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Production, optimization, characterization and antifungal activity of chitinase produced by *Aspergillus terrus*

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Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. Chitinase enzyme has received increased attention due to its wide range of biotechnological applications. *Aspergillus terrus* was found to be a good chitinase producer among the five fungi isolated from different soil samples from Al-Jouf city, Saudi Arabia. Maximum production of chitinase was obtained when using 2% of Shrimp-shell powder as a sole carbon source in the fermentation medium. The high level of chitinase production was observed in the culture medium with pH 5 at 30°C for five days at shaking conditions. Some properties of the crude chitinase were studied. In the present study, the antifungal activity of crude *A. terrus* chitinase was investigated against *Apergillus niger*, *Aspergillus oryzae, Pencillum oxysporium*, *Rhizoctonia solani*, *Fusarium oxysporium*, *Rhizopus sp.* and *Mucor* sp. and also estimated. The chitinase was found to inhibit the growth of some phytopathogenic fungi tested. The present work provides a suitable medium composition for enhancement of chitinase production by *A. terrus* and some properties of crude enzyme. Moreover, the study reflects the potential of *A. terrus* chitinase for biotechnological application.

Key words: Chitinase, *Aspergillus terrus*, optimization, antifungal activity.

INTRODUCTION

Chitin, a (β-1,4)-linked homopolymer of N-acetylglucosamine is the second most abundant biodegradable polymer, can be found as a part of fungi, plants, crustaceans, insects, arthropods, and algae components (Gohel et al., 2006). Approximately 75% of the total weight of shellfish, such as shrimp, crabs and krill are considered as waste, and chitin comprises 20 to 58% of the dry weight of the said waste (Wang and Chang, 1997). Microbial degradation of insoluble macromolecules such as lignin, cellulose, keratin and chitin depends on the production of extracellular enzymes with the ability to act on compact substrate surface. The use of chitin as a carbon source by bacteria and fungi has been a subject of sporadic interest. The enzymatic degradation of chitin by microorganisms occurs in two steps: first the hydrolysis by chitinase poly (1.4(N-acetyl-

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β-D-glucosaminide) glycanohydro-ase; EC3.2.1.14) to oligomer ss, mainly dimers, followed by their degradation to free N-acetylglucoseaminidase; EC3.2.1.30). Chitinolytic enzymes are able to lyze the cell wall of many fungi. The microorganisms that produce these enzymes are able to destroy the cell wall of many fungi, and capable of eradicating fungal diseases that are a problem for global agricultural production (Budi et al., 2000). Also, chitinase find widespread applications in the field of agriculture, medicine, biochemical processing engineering, waste management, pesticide control, in food and feed, sweeteners and cell wall degrading enzyme. In viruses, chitinases are involved in pathogenesis (Dahiya et al., 2006). Other application of chitinases is bioconversions of chitin waste to single cell proteins and ethanol and fertilizers. Industrial applications of chitinases have been governed mainly by key factors such as cost production, self-life stabilities and improvement in enzyme properties by immobilization (Daizo, 2005). Due to multiple applications of chitinases, they become interesting enzymes for study.

Several microorganisms, including bacteria such as *Serratia marcescens Bacillus lichiniformis, Bacillus subitils, Bacillus thuringiensis*, and *Vibrio alginolyticus* (Joshi et al., 1989; Sowmeya et al., 2001; Wang et al., 2006; Merina et al., 2012) and many species of fungi such as: *Asergillus* sp*., Myrothecium anisopliae, Streptomyces sp., Trichoderma harzianum, Trichoderma viride,* and *Verticillium lecanii* (De Siqueira et al., 1997; Mathivanan et al., 1998; Liu et al., 2003; Nampoothiri, et al., 2004; Gunalan et al., 2012; Sowmya et al., 2012) have a chitinase producing ability. Chitinase activity in plant (Gomes et al., 1996), animals (Mana et al., 2009) and human serum has also been described recently (Mathivanan et al., 1998).

Optimization of culture media is very important to maximize the yield and productivity of enzyme, and minimize the product cost (Abdel-Fattah et al., 2005). The aim of this work was to isolate the locally prominent chitinolytic fungi from different soil samples with unique properties and optimize their fermentation conditions for maximum chitinase production.

MATERIALS AND METHODS

Shrimp and crab-shell powder

Shells of shrimp and crab were collected, washed several times in warm tap water, then distilled water and dried in an air dried oven at 60°C for 24 h. After drying, the shells were hammer milled to fine particles.

Chitin and colloidal chitin

Demineralization of the chitinous wastes was done. They were treated with 1.75 M acetic acid at room temperature for about 12 to 15 h. The ratio of waste to solvents was maintained (1:15 w/v). The dematerialized material obtained was recovered by filtration, rinsed with de-ionized water and dried in forced hot air oven at 65°C. The dematerialized material was then deproteinized with 21 ml of 5% NaOH solution for 2 h at 100°C, and rinsed with cold water. Chitin was collected on a coarse sintered glass funnel and washed with deionized water to pH 7.0. Dried material (chitin) was powdered and sieved to fine size. Colloidal chitin was prepared as follows: 5 g of the chitin powder were homogenized in 100 ml of 12 M HCl and stirred at 40°C for 2 h. The chitin was added by NaOH and collected by centrifugation. The pellet was washed twice with distilled water and lyophilized (Trachuk et al., 1996).

