Polish Journal of Microbiology
2005, Vol. 54, No 2, 145–151

A Potent Chitinolytic Activity of *Alternaria alternata* Isolated from Egyptian Black Sand

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Received 6 October 2004, received in revised form 4 February 2005,
accepted 7 February 2005

Abstract

Eight fungal species characterized by chitinolytic activity were isolated from Egyptian black sand collected from Rosetta coast. Genus *Aspergillus* and *Alternaria alternata* exhibited the highest density (> 40% of the total count, each) on the isolation plates containing different treatments of native shrimp shell chitin. Genus *Aspergillus* was represented by *A. flavus*, *A. niger*, *A. foetidus* and *A. ungius*, with the former species being the most dominant. The other species were *Cladosporium herbarum*, *Fusarium equisitum* (5.71% of the total count, each) and *Dendryphiella vinosa* (3.21% of the total count). The isolated species were screened for chitinase production on agar plates containing 0.2% colloidal chitin. The chitinolytic activity of each individual was not always correlated with its density on the isolation plates. *Alternaria alternata* was the most promising species for chitinase excretion. The use of colloidal chitin (1.5%) as a sole carbon source was superior for the enzyme production by *A. alternata.* Maximum enzyme yield was obtained after 7 days incubation at 30°C with shaking (150 rev min^{-1}), with an initial pH value of the growth medium at 5.0. Presence of $NaNO_3$ (0.3%), the best nitrogen source, and $CaCl_2$ (100 μg/ml) stimulated the induction of the enzyme. The crude *A. alternata* chitinase revealed a potential insecticidal effect on the larvae of fruitfly (82% mortality) and could degrade crude shrimp shell waste.

Key words: chitinase, *Alternaria alternata*, chitinolytic activity, Egyptian black sand

Introduction

Egyptian black sand deposits occur in some locations along the mediterranean coast and extend from El Arish in the east to Abu Quir in the west. These black sands contain some radioactive elements like uranium, thorium and K^{40}, in trace safe amounts, together with high salt content and some heavy minerals of economic value (Dabbour, 1995). Black sand habitats were extensively studied geologically, but rarely evaluated microbiologically. It is expected that microorganisms isolated from black sand will be highly active, especially in enzyme production and activity, due to their existence in weak radioactive environment. One of these enzymes is chitinase which is recently facing more attention in the field of biotechnology as it degrades chitin.

Chitin is one of the most abundant polysaccharide on the earth. It is present in the cell walls of most fungi (Muzzarelli, 1977) and exoskeleton of arthropods. A lot of chitineous substances contained in shell of shrimp, crabs, lobsters and others are accounting for about 10% of global landings of the aquatic products (Nopakarn *et al*., 2002). However, these substances are discarded as wastes and its degradation is of great importance as it can contribute to both carbon and nitrogen cycles in the biosphere (Reguera and Leschine, 2003). Chitin is degraded by chitinase to N-acetyl glucosamine which can be utilized as a substrate in many industrial applications. Moreover, chitinase induces the bioconversion of chitineous wastes to cell, ethanol (Ferrer *et al*., 1996), fertilizer (Sakai *et al*., 1998) and production of chitoligomers for pharmaceutical or chemical purposes (Patil *et al*., 2000). Recently, chitinases could be exploided as a biocontrol agent for fungal phytopathogens (Palani and Lalithakumari, 1999; Giambattesta *et al*., 2001; Witkowska and Maj, 2002) and as an inhibitor for food spoiling moulds (Gad, 2003). Furthermore, the possible use of chitinase as insecticide was also elucidated (Mendonsa *et al*., 1996; Ghaly, 2003).

Accordingly, the present study aimed at isolation of chitinolytic fungi from Egyptian black sand. Emphasis was directed to chitinase production and factors which maximize the yield by the most potent producer *Alternaria alternata*. The chitinolytic activity of the crude enzyme was further tested on the larvae of fruitfly.

Experimental

Materials and Methods

Samples of black sand. Ten black sand samples were collected, in clean plastic bags from Rosetta coast in Alexandria, and thoroughly mixed into one composite sample.

