Biosynthesis of biologically active chitinase utilizing some Egyptian chitinaceous wastes and the properties of the synthesized enzyme

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Background and objective
Chitin-degrading enzymes have an utmost practical importance in many fields such as medicine, agriculture, and industry. These enzymes are used as effective antibacterial, antifungal, antihypertensive, and antioxidant agents and also as excellent food quality enhancers. The objective of the present article was to formulate the production medium and to pinpoint the proper growth conditions for the chosen microorganism producing highly active chitinase enzymes. The general properties of the crude enzyme preparation were determined to define the proper conditions for enzyme action. Under the specified conditions, the capability of the enzyme preparation for antimicrobial and antioxidant activities were decided.
Materials and methods
Eighteen recommended microbial strains were screened for biologically active chitinolytic enzymes productivity. Chitinase enzyme was determined, and also the important properties of the crude chitinase were duly pinpointed. Finally, biological activities of the crude enzyme were studied.
Results and conclusion
and conclusion Among all the 18 organisms, *Streptomyces halstedii* H2 was the most potent producer of an influential chitinase enzyme. The maximum chitinase activity of 49.5 U/ml was obtained from medium contains glucose 6 g/l, ammonium nitrate (0.9 g/l), and urea (0.64 g/l) at 30°C and pH 9.0. The important properties of the streptomycetal chitinase were duly pinpointed as follows: optimum enzyme and substrate concentrations were 1.6 mg/ml and 1.4% (w/v), respectively, and optimum reaction pH and temperature were 7.2 and 45°C, respectively. The crude preparation was stable for 60 min at pH 7.2 and 30°C and retained 92.6% of the original activity. Under the specified conditions, at varying concentrations, the enzyme preparation exhibited considerable 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity accompanied with low antimicrobial activity, pointing out the partial purification necessity of the crude enzyme preparation.

Keywords:
biological activities, chitinase, 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity, marine chitinaceous wastes, microorganisms

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Introduction

Marine wastes are considered the principal source of chitin, and more than 80 000 tons of chitin is produced from marine wastes every year [1]. The accumulation of large amounts of wastes from crustaceans has become a main concern in the industry of seafood processing. Therefore, the utilization of these wastes for production of high value-added products, such as chitin, chitosan, and their derivatives for valuable applications in different fields, has vital demands [2–4]. In Egypt, crayfish, crabs, and shrimp are the most important crustacean seafoods. Shrimp shell wastes which constitute ~40–50% of the total weight of shrimp become an environmental risk in Egypt owing to the recent increased consumption of shrimps [5–7].

Chitin is nitrogen-containing polysaccharide consisting of β-1,4-linked N-acetyl-D-glucosamine, which is chemically analogous to the cellulose, except that one of the hydroxyl groups of each glucoside residue is replaced by an acetylated or deacetylated amino group. Chitin comprises ~20–58% of the shellfish waste dry weight [8,9].

Chitinase (EC 3.2.11.14) enzymes are capable of hydrolyzing insoluble chitin to its oligo and monomeric components found in a variety of

organisms including viruses, bacteria, fungi, insects, higher plants, animals, and play important physiological roles depending on their origin [10,11]. Chitinases are constituents of several bacterial species; some of the best known genera include *Aeromonas*, *Serratia*, *Vibrio*, *Streptomyces*, and *Bacillus* [12–15] and some fungal species [16,17]. Chitinase applications in many fields, such as medical, agricultural, and industrial were comprehensively reported by many authors [18,19].

Materials and methods

Microorganisms

The following microbes were screened in this study: *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus subtilis*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Aspergillus niger*, *Aspergillus terreus*, *Dichotomous* sp., *Trichoderma harzianum*, *Trichoderma viride*, *Penicillium* sp., *Actinomadura* sp., *Nocardia* sp., *Streptomyces halstedii* H2, *Streptomyces* natalensis NRC1, *Streptomyces* NRC 35, and *Streptomyces* sp., which were provided from the Culture Collection Centre of the National Research Centre, Egypt, whereas the bacterial strain *B. subtilis* NRRL 1315 was imported from the National Centre for Agricultural Utilization Research (Peoria, Illinois, USA; NRS).

On the other hand, the pathogenic bacterial strains (*B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*) and the pathogenic fungal strains (*A. niger*, Candida albicans, *Fusarium oxysporum*, and *Rhizoctonia solani*) were provided from Microbiology Department, Faculty of Science, Ain Shams University, Egypt.

Chitinaceous waste samples

Different chitinaceous wastes (shrimp, prawn, crab shells, and mollusk valves) were collected from local fish market in Cairo governorate, Egypt.

Chitinase production media

The following media were employed for the production of chitinases and were in the form of g/l.

Bacterial production medium 1

Colloidal chitin, 20; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MnCl_2$, 0.001; $ZnSO_4$, 0.001; and $FeSO_4.7H_2O$, 0.01, with pH of 7.2±0.2 [20].

Fungal production medium 2

Colloidal chitin, 10.0; yeast extract, 4.0; $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 0.68; K_2HPO_4, 0.87; NH_4Cl, 1.0; and KCl, 0.2, with pH of 6.5 [21].

Actinomycetal production media (3–6)

Medium 3

Chitin, 5.0; glucose, 3.0; soya bean, 3.9; NH_4NO_3, 0.9; $MgSO_4.7H_2O$, 0.3 and trace element solution, 1 ml; pH, 9.0. The trace element solution composed of (g/100ml): $FeSO_4.7H_2O$, 0.5; $MnSO_4.2H_2O$, 0.16; $ZnSO_4.7H_2O$, 0.14; and $CaCl_2$, 0.2 [22].