Sample collection and isolation of chitinolytic fungi

Sediment samples were collected from different areas of Al-Jouf in Saudi Arabia. The samples were taken from 2 to 3 cm depth with the help of sterile spatula and put in sterile bags for further processing. For isolation of chitinase producing fungi, the agar medium amended with colloidal chitin was used. The mineral synthetic medium consists of (g/L) $(NH_4)_2SO_4$, 1: K_2HPO_4, 1; $MgSO_4.H_2O$, 0.5; KCl, 0.5; NaCl, 5.0; $CaCl_2$, 0.02; $FeSO_4.7H_2O$, traces; shrimp-shell powder, 20, agar, 15; and distilled water 1 L. The pH of the medium was adjusted to 5 with KOH and HCl. The medium were sterilized by autoclaving at 121°C for 20 min. Different types of fungi were isolated from the soil samples by enrichment method. The colonies showing clearance zones on creamish background were considered as chitinase-producing fungi.

Screening of chitinase producing fungi

Fungal isolates were selected on the basis of larger hydrolysis zones after three days of incubation and further screened for maximum enzyme production in MSM. The cultures were centrifuged at 6000 rpm for 15 min at 4°C and the supernatant was used for chitinase assay.

Preparation of the crude enzyme

At the end of incubation period, the fungal mat was separated by filtration in sintered glass filter (G3). The culture filtrates of several batches were collected and pooled. The culture filtrate was centrifuged at 6000 rpm for 15 min to remove any muddy material. The clear supernatant was considered as the crude enzyme source.

Assay of chitinase activity

Chitinase activity was determined spectrophotometrically by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The reaction mixture was composed of 0.5 ml of 1% colloidal chitin suspended in 0.02 M phosphate buffer (pH 7.0) and 0.5 ml of the enzyme solution. After 30 min incubation at 40°C, 0.75 ml of 3,5-dinitrosalicylic acid reagent (DNSA) (Joshi et al., 1989) was added to stop the reaction. The suspension was heated for 10 min at 100°C and centrifuged at 8000 rpm for 10 min. The supernatant absorbance at 530 nm was measured. A standard curve was obtained using N-acetylglucosamine as a standard. One unit of the chitinase activity was defined as the amount of enzyme which yields 1 µmol of reducing sugar as N-acetyl-D-glucosamine (Glc NAC) equivalent per minute.

Protein estimation

The extracellular protein of the culture was determined by Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

Optimization of enzyme production

Effect of using different sources of chitin

To find out the best substrate for highest enzyme production, the chitinase production was carried out by using different polysaccharides of marine origin, shrimp-shell powder (2%), crab-shell powder (2%), chitin powder (1%), colloidal chitin (1%), chitosan(1%), demineralized chitin (1%), deproteinized chitin (1%), agar (1%), alginate (1%), mannan (1%), K –carrageenan (1%).

Effect of using single and mixed carbon sources

The effect of using additional carbon source supplemented in media at a concentration of (1%) such as glucose, sucrose, lactose, maltose and N-acetyl-D-glucosamine (Glc NAC) for maximum enzyme production was also investigated. The supplemented media were inoculated with 2% inoculums and fermented at an optimized chitin source.

Effect of different concentration of chitin

50 ml of sterile production medium prepared with different substrate (shrimp-shell powder) concentration varied from 5 to 40 g/l, inoculated with 2 ml spore suspension of the culture and incubated at 30°C under shaking condition (120 rpm). The culture filtrate was harvested after five days and enzyme assay as well as the extracellular protein was measured.

Effect of incubation time on chitinase production

To determine the optimum incubation time for the chitinase production, inoculated flasks were incubated in a rotary shaker in 120 rpm at 30°C for about sseven days. Every 12 h the culture filtrate was collected and checked for the chitinase activity, dry weight as well as total extracellular protein.

Effect of nitrogen source on chitinase production

To investigate the influence of using various nitrogen sources on the production of chitinase, 50 ml of production medium with different nitrogen sources such as NH_4NO_3, $NaNO_3$, urea, KNO_3 and NH_4Cl was prepared, sterilized, inoculated and incubated at 30°C for optimum incubation period under the shaking conditions. The culture filtrate was harvested and the enzyme activity, dry weight as well as extracellular protein were measured.

Utilization of fungal biomass as a source of chitin for chitinase production

Pencillium chrysogenium, *F. oxysporium*, *A. oryzae A. niger* were grown in Czapek-Dox broth. After 15 days of incubation, the fungal mats were collected and sterilized by autoclave at 121°C for 20 min. The sterilized fungal mats were washed twice with sterile distilled water and dried in an oven at 80°C till constant weight (Goel et al., 2004). The dried fungal mat was powdered and used as chitin source (1 g/l) for the production of chitinase. Three sets of flasks were used: 1) MSM in which shrimp-shell powder was replaced by fungal mat (2 g/l), 2) MSM supplemented with fungal mat (2 g/l), and 3) control only with SMS without any fungal mat.

Characterization of crude chitinase enzyme

Some properties of the crude chitinase were studied. These include the enzyme concentration (varied from 0.198 to 1.980), substrate concentration (range from 1.0 to 20.0 mg), pH value of the reaction mixture (range from 3.6 to 8.0), and the temperature of the reaction (varied from 30 to 60°C) and substrate specificity.

Effect of crude chitinase enzyme on fungal cell walls

Test microorganisms

Different fungal strains were obtained from Fungi Center, Faculty of Science, Assuit University, Egypt. The cultures were subcultured, maintained on Sabouraud's dextrose agar (SDA) medium and stored in refrigerator at 4°C.

Inoculum preparation

Fungal inoculums were prepared by inoculating a looful of test organisms in 5 ml of Sabouraud's dextrose agar medium and incubated at 30°C for three days.

Antifungal activity by clearance zone

The antifungal activity was assayed *in vitro* by inhibiting the growth of fungus on Sabouraud's dextrose agar (SDA) medium. A spore suspension of pathogenic fungi was uniformly spread on plates of SDA medium. Discs that were soaked with the crude enzyme (50 U) were laid on the inoculated plates; the control was disc-soaked in boiled-enzyme extract. Fungal growth and the clearance zones were observed over two to five days of incubation at 30°C (Haggag and Abdallh, 2012).

