Fig. 2) were subjected for characterization and identification. The isolate was identified for level of genus as *Amanita sp.*

Fig. 1. Mature ascocarps of *Amanita sp.* growing in soil richened with grasses

Fig. 2. Basidiocarp of *Amanita sp.* annulus and the scales on the cap

In Jazan rejoin, nine genera of mushroom were observed including *Amanita*, *Agaricus*, *Boletus*, *Pleurotus*, *Podaxis*, *Lepiota*, *Coprinus*, and *Agrocybe* (Manzelat 2019), the highest frequency was associated to *Lepiota procera*, *L. rhacodes*, and *Agaricus bisporus.* However, in previous study (Abou-Zeid and Altalhi 2006), *Pleurotus ostreatus*, *Lepiota cristata*, and *Boletus* rarely occurred. Some species of mushrooms were firstly recorded by Jamal *et al.* (2019) in Saudi Arabia including *A. pediades*, *C. scabella*, *P. conopilea*, *T. fimbriatum*, and *P. demidoffii* from Al-Baha, while *C. palaeotropicum* and *C. macrospora* were collected from Jeddah and Al-Taif, respectively. The collected isolate was recorded previously as mentioned but not subjected for biotechnological application, particularly chitinase production. Therefore, it was tested for enzyme production at different conditions using different substrates.

From Fig. 3, two parameters including different temperatures up to 70 °C and different substrates (namely dead fungal biomass and chitin) were effective for chitinase production. It is clear that chitinase activity increased with increased temperature up to 50 °C, . Then it decreased and continued in activity until 70 °C. It is also evidenced from the Fig. 3 that the chitinase activity at 60 °C was higher than its activity at 20 and 30 °C. These results reflected growth of the producing fungus in the climatic condition of the Jazan region, which is characterized by high temperatures. In contrast, chitin encouraged the chitinase activity compared with fungal dead biomass at all tested temperatures but at a narrow spectrum. The activity was 1.2, 2.76, 3.2, 3.4, 4.98, 3.5, and 0.9 $U.mg^{-1}$ using chitin, whereas the activity was 0.9, 2.1, 2.98, 3.2, 4.6, 3.1, and 0.6 $U.mg^{-1}$ using dead fungal biomass at 10, 20, 30, 40, 50, 60, and 70 °C, respectively. Thermostable and denaturation-resistant chitinase was produced by *A. niveus* at 65 °C and pH 5.0 (Alves *et al.* 2018). Its stability was recorded at 60 °C for up to 2 h. According to a previous report, chitin induced the fungal chitinase with maximum activity at 1% chitin after 8 days (Alves *et al.* 2018). Fungal mycelia were utilized for chitinase production in numerous examinations. Mucha *et al.* (2006) mentioned that during mycoparasitism, chitinase production was observed by a number of ECM fungi including *Amanita muscaria*, *Laccaria laccata*, *Suillus bovinus*, and *S. luteus* using different enzyme substrates including colloidal chitin and different mycelia of fungi including *Mucor hiemalis*, *Trichoderma harzianum*, and *T. virens*. Earlier

investigation detected that the activities of root chitinase was encouraged by ECM *Pisolithus tinctorius* against phytopathogenic *Phytophthora cinnamomi* (Albrecht *et al.* 1993).

Thermal stability results indicated that chitinase produced using the two substrates was stable up to 60 °C, and then it gradually lost its activity (Fig. 4). There was no relationship among optimum temperature of enzyme activity and thermal stability. For instance in the current study, 50 °C yielded optimal enzyme activity, while thermal stability continued up to 60 °C. The property of enzyme thermal stability may depend on the fungus producer as previously mentioned and exposure time to temperature (Xia *et al.* 2001; Farag *et al.* 2016). Thermal stability studies indicated that chitinase of *C. cladosporioides* (Al Abboud *et al.* 2022), *Trichoderma viride* (Ekundayo *et al.* 2016), *Penicillium chrysogenum* (Atalla *et al.* 2020), and *A. flavus* (Beltagy *et al.* 2018) was stable up to 50 ºC. The high activity of thermostable chitinase at higher temperatures, in agreement with Guo *et al.* (2008), gave it remarkable benefits over industrial catalysis. For instance, the creation of chito-oligosaccharides from the waste of chitin conducted at elevated temperature can decrease the viscosity and contamination of the medium. Additional probable utilization is in control agents involving chitinase to fight plant infections by molds as well as insects that should be applied in the fields for long times at high temperatures (Shahidi and Abozaytoun 2005; Bakri *et al.* 2022). The conservation of the enzymatic activity beneath adverse environments is vital to achieve affirmative effects in the protection of plant from pathogens invasion.