Medium 4

Na-carboxy methyl cellulose, 10.0; peptone, 5.0; beef extract, 3.0; and NaCl, 5.0, with pH 7.2±0.2 [23].

Medium 5

Chitin, 10.0; KH_2PO_4, 1.36; yeast extract, 0.5; $MgSO_4.7H_2O$, 3.0; NaCl, 10.0; and $(NH_4)_2SO_4$, 1.0, with pH of 7.2±0.2 [24].

Medium 6

Chitin, 2.0; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1.0; KCl, 0.5; $(NH_4)_2SO_4$, 2.0; and $FeSO_4$, 0.01, with pH of 7.2 [25].

Buffer solutions

The following two buffers were applied for different reaction pHs: phosphate buffer (pH 5.7–8.0) and glycine-NaOH buffer (pH 9.0–10.4).

Chemicals

Chitin and *N*-acetyl-D-glucosamine were purchased from MP Biomedical Inc, rue Geiler de Kayserberg 67402 illkirch, France and all the other chemicals were of analytical grade.

Sample preparation

Different crustaceous wastes including shells and valves obtained from shrimps, prawns, crabs, and mollusks were washed under running water to remove organics, proteins, and other impurities. The specimens were then boiled in water for 0.5 h to remove the tissue, and followed by drying in an oven at 100°C for 2 h to break down the crystallinity of chitin. At the end, the dried shells were crushed in a blender with a sieve of 0.1 mm diameter. The raw material obtained is stored in bags and placed in a cold at 4°C to be used for microbial chitinase enzyme production [26].

Maintenance of the tested microorganisms and stock cultures

The tested bacteria were maintained on the nutrient agar slants and incubated at 37°C for 24 h, whereas the fungi were maintained on potato-dextrose-agar medium slants and incubated at 30°C for 7 days; other than that, the actinomycetes were maintained on yeast extract-malt extract medium (ISP_2) slants and incubated at 30°C for 5 days.

Preparation of colloidal chitin

The colloidal chitin was prepared by the method described by Reid and Ogrydiazk [27] as follows: 5 g chitin was soaked in 100 ml of 85% (w/v) phosphoric acid at 4°C for 24 h. It was well mixed in an electric blender and diluted with distilled H_2O, centrifuged at 2300 g for 20 min, washed several times with dist. H_2O to remove of the excess acid, and then pH was adjusted to 7.0, using 1 N-NaOH. The colloidal chitin was dried at 45°C and kept in the refrigerator till use.

Protein content

The protein of any solution was determined colourimetrically by Folin-Ciocalteu phenol reagent (Merck Company, Schuchardt, Germany) according to the method by Lowry *et al*. [28]. The standard curve of protein was constructed using bovine serum albumin.

Chitinase activity assay

Chitinolytic enzyme activity was determined according to Stauff and Jaenicke [29] modified method, and the enzymatic products were measured applying the modified method of Neish [30], which was based on those described by Nelson [31] and Somogyi [32]. This was done by incubating 1 ml of enzyme solution with 1 ml of (1%, w/v) colloidal chitin suspension in 0.2 M-phosphate buffer, at pH of 6.5. The reaction mixture was incubated in shaking water bath (70 rpm) at 40°C for 30 min, and then 1 ml of the mixture was withdrawn and added to 2 ml of copper reagent, boiled for 20 min, and cooled in running tap water, and then 2 ml of arsenomolybdate reagent was added and finally made up to 25 ml with distilled water. The released reducing sugars were colorimetrically measured at 520 nm. Standard curve was constructed using *N*-acetylglucosamine as standard. One chitinase activity unit was defined as 1 µmole *N*-acetylglucosamine released per one hour reaction at the specified conditions.

Optimization of enzyme production

Each of fermentation period, culture medium composition, initial pH, N and C sources, some saccharides addition, inoculum size and age, agitation rate, and incubation temperature were duly studied for microbial chitinase production optimization.

Employment of some pretreated chitinaceous marine wastes on microbial chitinase enzyme production

Equal weights of marine chitinaceous waste (shrimp shells, prawn shells, crab shell pieces, crab shells powder, or mollusk valves powder) were physically, chemically, and physicochemically pretreated, and then separately applied instead of the pure chitin in the optimized production medium.

General properties of the crude chitinase determination

Effects of enzyme protein and substrate (colloidal chitin) concentration, reaction temperature, and reaction pH on enzyme activity were studied, besides the enzyme pH stability.

Biological activities of the crude enzyme

Free radical scavenging activity

Free radical scavenging activity of the crude chitinase enzyme was determined by simple, rapid, and inexpensive method involving the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) with some modification [33]. DPPH is a free radical of violet color. The antioxidants present in the sample scavenge the free radicals and turn it into yellow color. The color change from violet to yellow is proportional to the radical scavenging activity. In brief, the crude enzyme stock ethanolic solution (10.0 mg/ml) was diluted with ethanol to final concentrations ranged from 0.25 to 9.0 mg enzyme (wt/ml). Add 0.5 ml of a 0.3 mM-DPPH ethanolic solution separately to 0.5 ml of each sample solution, and the reaction mixture was vortexed and incubated for 1 h in room temperature. The absorbance of the solution was measured at 518 nm. Ascorbic acid was used as standard. The inhibitory percentage of DPPH was calculated according to the following equation:

$$I = \left\{ \frac{(\text{ABS CONTROL} - \text{ABS TEST})}{\text{ABS CONTROL}} \right\} \times 100.$$

where I=DPPH inhibition %, ABS CONTROL=absorbance of control (t=0 h), and ABS TEST=absorbance of a tested sample at the end of the reaction (t=1 h). Each assay was carried out in triplicate, and the results were averaged.