Antifungal activity by measuring the libration of sugars

Fungal mycelia were homogenized by an ultraturrax blender for 15 s washed three times with 300 ml deionized water on filter paper, and then suspended in 15 to 20 ml of deionized water. These mycelia were used for estimation of sugar libration from whole cells (Beyer and Diekmann, 1985).

Statistical analysis

All results are made in triplicates, and the values are the mean values. An analysis of variance (ANOVA) for mean comparison was used.

RESULTS AND DISCUSSION

A total of 10 morphologically different chitinolytic fungi were isolated from different soil samples from the market of fishery in Al-Jouf, Saudi Arabia. On the basis of colloidal chitin degradation and zone of clearance, five isolates were selected for secondary screening in mineral synthetic broth medium and tested for chitinase activity. All isolates are identified by Faculty of Science, Assiut University, Egypt. Based on maximum chitinase production (4.82 U/mg protein) after 5 days of incubation, the *A. terrus* was selected for further and more detailed

Table 1. Chitinolytic activity of the tested isolates grown on shrimp-shell Powder as a sole carbon source.

Isolated strain	Final pH	Extracellular protein (mg/ml)	Chitinase activity (U/mg protein)
Aspergillus terrus	5.6	1.13	4.82±0.011
Aspergillus oryzae	5.8	1.10	4.27±0.011
Aspergillus niger	5.9	1.03	3.87±0.023
Pencillium chrysogenium	6.2	0.96	2.76±0.030
Fusarium sp.	6.0	0.90	2.05±0.050

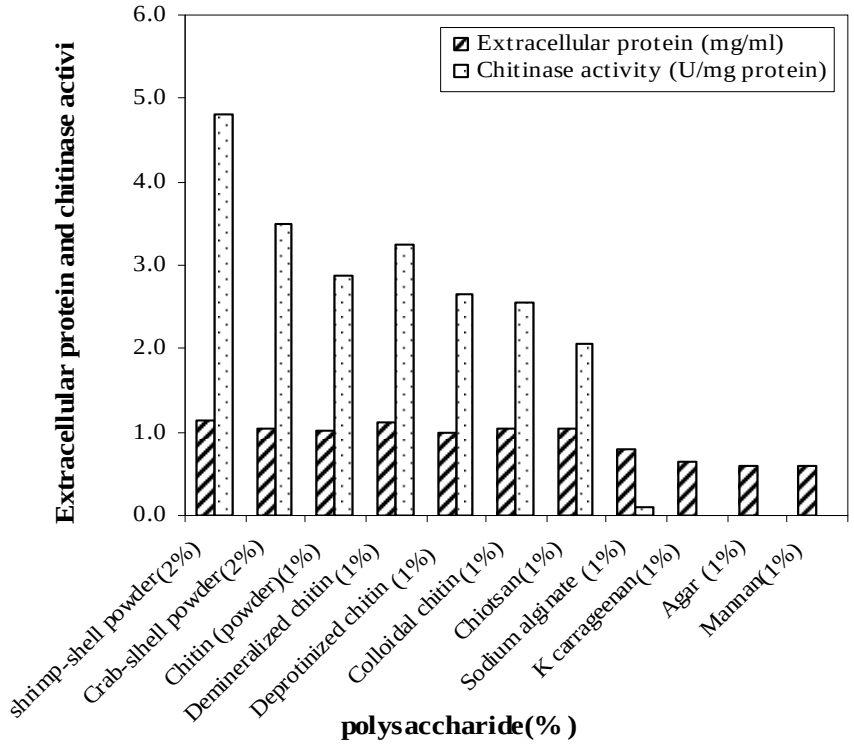

Figure 1. Extracellular cellular and chitinase activity of *Aspergillus terrus* as grown on some polysaccharides of marine origin.

(Table 1). Among the various substrates like shrimp-shell powder, crab-shell powder, chitin powder, colloidal chitin, chitosan, demineralized chitin, deproteinized chitin, sodium alginate, k carrageenan, agar, and mannan, the shrimp-shell powder (2%) was found to be the best substrate for maximum chitinase production (4.82 U/mg protein) (Figure 1). Similar observation has also been reported with *Bacillus amyloliquefaciens* (Sabry, 1992), *Beauveria brassiana* (Suresh and Chandrasekaron, 1999) and *Verticillum lecanii* (Matsumoto et al., 2004). On the other hand, Kuddus and Ahmed (2013) used colloidal chitin for the production of chitinase by *Aphanocladium.*

To optimize the shrimp-shell powder concentration, the effect of various levels was investigated and the results are shown in Figure 2. The activity obtained in the culture filtrate increased by 4.9-fold by increasing shrimp-shell powder concentration from 5 to 20 g/l. Higher shrimp-shell powder concentrations increased the viscosity of the medium and thus reduced the oxygen supply which yield lower growth and enzyme production. These results are in good agreement with many reported for the production of chitinase by other microbial strains (Trachuck et al., 1996; Wang and Chang, 1997).