Preparation of native shrimp shell chitin. Native shrimp shell wastes were collected from El-Uboor market. Chitin was prepared from the waste in three treatments: crude, treated or colloidal chitin. The crude chitin was prepared by washing part of the shrimp shell waste, drying up in sun and then grinding it (dehydrated ground shrimp shell chitin). Another part of the waste, after washing, was alternatively pretreated with NaOH and HCl (Jeuniaux, 1966) for several times, dried up and ground to obtain treated chitin. Colloidal chitin was prepared from treated chitin using phosphoric acid (H_3PO_4) according to Reid and Ogrydiazk (1981).

Isolation of chitinolytic fungi. The soil dilution plate method (Johnson *et al*., 1960) was followed for isolation of chitinolytic fungal species from black sand. A basal mineral salt medium containing (g%) $NaNO_3$, 0.2; KH_2PO_4, 0.1; $MgSO_4 \times 7H_2O$, 0.05; KCl, 0.05; agar, 2 and streptomycin, 0.003 was supplied with the different forms of 0.5% chitin (crude, treated or colloidal, separately) as a sole carbon source. The pH was adjusted to ~5. One ml of black sand suspension, at the proper dilution, was placed on the plates (5 replicates, each). The plates were swirled and incubated at 28°C for 2 weeks. The developing fungal colonies were counted and identified according to Raper and Fennell (1965), Moubasher (1993) and Ellis (1971, 1976). The percentage of relative density (RD %) was calculated.

Qualitative screening for chitinolytic activity. The isolated fungal species were screened for chitinolytic activity on chitin-agar medium containing 0.2% colloidal chitin in citrate phosphate buffer (pH 5.0). A fungal disc (6 mm) cut from the periphery of 7 days old culture, grown on Dox·s medium, were inoculated on the plates (triplicates). The plates were incubated for 5 days at 28°C. The diameters of clearing zones were measured (mm) and means were calculated and taken as an evidence for chitinolytic activity.

Production of chitinase enzyme by *Alternaria alternata.* The fungal isolate *Alternaria alternata* which exhibited the greatest clearing zone on the agar plates was selected for further and more detailed studies. A basal growth medium containing (g%) colloidal chitin, 0.5; $NaNO_3$, 0.2; KH_2PO_4, 0.1; Mg $SO_4 \times 7H_2O$, 0.05 and KCl, 0.05 was used for chitinase production. The pH was adjusted to 5.0. The flasks (triplicates) were inoculated with 1 ml spore suspension (~10^6 ml^{-1}) of 7-day old cultures. The inoculated flasks were incubated at 30°C under shaking (100 rev min^{-1}) for 7 days. The culture broth was centrifuged, undercooling, and the clear supernatant was used as a source for crude chitinase.

Enzyme assay. One ml of 0.5% colloidal chitin (suspended in citrate phosphate buffer, pH 5.0) was incubated with 1 ml of the crude chitinase at 40°C for 90 minutes. The reducing sugar was estimated according to Reissig *et al*. (1955) using a standard curve of N-acetyl glucosamine. One unit of enzyme activity is the amount of the enzyme which releases 1 mmol of N-acetyl glucosamine under the assay conditions.

Cultural conditions controlling chitinase biosynthesis by *Alternaria alternata*

a) Environmental conditions. The effect of incubation period (2–9 days), incubation temperature (20°C–60°C), pH value of the growth medium (3–8) as well as shaking speed (100–250 rev mn^{-1}) on chitinase production were investigated.

b) Nutritional conditions. The following nutrients were studied for their effect on chitinase production: different carbon sources (1 g%) as well as crude, treated and colloidal chitin (0.5 g%, each), colloidal chitin concentration (0.5–3.0 g%), different inorganic N sources (at equimolecular bases), organic N sources (0.2 g%), concentration of $NaNO_3$ (0.1–0.4 g%) and salts of some microelements (100 µg/ml).

Effect of crude chitinase on fruitfly larvae. Two µL of the crude enzyme was pipetted on the larvae (50) of the fruitfly. Control was treated with distilled water instead of the enzyme. The larvae were left for 24 hrs at room temperature in beakers covered with a piece of cloth. Percentage of mortality was calculated.

Statistical analysis. Summary statistics were used to obtain the means and standard errors (SE). One way analysis of variance (ANOVA) was detected to evaluate the significant differences between means using SPSS statistical software ($P<0.01$).