Percentage of radical scavenging activity was plotted against the corresponding concentration of the crude enzyme to obtain IC_{50} value. IC_{50} is the maximal concentration of the compound to cause 50% inhibition.

Antimicrobial activity

The antimicrobial activity of the tested crude *S. halstedii* H2 chitinase was carried out against gram-positive and gram-negative bacteria (*B. subtilis*, *S. aureus* and *E. coli*, and *P. aeruginosa*) and fungi (*A. niger*, *C. albicans*, *F. oxysporum*, and *R. solani*). Antimicrobial activity was determined by well diffusion method [34] with slight modification. Measurement of the inhibition zones was done by placing 0.1 ml of the crude preparation separately, of

each one in 10-mm diameter wells cut in nutrient agar plates seeded by test bacteria and potato dextrose agar plates seeded by test fungi. After that, incubate all plates at 37°C for 24 h for bacteria and 30°C for 72 h for fungi, and the zone of inhibition was measured in mm diameter.

Statistical analysis

All experiments were repeated three times, the data shown in the corresponding tables and figures were the mean values of the experiments, the data statistics were analyzed and the Standard Error Mean (SEM) was determined.

Results and discussion

Screening for chitinase production by some recommended microorganisms

The screening of eighteen recommended microorganisms (six bacteria, six fungi, and six actinomycetes) for the production of chitinase enzymes in shaken cultures lasting for different periods (1–5, 3–7, and 1–6 days for bacteria, fungi, and actinomycetes, respectively), Tables 1–3, was performed. The data showed that with most cultures of different ages, the protein level was in the usual range and had no consistent relationship with both the microbial growth and chitinase activity. For all cultures, the final pH varied within a very limited range from the neutral to the slightly alkaline. It was noticed the prevalence of some tested actinomycetal followed by fungi to the bacterial organisms in chitinase enzymes production. Among the tested microorganisms, 5-day shaken *S. halstedii* H2 culture afforded the highest chitinase productivity (7.20 U/reaction) followed by the fungal species *T. harzianum* (4.12 U/reaction) on 3-day shaken culture. The other actinomycetes afforded in 2- and 3-day shaken cultures moderate chitinase productivity (2.26 U/reaction). In this respect, many streptomycetes were reported to be potent chitinase producers [25,35,36]. On the contrary, in a 3-L bench-top bioreactor, the fungal strain *Penicillium*

Table 1 Screening of some bacteria for chitinase activity during different incubation periods applying shaken culture technique

Incubation period (day)	1		3		5	
Bacteria	PC[a]	Activity[b]	PC	Activity	PC	Activity
Bacillus licheniformis	0.05	0.07	0.20	2.28	0.14	1.24
Bacillus Megaterium	0.13	0.22	0.13	0.13	0.05	0.11
Bacillus subtilis NRRL 1315	0.07	0.22	0.06	0.15	0.04	0.02
Bacillus subtilis	0.08	0.12	0.04	0.12	0.01	0.02
Pseudomonas putida	0.06	0.10	0.04	0.21	0.01	0.04
Pseudomonas fluorescens	0.04	0.08	0.02	0.09	0.02	0.04

Values are expressed in unit: U/reaction=U/ml enzyme. [a]PC, protein content of culture filtrate (mg/ml). [b]Activity, chitinase activity (U/reaction).

Table 2 Screening of some fungi for chitinase activities during different incubation periods applying shaken culture technique

Incubation period (day)	3		5		7	
Fungi	PC	Activity	PC	Activity	PC	Activity
Aspergillus niger	0.05	0.07	0.14	0.28	0.12	0.24
Aspergillus terreus	0.19	0.22	0.13	0.15	0.05	0.11
Dichotomous sp.	0.15	0.22	0.06	0.15	0.02	0.02
Trichoderma harzianum	0.80	4.12	0.60	3.12	0.51	2.02
Trichoderma viride	0.60	2.10	0.40	2.21	0.21	1.04
Penicillium sp.	0.04	0.08	0.02	0.09	0.00	0.04

Table 3 Screening of some actinomycetes for chitinase activity during different incubation periods applying shaken culture technique

Incubation period (day)	1		2		3		4		5		6	
Actinomyces	PC	Activity	PC	Activity	PC	Activity	PC	Activity	PC	Activity	PC	Activity
Actinomadura sp.	0.36	0.03	0.40	1.09	0.45	2.26	0.60	2.57	0.63	4.03	0.50	2.70
Nocardia sp.	0.08	0.06	0.13	0.48	0.17	0.55	0.19	1.68	0.27	2.86	0.26	2.41
Streptomyces halstedii H2	0.18	1.03	0.26	1.62	0.60	2.73	0.63	3.98	0.75	7.20	0.55	3.05
Streptomyces natalensis NRC1	0.07	0.05	0.13	0.36	0.15	0.55	0.19	0.80	0.19	0.73	0.10	0.43
Streptomyces NRC 35	0.27	0.04	0.23	0.09	0.19	0.27	0.19	0.55	0.15	0.23	0.09	0.15
Streptomyces sp.	0.11	0	0.33	0.09	0.35	1.44	0.35	1.98	0.28	2.33	0.20	1.65

janthinellum A8 produced highly active chitinase after 7 days [37].

Accordingly, among all the screened eighteen organisms, the *S. halstedii* H2 was chosen as the most potent chitinase producer in 5-day shaken culture and applied in the succeeding experiments.