To determine the effect of using single and mixed carbon sources on chitinase production, experiments were carried out with *A. terrus* in which chitin medium was supplemented with additional carbon source in a concentration of 1% (w /v). Data on the effect of several carbon sources on chitinase production by *A. terrus* are represented in the Table 2. Enzyme production was supported in the presence of all sugars in the medium alone or with the chitin as compared to control. Joo

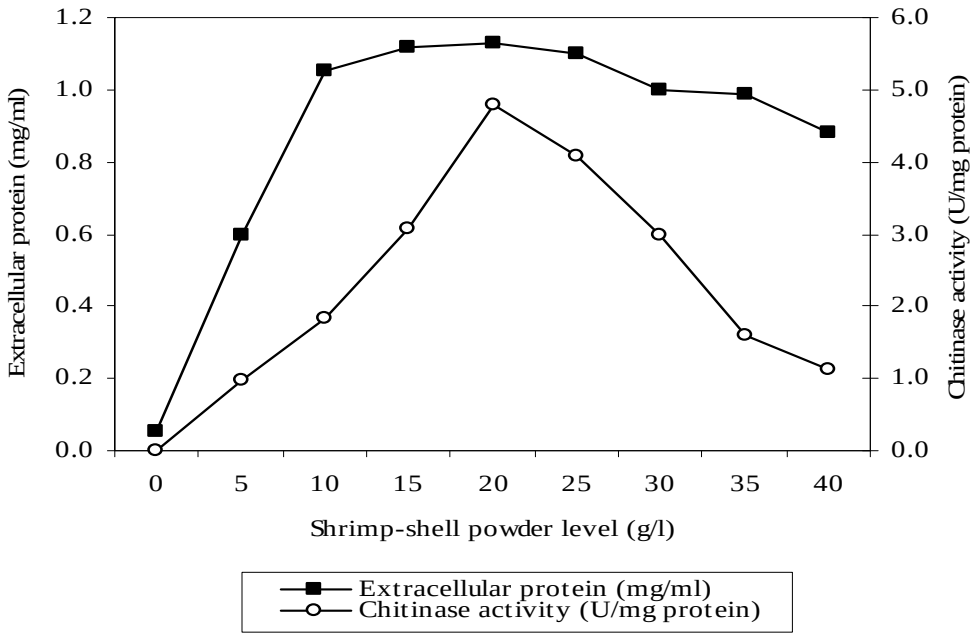

Figure 2. Chitinase activity and extracellular protein of *Aspergillus terrus* culture grown in different concentration of shrimp- shell powder.

Table 2. Effect of using single and mixed carbon sources on chitinase production *by A. terrus* cultures.

Carbon source	Final pH	Extracellular protein (mg/ml)	Chitinase activity (U/mg protein)
Glucose (1%)	6.2	2.25	0.86± 0.11
Shrimp-shell powder + glucose	6	2.55	2.98± 0.06
Sucrose	6.5	2.16	0.75± 0.04
Shrimp-shell powder + sucrose	6.2	2.52	2.55± 0.04
Lactose	6.4	1.85	0.5± 0.01
Shrimp-shell powder + lactose	5.9	2.11	1.98± 0.03
Maltose	5.8	1.65	0.45± 0.02
Shrimp-shell powder + maltose	5.8	1.89	1.55± 0.04
N-acetylglucosamine	5.8	0.99	0.35± 0.04
Shrimp-shell powder + Glc-NAGA	5.8	1.05	0.98±0.02

(2005) reported that glucose (0.4%) along with chitin induced high levels of chitinase by *Streptomyces halsteolii*. Narayana et al. (2007) found that amended of starch 0.2% to the colloidal chitin medium increased chitinase production by *Streptomyces* sp. ANU6277.

The effect of various nitrogen sources on chitinase production was studied. The nitrogen source constituent was replaced on equal nitrogen basis, by NH_4NO_3, $NaNO_3$, urea, KNO_3 and NH_4Cl. As shown in Figure 3. The production of chitinase was influenced by the nitrogen source then incorporated into the medium. The highest activity (6.28 U/mg protein) was recorded at ammonium sulphate at a concentration of 1%. Ammonium sulphate is a cheap nitrogen source and represented an advantage for the production of chitinase by low cost fermentation. These results are in good agreement with those reported for the production of chitinase by *Aspergillu*s sp. SI.13 (Rattanakit et al., 2002).

Role of fungal biomass on chitinase production

The cell wall of most fungi contains chitin as the major component, chitinase enzymes are well known to lyse the cell wall of both live and dead fungi (Ueno et al., 1990). Utilization of dead mass of *F. oxysporium*, *A.* oryzae, *A. niger* and *Pencillium citrinum* by *A. terrus* for chitinase

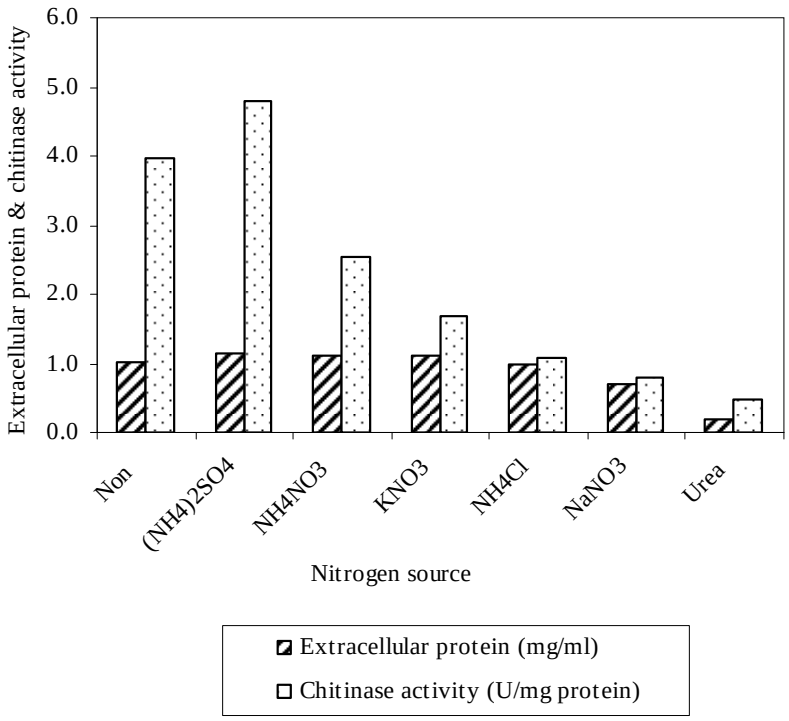

Figure 3. Chitinase activity and extracellular protein of *Aspergillus terrus* cultures grown in different nitrogen source.