Results and Discussion

The total count of chitinolytic fungi isolated from Egyptian black sand was 335 colonies/g dry soil (Table I). Fungi belonged to 8 species belonging to 5 genera. Genus *Aspergillus* and *Alternaria alternata* showed the highest density on the isolation plats (RD > 40%, each). Genus *Aspergillus* comprised *A. flavus*, *A. foetidus*, *A. niger* and *A. ungius*. *A. flavus* and *A. alternata* were developed on the plates containing colloidal, treated and crude chitin, exhibiting a strong chitinolytic activity. However, the other *Aspergillus* species were developed on plates which contained colloidal chitin only, showing less activity. *Cladosporium herbarum* and *Fusarium equisitum* came next in density (RD 5.7%, each). They were isolated from plates containing colloidal chitin, with the former species being more active as it could be also recovered from treated chitin. The least dominant *Dendryphiella vinosa* (RD 3.2%) appeared on colloidal chitin plates only, and showed low activity.

Table I
Total count (colonies/g dry soil) and relative densities (RD %) of chitinolytic fungi isolated from Egyptian black sand on media containing different treatments of native shrimp shell chitin

Fungal species	Treatments of chitin			Total count (colonies/g) dry soil	Relative density (RD%)
	Crude	Treated	Colloidal		
Aspergillus spp.	23	38	84	145	43.3
A. flavus	23	38	38	99	29.6
A. foetidus	–	–	8	8	2.4
A. niger	–	–	23	23	6.8
A. ungius	–	–	15	15	4.5
Alternaria alternata	19	80	42	141	42.1
Cladosporium herbarum	–	11	8	19	5.7
Dendryphiella vinosa	–	–	11	11	3.2
Fusarium equisitum	–	–	19	19	5.7
Total	57	91	187	335	100

The chitinolytic activity of fungal species isolated from Egyptian soil was demonstrated by El Naghy *et al.* (1985), Sherief *et al*. (1999), Nour El-Dein *et al*. (1999), Shindia *et al*. (2001), Ali and Ibrahim (2003) and Ghareib *et al*. (2004). They reported that the chitinolytic fungi showed great variations towards chitin degradation ranging from weak to strong activity, depending on inherited characters.

It is evident that all the isolated species were recovered from medium containing colloidal chitin, *i.e.* they could degrade this form of chitin, whereas *C. herbarum*, *A. flavus* and *A. alternata* could also utilize the treated one (Table I). On the other hand, the last two species only were able to degrade the crude chitin, an observation which indicated the availability of the used form of chitin for degradation and/or the rate of chitinolytic activity of each individual. In this connection successive pretreatment of crude shrimp shell chitin with NaOH followed by HCl resulted in deproteinization (Chang and Tsai, 1997) and removal of most of $CaCO_3$ from the shell (Cosio *et al*., 1982), respectively. This treated form of chitin is more available for degradation than the crude one. Moreover, when this treated chitin was converted to the colloidal form (chitodextrin) it became swollen and most available for attack (Monreal and Reese, 1969).

The results of qualitative screening on agar plates revealed that the chitinolytic activity of each species was not always correlated with its density on the isolation plates (Table II). *A. alternata* followed by *A. flavus* exhibited the greatest clear zones (37 and 32 mm, respectively) which confirmed their higher chitinolytic activity. *A. foetidus* and *F. equisitum* showed a moderate activity and their clear zones measured 26 and 21 mm, respectively. The other species showed lower activity and achieved clear zones ranging from 10 to 18 mm. Therefore, *A. alternata* was selected for maximization of chitinase production.

Chitinase biosynthesis by *A. alternata* was gradually increased by increasing the incubation period up to the 7th day (2.467 units/ml, Fig. 1a). However, prolonged incubation reduced the enzyme productivity. Similar result was obtained by Sherief *et al*. (1991) for chitinase of *A. carneus*. Neverthless, different incubation periods were recorded for chitinase production by filamentous fungi where it was 2–4 days for *Talaromyes emersonii* (McCormack *et al*., 1991), 4 days for *Chaetomium thermophilum* and 8 days for *Cunninghamella echinulata* and *Thermomyces lanuginosus* (Nour El-Dein *et al*., 1999 and Ali and Ibrahim, 2003, respectively).