Effect of fermentation period on enzyme production by *Streptomyces halstedii* H2

The highest chitinase activity and growth were achieved by *S. halstedii* H2 after 5 days of incubation (7.18 U/reaction) and gradually decreased at extended periods, where 6.4% chitinase productivity was lost after the seventh day, and this may be attributed to the enzyme digestion by proteases, when the enzyme substrate in the culture medium was consumed [38]. This finding was more or less in accordance with those reported by Nawani and Kapadnis [35] and Suzuki *et al*. [25].

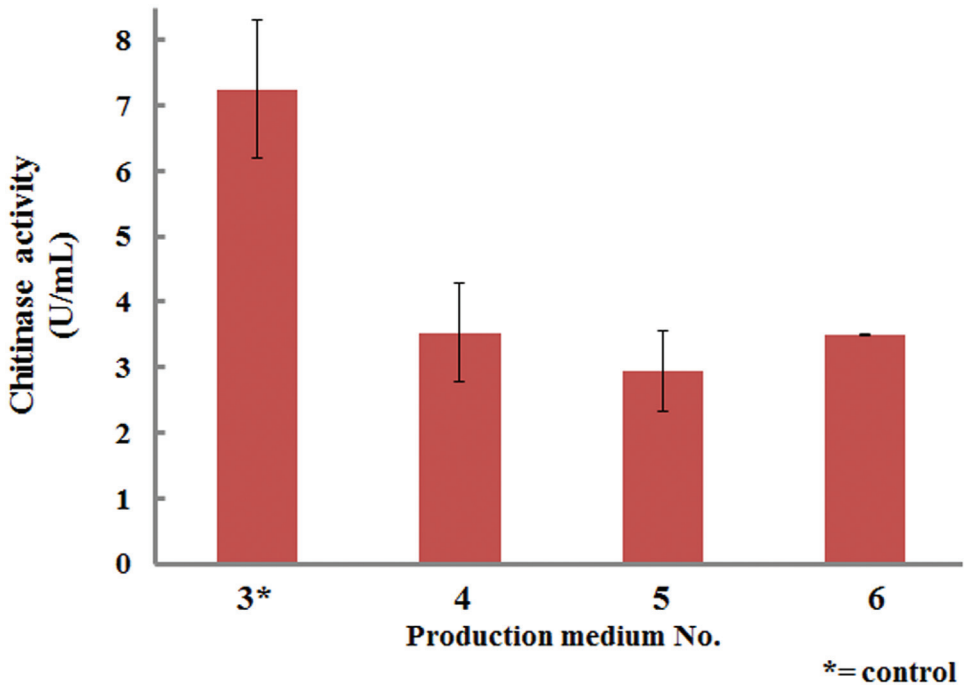
Effect of culture medium

Four culture media of varying formulations and including the foregoing basal medium 3 as control were examined to judge any was the most favorable and affording the highest chitinase productivity (Fig. 1). The basal production medium 3 was the most favorable and led to the maximal *S. halstedii* H2 chitinase productivity (7.25 U/reaction). The other media 4 and 6 had almost lower similar effect on chitinase productivity, and more than 49% productivity decrease was recorded with medium 5. It was noticed that medium 4 enhanced *S. halstedii* H2 growth rather than the enzyme production. Accordingly, the culture medium 3 was chosen for chitinase production by *S. halstedii* H2. In this concern, similar formulation was designed by Suzuki *et al*. [25] for streptomycetal chitinase production by *S. halstedii* MF 425 applying the shaken technique. As medium 4 contained no chitin, the streptomycetal strain was able to produce considerable chitinase, indicating its constitutive nature, concluding that chitinase could be produced either adaptively or constitutively by *S. halstedii* H2.

Effect of initial pH

The effect of the culture medium initial pH on S. *halstedii* H2 chitinase productivity under wide pH range (5.7–10). The initial pH 9 afforded both the highest chitinase productivity and maximal dry weight of biomass (7.25 U/reaction and 750 mg/culture, respectively). The acidic pH or even slight acidic (5.7–6) had the most adverse effect on the enzyme production, and more than 67.6% loss in enzyme productivity was recorded. The optimum initial pH range 7–9 resulted in final alkaline pH range from 8.7 to 8.8. Conclusively, it seemed that the final alkalinity is inevitably needed for active chitinase production by

Figure 1



Effect of different culture media on *Streptomyces halstedii* H2 chitinase productivity applying 5-day-old shaken cultures; each value is presented as mean±SD (*n*=3).

S. halstedii H2. On the other hand, the changes in the protein value with the initial pH changes were limited. This was accorded with those reported by Mejía-Saulés *et al.* [39], who applied an initial pH from 6 to 9 for effective chitinase production by *Serratia marcescens* WF. In addition, initial pH of the production medium at 7–7.2 for optimal streptomycetal chitinase production was reported by Nawani and Kapadnis [35] and Suzuki *et al.* [25].

Effect of nitrogen source

Two groups of N sources on equal N basis were employed, that is, inorganic (ammonium nitrate, ammonium sulfate, and sodium nitrate) and organic (soya bean and urea). The data (Fig. 2) disclosed that the replacement of ammonium nitrate in the basal medium 3 with ammonium sulfate or sodium nitrate led to 51.4 and 19.4% productivity loss. On the contrary, replacement of soya bean with urea led to noticeable jump in streptomycetal chitinase productivity, which was more than 4.7-fold as that of the basal medium. However, Nawani and Kapadnis [35] recommended both yeast extract and ammonium sulphate for streptomycetal chitinase production, and also Sherief *et al.* [40] decided the priority of peptone, yeast extract, and meat extract than urea for the fungal chitinase synthesis.