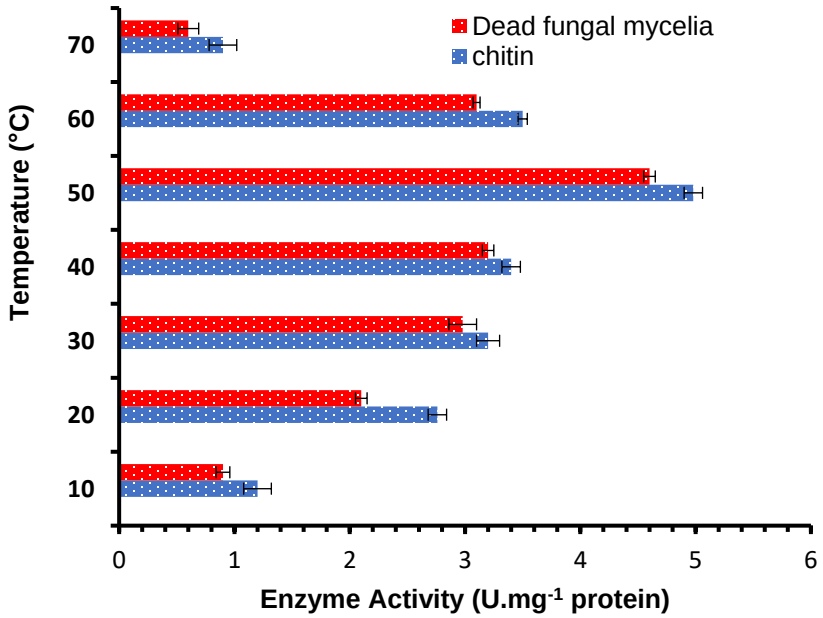

Fig. 3. Chitinase production at different temperatures using two substrates chitin and dead fungal mycelia