production was studied (Figure 4). The production of chitinase enhanced in MSM amended with dried dead fungal mats over MSM. On the other hand, the enzyme production decreased when the fungal mats replaced shrimp-shell powder from MSM. An increase in chitinase production was also observed from *Streptomyces aureofaciens* and *Streptomyces halstedii* when cultured in a medium containing colloidal chitin supplemented with fungal cell wall preparations (Joo, 2005; Kumar and Gupta, 2006). Temperature affects various biological processes, therefore the growth of fungi and enzyme production are also affected with the change in incubation temperature. In the present investigation, the increased of incubation temperature from 25 to 35°C increased the growth and the production of the enzyme where maximum enzyme activity (14.88 U/mg protein) obtained at an optimum temperature of 30°C (Figure 5). These results are in good agreement with those reported by other workers (Sandhya et al., 2004, Sowmya et al., 2012). On the other hand, the highest production of chitinase by other fungi was carried out at optimum temperature ranging from 26 to 28°C (Sherief et al., 1991; Brzezinska and Jankiewicz, 2012).

The results showed that an initial pH range from 4 to 9 seems appropriate for both growth and chitinase production. However, pH 5 for *A. terrus* was the optimum value for the highest enzyme activity (Figure 6). Higher or lower pH values showed an adverse effect on the enzyme production. These results are in line with other chitinase production by *Aspergillus carneus* (Sherief et al., 1991). In contrast, the high level of *A. terrus* chitinase is observed in culture medium with pH 6.5 (Ghanem et al., 2010).

Some properties of crude chitinase enzyme

The effect of enzyme concentration on the activity of chitinase is shown in Figure 7, the maximum activity was obtained using 1.188 mg of the crude chitinase extract. The effect of colloidal chitin concentration on the chitinase enzyme (Figure 8) shows that maximum chitinase activity was obtained at 7.5 and 10.0 after which the activity falls slightly. A decrease in the chitinas activity 12.5 mg colloidal chitin may indicate accumulation of intermediates that results from chitin decomposition into the medium which make up a synthetic inhibitor of chitinase itself. The chitinase activity was determined using crude enzyme concentration (1.188 mg) and colloidal chitin as a substrate (7.5 mg) at different temperature ranging from 30 to 60°C is shown in Figure 9. The optimum temperature for chitinase activity was found at 40°C, above that the activity was decreased. A drop in the activity may due to heat inactivation of the enzyme. Similar temperature values were determined for chitinase from different microorganisms (Sherief et al.,

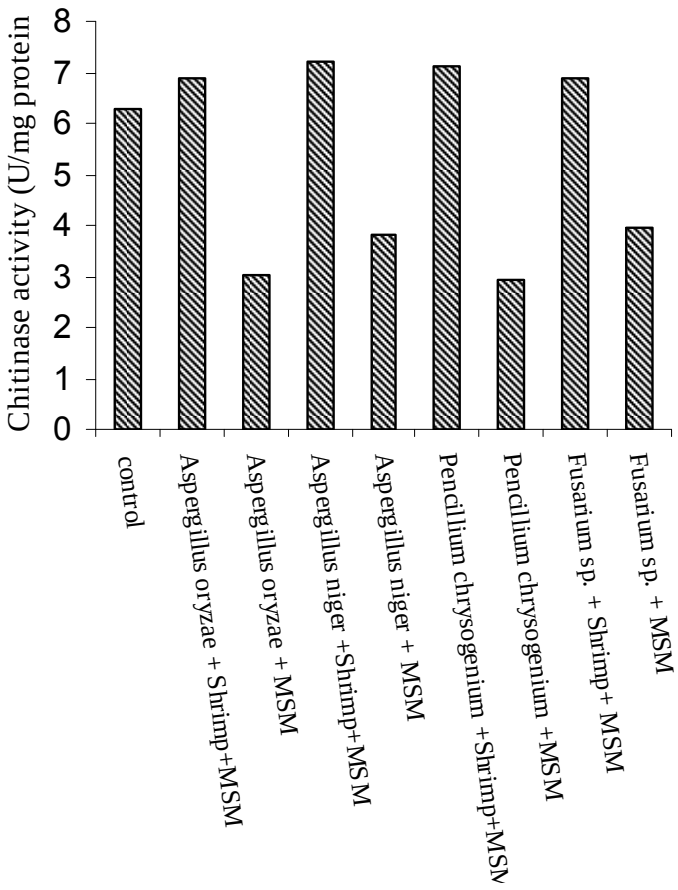

Figure 4. Utilization of fungal mate for chitinase production by *Aspergillus terrus* (values are means of three replicates + SD).