Effect of carbon source

The data in Fig. 3 clarify that none of the added saccharides to the optimized medium favored S. *halstedii* H2 chitinase production as glucose did, except starch and lactose, which led to moderate enzyme yield. These findings were more or less coincided with those reported by many authors on the reverse effects of many carbon sources rather than chitin and chitin compounds, those including *N*-acetylglucosamine, glucose amine, glucose, galactose, mannose, and others on chitinase enzymes system of many microflora under controlled conditions [41,42]. Nawani and Kapadnis [35] found 1% (w/v) chitin flakes from crab shells were proper for chitinase production by *Streptomyces* spp. NK528 and NK951, whereas Macagnan *et al.* [43] recommended the colloidal chitin for chitinase production from many streptomycetal species. Kuddus and Ahmed [44] found the same for *Aeromonas hydrophila* HS4 and *Aeromonas punctata* HS6 as the previous authors.

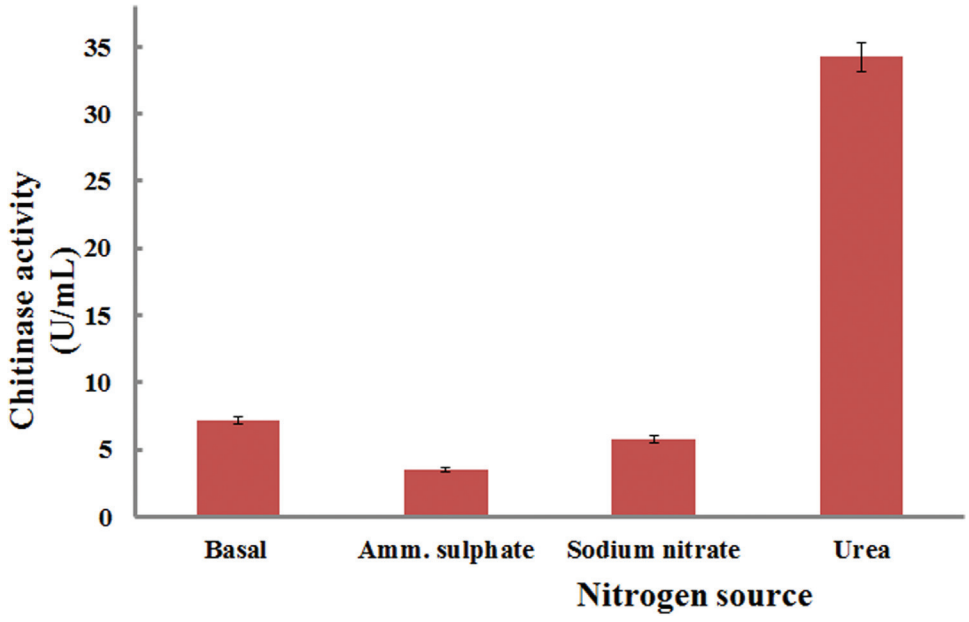
Effect of some additional saccharides

The effect of additional saccharides (glucose, lactose, and starch) in equal C basis in chitinase production medium was studied. Among the three sugars added, the highest chitinase productivity was obtained by the additional glucose (49.65 U/reaction) affording more than 44.75% productivity increase. In this respect, Kumar *et al.* [45] tested a mixture of different chitin forms (chitin and colloidal chitin) and found the ratio of 1 : 1.5 wt/wt, respectively, enhanced chitinase productivity by *Humicola grisea* ITCC.

Effects of inoculum size, age, and agitation rate

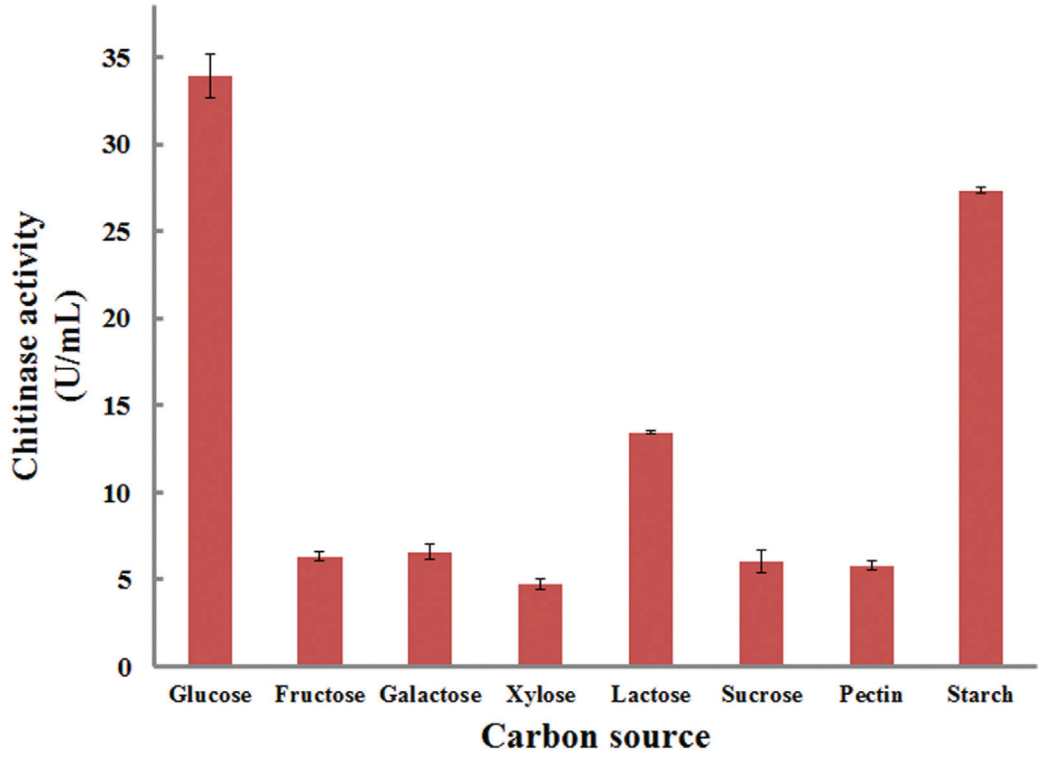
The effect of *S. halstedii* H2 inoculum size (5–15%, v/v) and age (12–72 h) was studied. 10% (v/v) inoculum