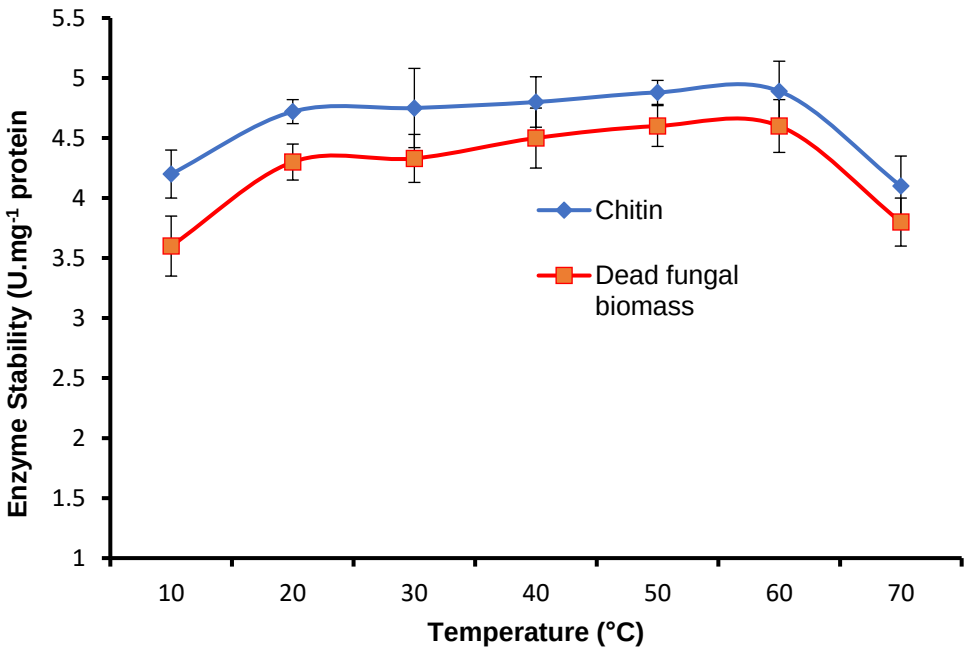

Fig. 4. Chitinase thermal stability produced using two substrates at different temperatures

Activity of chitinase at different pH was visualized in Fig. 5 using chitin and fungal dead biomass. The results showed that pH 6 was optimal for chitinase activity using the two substrates. As can be seen from the results, acidic conditions at pH 4 and 5 were preferable than alkaline conditions at pH 8 and 9 for enzyme activity. Additionally, chitin compared with dead fungal biomass was more efficient for chitinase production. This may be because of the occurrence of other components in dead fungal mycelia that interfere with the secretion of enzyme. Some investigators reported that pH 5 and 6 were optimum for fungal hydrolases enzymes (Abd El Monssef *et al.* 2016; Bhamare *et al.* 2018).

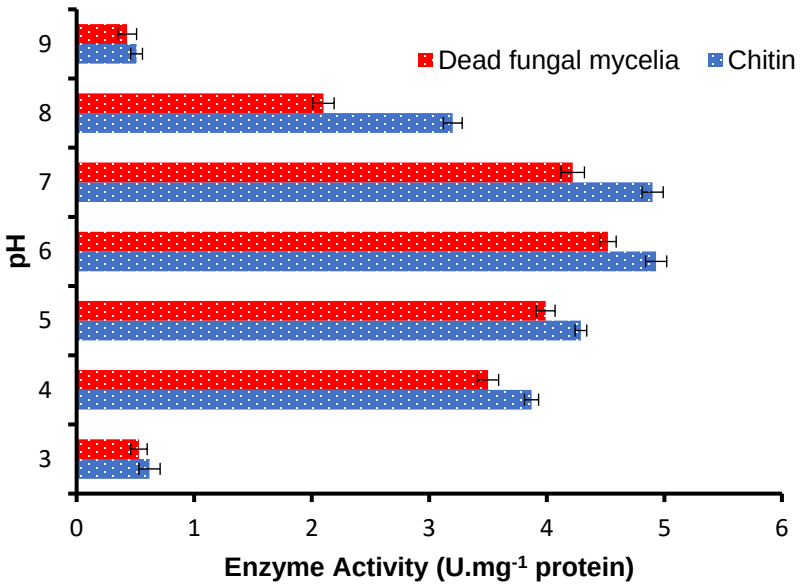

Fig. 5. Chitinase production at different pH using two substrates chitin and dead fungal mycelia