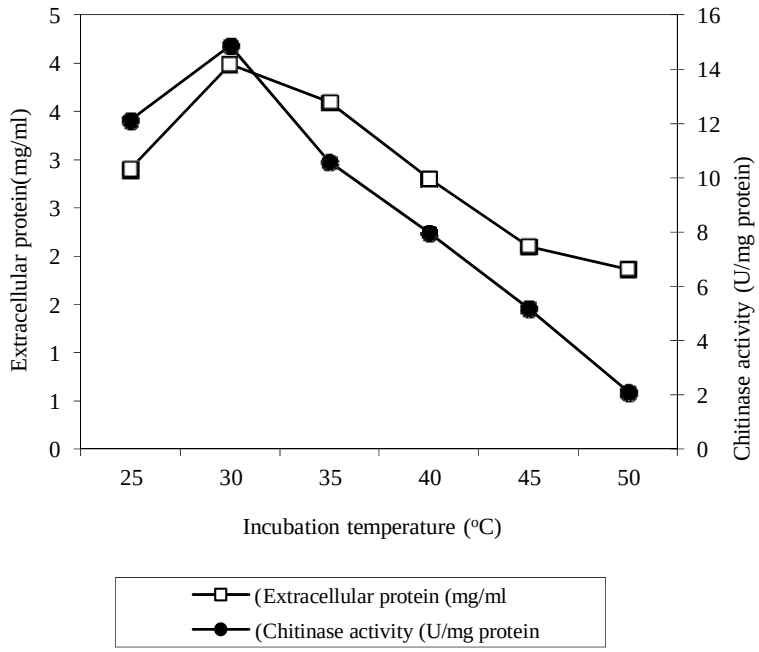

Figure 5. Chitinase activity and axtracellular protein of *Aspergillus terrus* cultures at different incubation temperature.

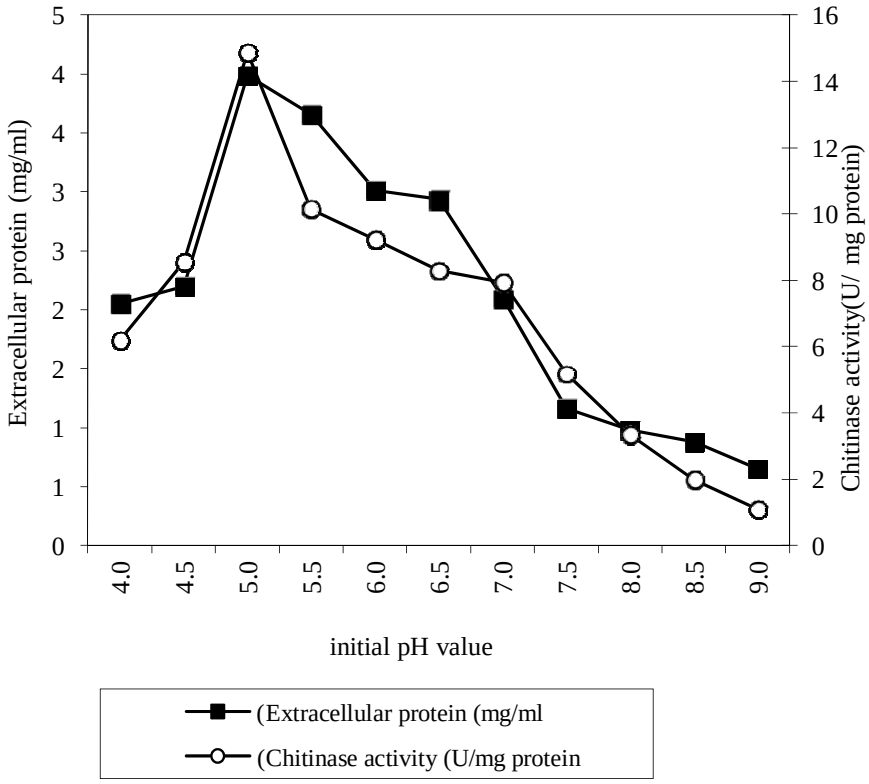

Figure 6. Chitinase activity and extracellular protein of *Aspergillus terrus* cultures at different incubation temperature**.**

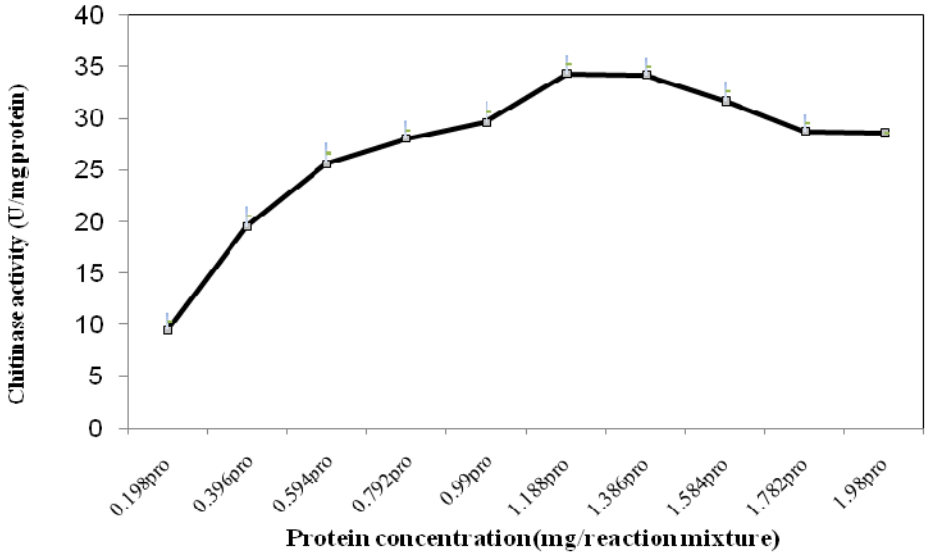

Figure 7. Effect of enzyme protein concentration on the activity. Each data point represents means ± standard deviation (n=3).