Figure 2



Effect of N sources in the culture medium on *Streptomyces halstedii* H2 chitinase productivity.

Figure 3



Effect of C source in the culture medium on *Streptomyces halstedii* H2 chitinase productivity.

(control experiment) was the most proper and led to the highest chitinase productivity, which decreased at above or below this concentration. Moreover, the inoculum age of 48 h was appropriate for the maximal chitinase productivity by *S. halstedii* H2 and the younger inoculum than 48 h produce lower enzyme yields. Suzuki *et al.* [25] inoculated *S. halstedii* culture medium with 4% (v/v) 47-h-old inoculum for optimal chitinase productivity. Concerning the effect of culture agitation rate, the rates from 150 to 250 rpm were applied in a bench-top thermostatic shaker, comparing it with a stationary culture, the shaking speed of 200 rpm resulted in the maximal chitinase activity, protein contents, well as dry weight of biomass, above which the productivity considerably decreased, and this was similar to those decided by EL-Masry [42].

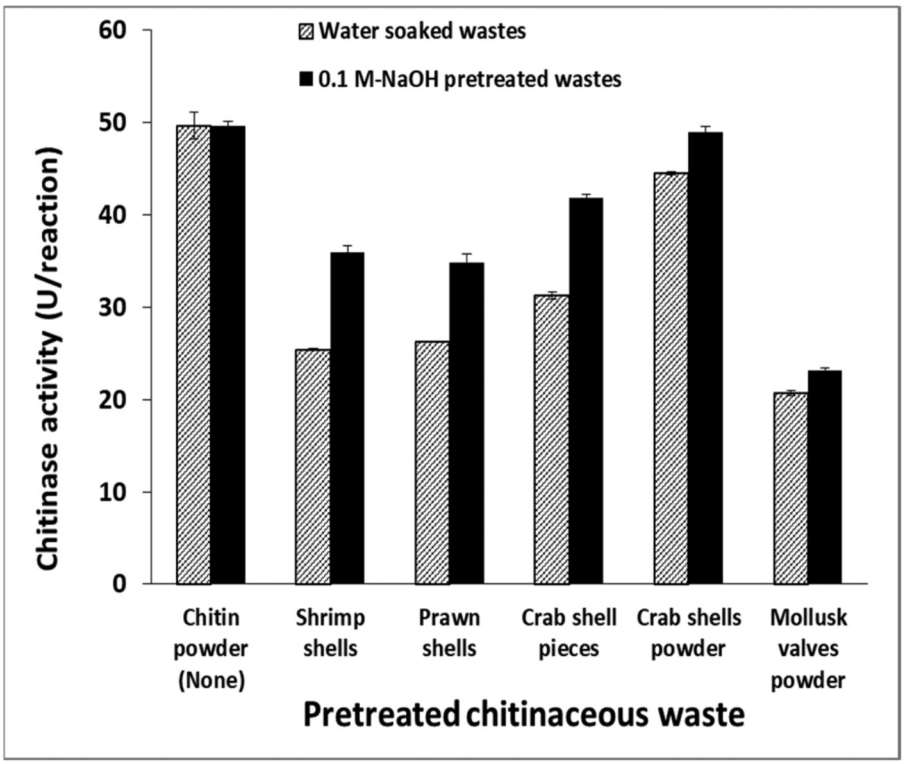
Incubation temperature

Different incubation temperatures (25–50°C) effect on the streptomycetal chitinase productivity was followed up. The maximal productivity was achieved at 30°C. The same optimum incubation temperature (30°C) was reported in shaken cultures for other streptomycetal chitinase enzymes [25,35], whereas Sakai *et al.* [46], Yuli *et al.* [47], and EL-Masry [42] decided higher temperatures (58, 55, 50, and 40°C) for chitinase production by *Bacillus* sp. MH-1, *Bacillus* sp.13.26, *Brevibacillus* sp. RPSG-NRC KX350091, and *Actinomadura* sp., respectively.

Employment of physically and chemically pretreated marine chitinaceous wastes for streptomycetal chitinase production

The employment of some physically, chemically, and physicochemically pretreated marine chitinaceous wastes, namely, shrimp, prawn, crab shells (pieces and powder), and mollusk valves powder instead of chitin powder in the optimized fermentation culture medium was implemented in 0.5% (w/v) concentration as recommended by many authors [48,49]. Physically pretreated (crushed and soaked overnight in distilled H_2O) wastes were tested for the extracellular chitinase enzymes production by *S. halstedii* H2. The data (Fig. 4a) declared that in all cases chitin powder was the most favorable by *S. halstedii* H2, and led to the highest chitinase enzyme production (49.65 U/reaction), followed by crab shells powder, crab shells pieces, prawn shells, shrimp shells, and mollusk valves powder (44.52, 31.28, 26.24, 25.40, and 20.68 U/reaction, respectively). The alkali-treated previous wastes crushed and soaked overnight in 0.1 M-NaOH were also examined (Fig. 4b); nevertheless, chitin powder was still the most favorable to *S. halstedii* H2 and led to the highest chitinolytic activity (49.65 U/reaction). Physically (crushed and

Figure 4



Effect of some (a) physically pretreated and (b) alkali-treated marine chitinaceous wastes on Streptomyces halstedii H2 chitinase production.