Maximal chitinase production was obtained when the fungus was incubated at 30°C (Fig.1b). Any shift below or above this temperature was followed by retardation in chitinase excretion. Similar results were obtained by Kabat *et al*. (1996) and Shindia *et al*. (2001). However, 45°C was found optimum for the enzyme production by *Ch. thermophilum* (Ali and Ibrahim, 2003).

Table II
Chitinolytic activity of fungal species isolated from Egyptian black sand on agar plates as determined by diameter of clearing zone

Fungal species	Diameter of clearing zone (mm ± SE)
Alternaria alternata	37 ± 2.8
Aspergillus flavus	32 ± 3.0
A. foetidus	26 ± 2.4
A. niger	18 ± 1.7
A. ungius	14 ± 1.3
Cladosporium herbarum	16 ± 1.2
Dendryphiella vinosa	10 ± 1.1
Fusarium equisitum	21 ± 1.5

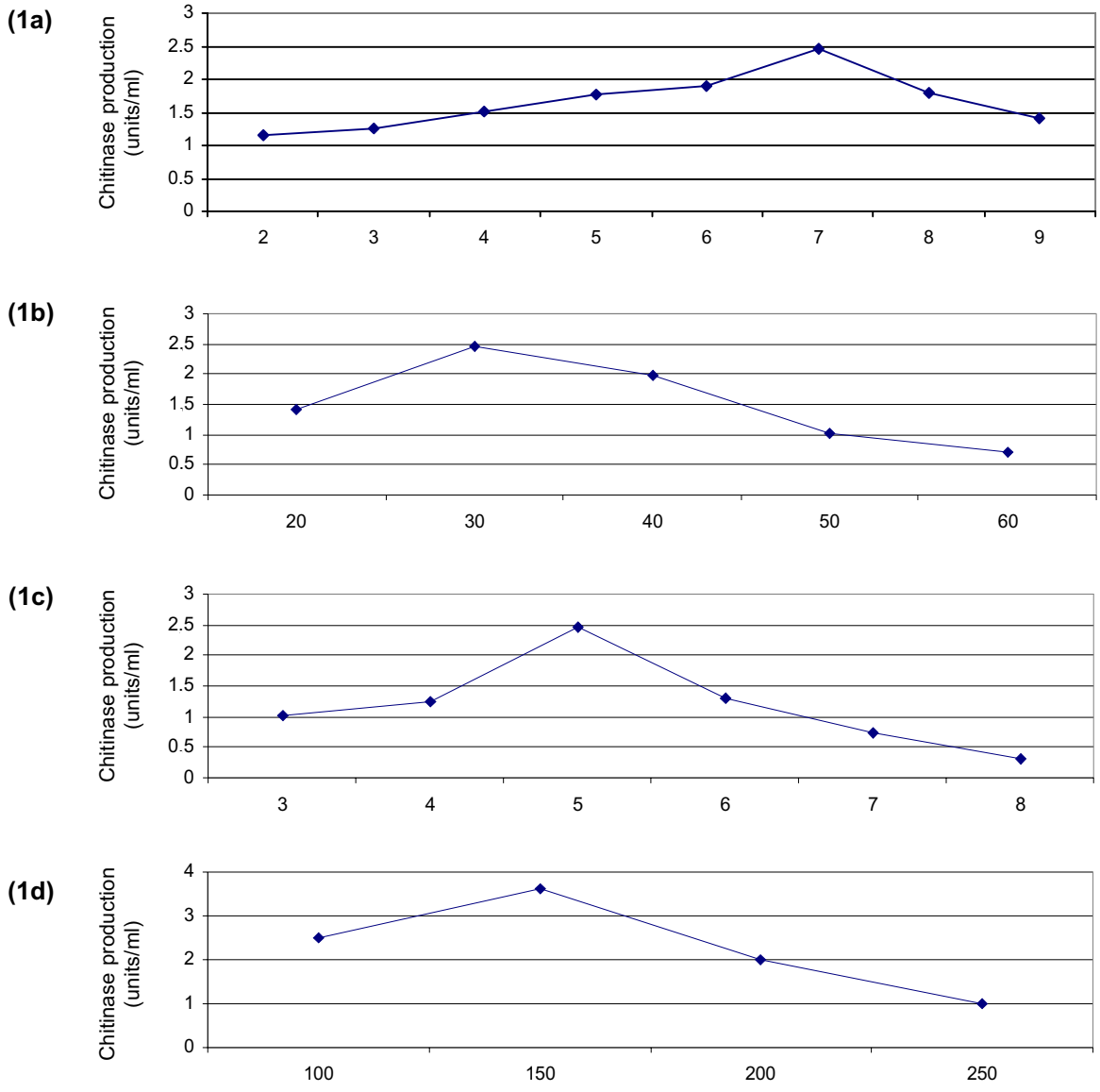

Fig.1. Influence of environmental factors affecting production of chitinase by *A. alternata*
a) Incubation period (days), b) Incubation temperature (°C), c) Initial culture pH, d) Shaking speed of the culture medium (rev min^{-1})

In the present study, the pH of the growth medium exerted a significant effect on chitinase biosynthesis ($P<0.01$). The optimum pH was 5 (2.478 units/ml), and any shift exerted remarkable reduction in enzyme productivity (Fig. 1c). This results were in complete accordance with those of Tweddell *et al*. (1994), Nour El-Dein *et al*. (1999) and Shindia *et al*. (2001). On the other hand, the optimum pH was found to be 7 for chitinase production by *Stachybotrys elegans* (Taylor *et al*., 2002), *Aspergillus* sp. S1-13 (Nopakarn *et al*., 2002) and *Penicillium janthinellum* (Giambattesta *et al*., 2001).