1991; Kinz et al., 1992, Brzezinka and Jankiewcz, 2012). The effect of pH of the reaction on the chitnase activity is shown in Figure 10. It shows that the maximum activity was detected at pH 5.2. These results are in good agreement with other microbial chitnases (Dahyia et al., 2005; Sandhya et al., 2004). On the other hand, the optimal pH for the crude chitinase produced by *A. niger* LOCK62 was found to be 6.5 and for *A. carneus* was 4.5

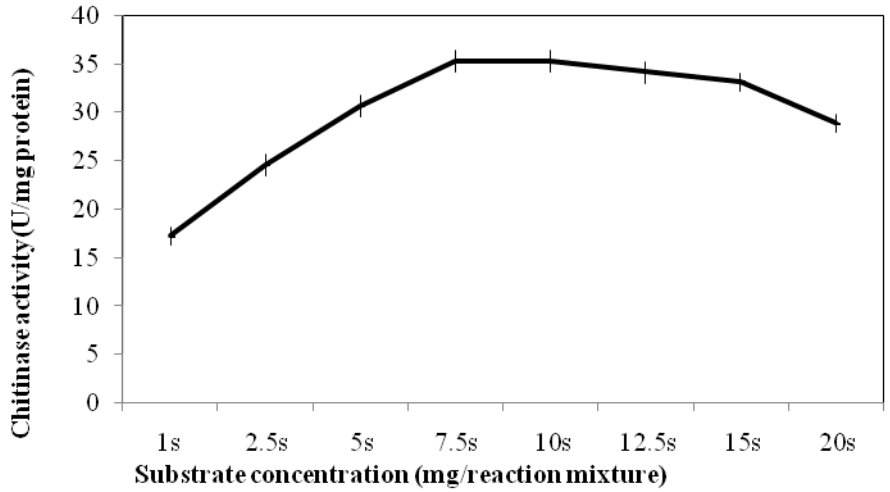

Figure 8. Effect of substrate concentration on the crude chitinase activity. Each data point represents means ± standard deviation (n=3).

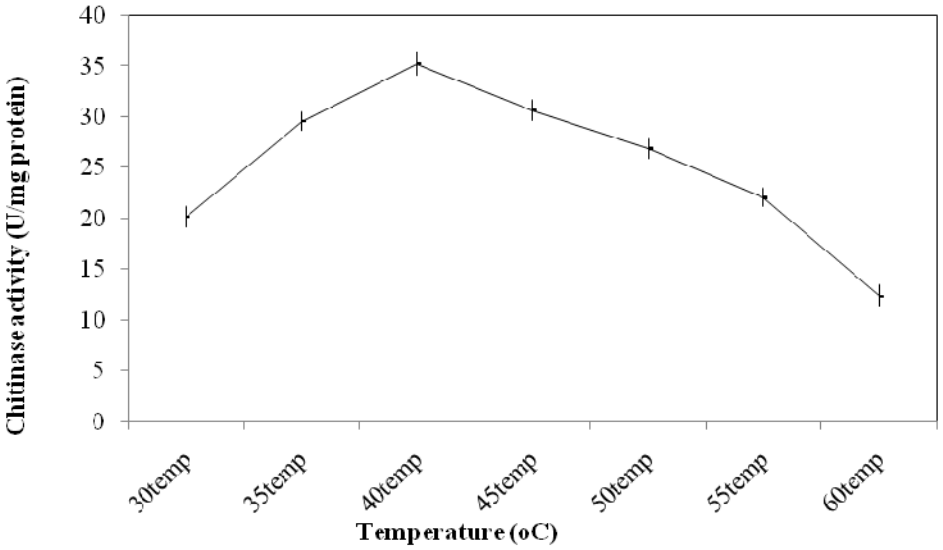

Figure 9. Effect of temperature of the reaction on the crude chitinase activity. Each data point represents means ± standard deviation (n=3).

(Brzezinka and Jankiewcz, 2012).

The ability to degrade several polysaccharides is an important criterion of chitinase potency. Table 3 compares the digestive capability of this chitinase on nine substrates. It is evident that the chitinase had better digestive ability on colloid chitin than other polysaccharides under the same assay condition. The relative activity of chitinase only reached 52.6% for chitin powder and 8.3% for chitosan substrate that when colloid chitin was used as the control. On the other hand, no any chitinase activity with n-carboxymethyl cellulose, cellulose, starch, sodium alginate, agar, and K-carragennan. From these results, *A. terrus* chitinase has high specificity which could only degrade glycosidic bond GlcNAc-GlcNAc. Similar results are reported from chitinase of thermophilic *Bacillus* sp. HU1 (Dai et al., 2011).

Antifungal activity of crude chitinase enzyme

In the present study, the antifungal activity of chitinase was tested against the seven different plant pathogenic fungi, *A. niger*, *A. oryzae, P. oxysporium*, *R. solan*i, *F.*

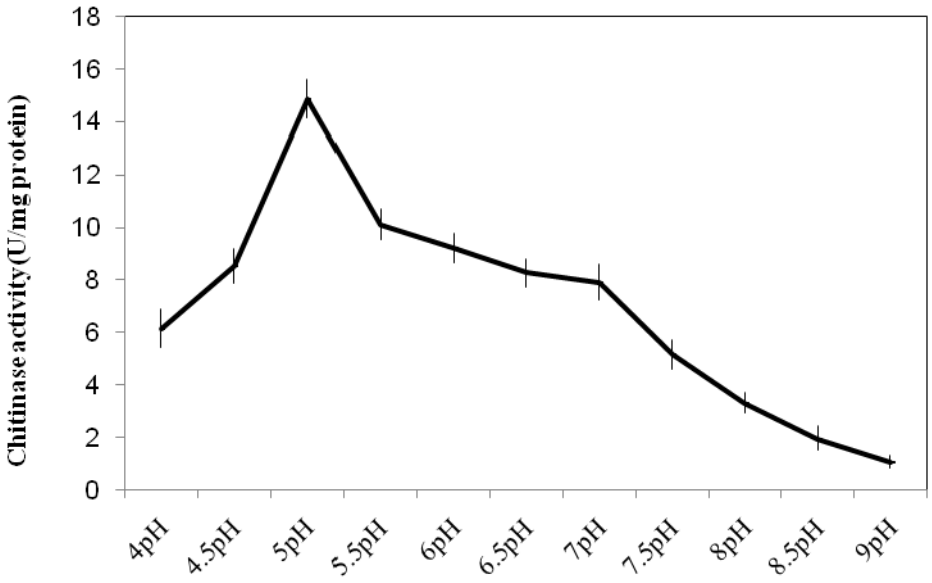

Figure 10. Effect of pH of the reaction on the crude chitinase produced by *Aspergillus terreus.* Each data point represents means ± standard deviation (n=3).