H_2O soaked overnight) or physicochemically (crushed and soaked in 0.1 M-NaOH overnight) pretreated crab shells to a great extent favored the streptomycetal chitinase synthesis as the pure chitin powder did (Fig. 4a and b).

In this concern, the utilization of the marine physically pretreated prawn and crab shells powder for microbial chitinase production was reported by many authors [39,50,51]. In addition, steam, drying, and milling were proceeded on prawn shells, crab shells, and squid pens to be utilized for the production of many value-added products including enzymes (chitinases, proteases, and nattokinase) through bioconversion means [52].

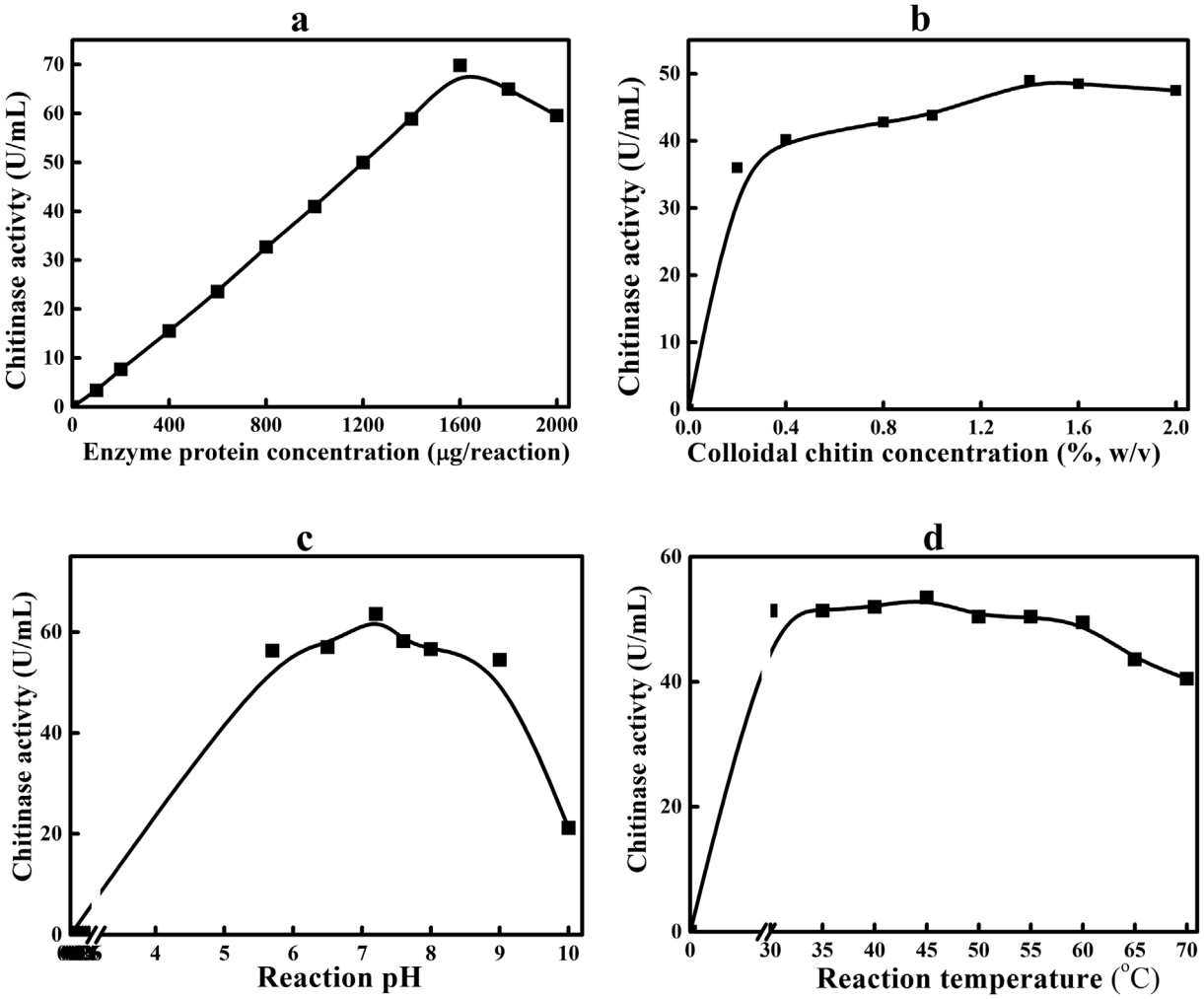
General properties of crude enzyme

The general properties of the crude streptomycetal chitinase include the effects of the enzyme protein concentration, substrate (colloidal chitin) concentration, the reaction pH, the reaction temperature, and pH stability. The results (Fig. 5a) indicated that 100 μg enzyme protein in the reaction mixture was able to proceed the reaction yielding 3.36 U/ml after 30 min at 40°C and pH 6.5. However, 200 μg/reaction led to a noticeable jump in enzyme activity, which reached 2.3-fold as that afforded by the lower enzyme concentration. The enzyme activity parallelly increased with the enzyme concentration till 1.6 mg enzyme/reaction, which achieved the highest activity (69.8 U/ml). This pointed out that 1.6 mg enzyme protein/reaction was enough to consume all the substrate applied in the reaction.