In this work, the impact of chitinase on the growth of *Fusarium oxysporum*, *Aspergillus flavus*, *Aspergillus terreus*, *Curvularia lunata*, *Cladosporium cladosporioides*, and *Alternaria alternata* was studied (Table 5). The obtained results indicated that all growth of the tested fungi decreased remarkably according to substrate used. In addition, the decrease in the growth was variable according to species. All results of fungal growth inhibition by chitinase were compared to fungal growth for control that were without treatment with chitinase. Antifungal activity of produced chitinase at 50 ºC was more than at 60 ºC, where the colony radius was less at 50 ºC than at 60 ºC. For instance, *Alternaria alternata* colony radius was 3.50 ± 0.07 and 2.26 ± 0.05 cm at 50 ºC, while it was 4.35 ± 0.07 and 4.13 ± 0.05 cm at 60 ºC using dead fungal mycelia and chitin as as substrate of enzyme, respectively. These results indicated that the chitinase has thermophilic properties. Although the chitinase activity towards the tested fungi decreased at 60 ºC, it did not lose activity. These results confirmed earlier results about the fungistatic activity of chitinase (Al-Rajhi *et al.* 2022a). Additionally, for example, Pandya and Saraf (2015) reported that the mechanism of chitinase activity against plant disease-causing fungi, such as *Macrophomina phaseolina* (60% inhibition) and *Rhizoctonia solani* (73% inhibition), *via* lysis of cell wall construction. Different levels of the antifungal potential of chitinase produced by *Aspergillus niveus* were observed with different values of MIC against different fungi (21 µg/mL for *A. fumigatus* and *Paecilomyces variotii*), (24 µg/mL for *A. flavus* and *A. phoenicis*), (84 µg/mL for *A. niger*) (Alves *et al.* 2018). A deficiency of *A. niger* chitinase activity was recorded after 3 h at 60 °C (Brzezinska and Jankiewicz 2012). In another investigation (Beltagy *et al.* 2018), the activity of *A. flavus* chitinase was stable for 15 min at 50 °C, while the activity of *Penicillium chrysogenum* chitinase was stable for 1 h at 50 °C (Atalla *et al.* 2020).

Table 1. Effect of Chitinase at Different Temperatures for 30 min on Fungal Growth

Substrate	***A. alternata* Colony Radius (cm)**		***C. cladosporioides* Colony Radius (cm)**	
	At 50 ºC	**At 60 ºC**	**At 50 ºC**	**At 60 ºC**
Control	(7.95 ± 0.18)*	(7.95 ± 0.18)*	(7.95 ± 0.14)*	(7.83 ± 0.03)*
Dead fungal mycelia	(3.50 ± 0.07)* (55.98 %)**	4.35 ± 0.07)* (59.28 %)**	4.80 ± 0.04)* (39.63 %)**	(6.50 ± 0.07)* (16.99 %)**
Chitin	(2.26 ± 0.05)* (71.57 %)**	(4.13 ± 0.05)* (48.05 %)**	3.20 ± 0.08)* (59.75 %)**	(4.88 ± 0.05)* (37.68 %)**
Substrate used	***C. lunata* Colony Radius (cm)**		***F. oxysporium* Colony Radius (cm)**	
0	(6.20 ± 0.10)*	(6.20 ± 0.10)*	(7.50 ± 0.18)*	(7.50 ± 0.06)*
Dead fungal mycelia	(4.50 ± 0.08)* (27.41 %)**	(5.10 ± 0.07)* (17.47 %)**	(6.39 ± 0.02)* (14.5 %)**	(6.70 ± 0.12)* (10.67 %)**
Chitin	(3.20 ± 0.10)* (48.39 %)**	(3.73 ± 0.05)* (39.84%)**	(3.10 ± 0.11)* (58.67 %)**	(4.43 ± 0.05)* (40.93 %)**
Substrate used	***A. flavus* Colony Radius (cm)**		***A. terreus* Colony Radius (cm)**	
0	(6.20 ± 0.03)*	(6.20 ± 0.03)*	(6.50 ± 0.14)*	(6.50 ± 0.14)*
Dead fungal mycelia	(3.85 ± 0.02)* (37.91 %)**	(4.26 ± 0.04)* (31.29 %)**	(4.20 ± 0.05)* (35.38 %)**	(4.50 ± 0.04)* (30.77 %)**
Chitin	(3.43 ± 0.05)* (44.68 %)**	4.48 ± 0.05)* (27.74 %)**	(3.50 ± 0.06)* (46.15 %)**	(3.95 ± 0.05)* (39.23 %)**

*****Colony Radius (cm); **inhibition % regarding to control as 0% inhibition

CONCLUSIONS

1. *Amanita sp.* was able to produce thermostable chitinase at 60 °C using chitin and dead fungal mycelia as enzyme substrates.
2. The produced chitinase at high temperature play a critical role in control of phytopathogenic fungi.
3. Acidic conditions at pH 4 and 5 were more effective in increasing chitinase production.

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