Interestingly, increasing the shaking speed of the growth medium up to 150 rev min^{-1} was followed by a significant increase ($P<0.01$) in chitinase excretion by *A. alternata* cells, with the release of 3.632 units/ml enzyme (Fig. 1d). Shaking increases oxygen solubility, allows higher and equal exposure of the substrate to be attacked by the fungus and keeps it in the colloidal form.

Several carbon sources were tested for the production of chitinase by *A. alternata* (Table III). Colloidal chitin was superior for the enzyme induction (3.710 units/ml) followed by treated chitin (1.843 units/ml). Also, the enzyme was induced in the presence of the crude shrimp shell chitin, a phenomenon which indicated a strong chitinolytic activity of the tested fungus. However, the enzyme was not produced in the presence of either free sugars (glucose, sucrose, fructose, lactose and galactose) or polysaccharide (cellulose). This indicated the induced nature of the present chitinase. These results were in harmony with the previous findings of Ulhoa and Peberdy (1991) and Haran *et al*. (1995). They elucidated that the preference

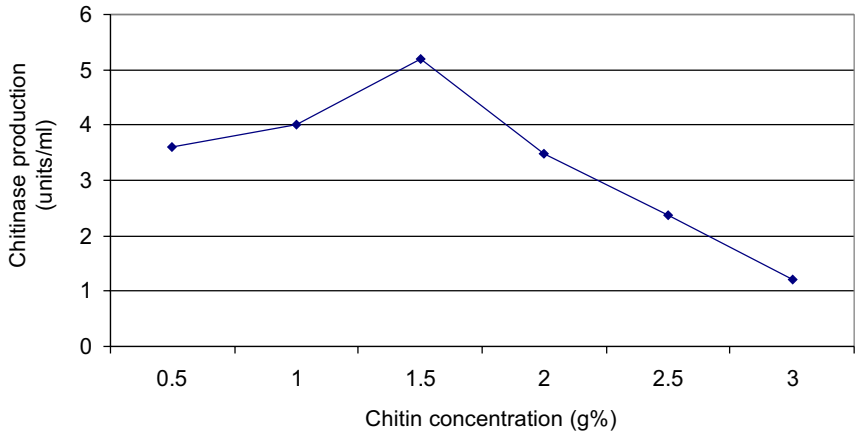

Fig. 2. Effect of different chitin concentrations on chitinase productivity by *A. alternata*