Table 3. Chitinase activity on various polysaccharide substrates.

Substrate	Relative activity (%)
Colloidal chitin	99.97±0.011
Powder chitin	52.8±0.015
Chitosan	8.3±0.050
Carboxymethyl cellulose	0
Cellulose	0
Starch soluble	0
Sodium alginate	0
Agar	0
K-carrengeen	0

Table 4. Antifungal activity of crude chitinase of *Aspergillus terrus.*

Fungi	Diameter of the zone of inhibition (mm)
Aspergillus niger	20±0.090
Aspergillus oryzae	15±0.050
Pencillum oxysporium	12±0.032
*Rhizoctonia solan*i	7±0.050
Fusarium oxysporium	5±0.035
Rhizopus sp.	0
Mucor sp.	0

oxysporium, Rhizopus sp and *Mucor* sp. Among them, the chitinase showed maximum inhibitory activity against *A. niger*. (20 mm in diameter), followed by *Aspergillus versicolor* (15 mm in diameter*) P. oxysporium* (12 mm in diameter) and *R. solan*i (7 mm in diameter). The fungus *F. oxysporium*. (5 mm in diameter) recorded the least inhibitory activity (Table 4). The growth of *Rhizopus* sp. and *Mucor* sp. was not inhibited. These results are in partial agreement with Brzezinska and Jankiewicz (2012) who found that the chitinase produced from *A. niger* LOCK 62 has an antifungal activity against many phytopathogens fungi. Many authors studied the antifungal activity of chitinase enzyme from different microorganisms (Howell, 2003; Kavitha et al., 2005; Kim and Chung, 2004; Patel et al., 2004; Zhao et al., 2010).

Another method for determination of the effectiveness of an enzyme preparation in degrading fungal cell walls was done by the estimation of the rate of sugar libration (Thomas et al., 1979). The libration of glucose and N-acetylglusoamine from the cell walls of 6 different fungi by the crude chitinase was determined as shown in Figure 11. The degradability of the cell wall polysaccharides can be compared by taking the rate of sugar libration; higher sugar libration activities were observed with ascomycetes and deuteromycetes , while no sugar libration with zygomycetes. Our results are in line with Farag (2004) who found that the *Bacillus brevis* chitinase could degrade the cell walls of filamentous ascomycetes and deteuromycetes, but was inactive with zygomycetes. Also, it was in a good agreement with the results observed by Beyer and Diekmann (1985).

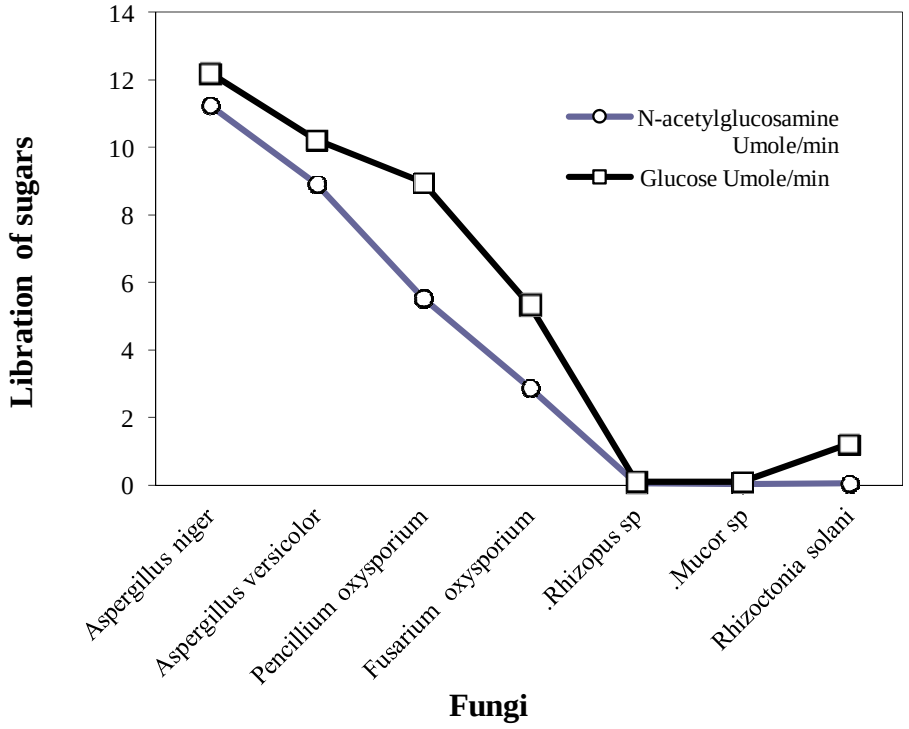

Figure 11. Sugar liberation from the hydrolysis of fungal cell walls.

Conclusion

From the present investigation, it is confirmed that strains isolated from the soil as *A. terrus* had chitinase activity and thus the enzyme extracted from these strains can be used as a catalyst for the degradation of chitin which is abundant in polysaccharides after cellulose. The result concluded that *A. terrus* chitinase has the ability to degrade the cell wall of many fungi, so it can be used as antifungal for phytopathogenic fungi.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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