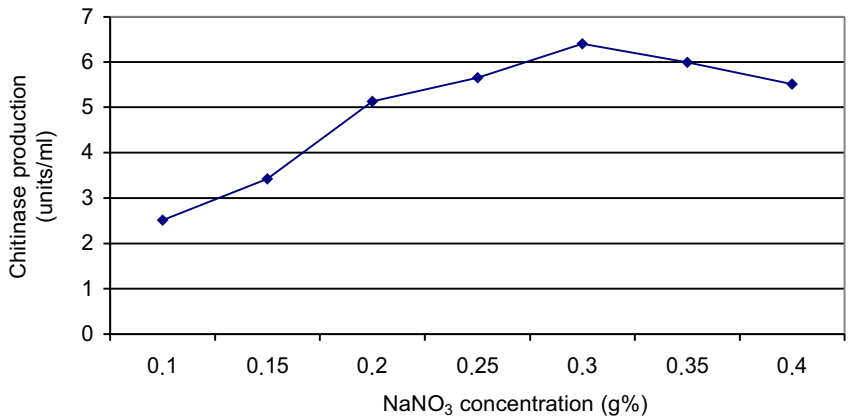

Fig. 3. Effect of $NaNO_3$ concentrations on chitinase production by *A. alternata*

of chitin for chitinase production was monitored by repressor-inducer system where chitin or its products of degradation (oligomers) act as inducers. Also, Donzelli and Harman (2001) claimed that chitinase expression and secretion was repressed by glucose and induction of chitinase required the presence of chitin.

Chitinase induction was significantly stimulated ($P<0.01$) by the increase in colloidal chitin concentration up to 1.5 g% where the yield reached 5.484 units/ml (Fig. 2). However, above this concentration the yield was declined. Similar results were reported by Shindia *et al*. (2001) and Ghaly (2003) but Monreal and Reese (1969) and Sherief *et al*. (1991) used 2 and 1% colloidal chitin for maximum chitinase production, respectively. The drop in enzyme production at higher levels of the substrate could be related to saturation with the inducer and/or the end product suppression mechanism (Monreal and Reese, 1969).

Interestingly, addition of different organic or inorganic nitrogen sources to the growth medium stimulated the induction effect of chitin, with exception of ammonium nitrate and ammonium sulphate. The best stimulator was sodium nitrate ($NaNO_3$) followed by peptone, yeast and beef extracts (data not shown). The stimulating effect of $NaNO_3$ was matched linearly with the increase in its concentration up to 0.3 g% (6.405 units/ml) but above it an inhibition effect was observed (Fig. 3). Donzelli and Harman (2001) explained that the inductive effect of chitin is altered by the level of ammonium in the growth medium of *Trichoderma atroviride* and its starvation resulted in early expression and secretion of chitinase.

Obviously, secretion of *A. alternata* chitinase was influenced by the presence of metal salts in the growth medium. Of them $HgCl_2$, $CuCl_2$ and $PbCl_2$ were strong inhibitors whereas $CaCl_2$ followed by $FeSO_4$

Table III
Effect of different carbon sources on chitinase production by *A. alternata*

Substrate	Enzyme production (units/ml)
Colloidal chitin	3.710 ± 0.200
Treated chitin	1.843 ± 0.135
Crude chitin	0.916 ± 0.041

The remaining substrates (sucrose, glucose, fructose, lactose, galactose and cellulose) were excluded as giving negative results

Table IV
Insecticidal activity of *A. alternata* crude chitinase on the larvae of fruitfly

Sample	Mortality (%)
Control	11
Treated	82

were activators. Other metal salts such as $CoSO_4$, $ZnSO_4$ and $MnSO_4$ were non-effective for chitinase production (data not shown). It was suggested that Ca ions, beside their activating effect, might play a role in the enzyme stability (Fenton and Eveleigh, 1981; Saad and Hamdy, 2004). On the contrary, Hg ions could possibly interfere with the active site of the enzyme (Rodriguez *et al*., 1995; Ghareib *et al*., 2004).

It is worthy to mention that treating of fruitfly larvae with *A. alternata* crude chitinase resulted in 82% mortality (Table IV). Chitinase of *Serratia marcescens* and *Streptomyces grisea* was able to exert premature hatch of *Melidogyne* nematodes eggs (Mercer *et al*., 1992). Also, chitinase of *Streptomyces albovinaceus* caused 100% mortality of ants but not affected the housefly and cockroach (Ghaly, 2003).

It could be suggested that Egyptian black sand is a good source for isolation of active microorganisms which can produce potent biotechnological beneficial enzymes. The present chitinase can be utilized in degradation of shrimp shell wastes that may help in reduction of some environmental problems. Furthermore, it can be used as effective insecticide, particularly against larvae of fruitfly, replacing the chemical criticized for their hazardous effects on human health.

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