The effect of substrate (colloidal chitin) concentration on chitinase was illustrated in Fig. 5b, where the substrate concentration enhanced the reaction rate according to the mass action law, to reach the maximal at 1.4% (w/v) colloidal chitin indicating the saturation of all the available enzyme active sites with chitin molecules. In this connection, Ghaly [53], Nampoothiri *et al.* [54], and Sandhya *et al.* [55] reported that the optimum colloidal chitin concentration for many streptomycetal and other microbial chitinases was from 0.5–1.5% (w/v).

The data in Fig. 5c display the effect of reaction pH on the *S. halstedii* H2 chitinase at different pH values ranging from 5.7 to 10, applying 0.2 M-phosphate buffer pH (5.7–8.0) and 0.2 M-glycine-NaOH buffer pH (9.0, 10.0). The data proved that alkaline pH (7.2) was the most favorable for the enzyme performance, and above or below this pH resulted in a gradual loss in the

Figure 5



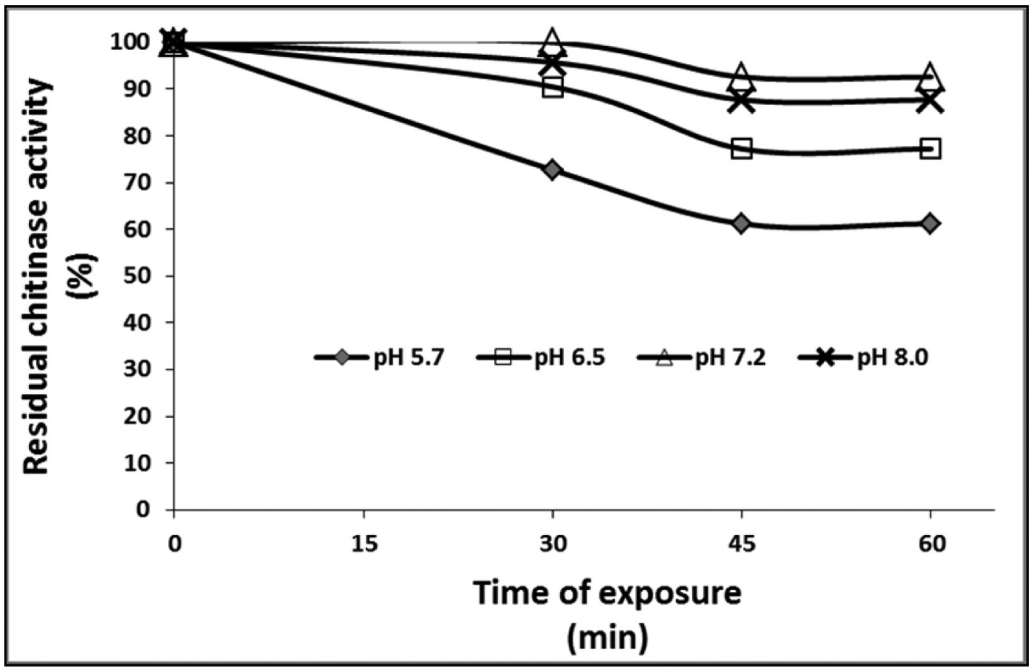
(a) Effect of enzyme protein concentration on the crude *Streptomyces halstedii* H2 chitinase activity. (b) Effect of colloidal chitin concn (%, w/v) on the crude *Streptomyces halstedii* H2 chitinase activity. (c) Effect of pH on the crude *Streptomyces halstedii* H2 chitinase activity. (d) Effect of reaction temperature on the crude *Streptomyces halstedii* H2 chitinase activity.

enzyme activity. It is worthy to note that at extreme alkaline pH (10.0), the enzyme still retained 71.2% of the optimum (at 7.2), indicating the good alkalinity tolerance of the streptomycetal chitinase. In this respect, the optimum pH of different chitinases from five strains of *Streptomyces* ranged from 4 to 7 [56].

The data in Fig. 5d disclose that heat till 45°C activates the enzyme reaction, and above which the enzyme activity slowly decreased. Elevation of temperature form 40°C (control) to 45°C led to more than 2.9% activation, indicating that 45°C was the optimum. At higher temperatures (55°C), the crude enzyme retained most its activity, which exceeds than 96.9% of the control (40°C). The crude enzyme retained also 83.8% at 65°C, indicating its high thermostability. The residual activity detected at temperatures higher than 65°C might be owing to the stabilizing effect of its substrate in the enzyme–substrate complex form [38]. In this respect, temperature 40°C was reported for streptomycetal chitinases by El-Sayed *et al.* [57] from *Streptomyces albovinaceus* S-22 and by Gomes *et al.* [58] from *Streptomyces* sp. RC1071. In addition, temperature 50°C was reported for chitinases of some *Streptomyces* strains [56].

The pH stability study was carried out on the crude enzyme preparation of the optimized concentration (1.6 mg protein/reaction), where it was heated alone at 30°C and different pH values (5.7, 6.5, 7.2, and 8) applying 0.2 M-phosphate buffer for different incubation periods (30, 45, and 60 min). At the end of each incubation period, the pH of the enzyme solution was readjusted to the optimum reaction pH (7.2) by adding 0.1 M-NaOH or 0.1 M-HCl, and then used in the reaction as usual manner. The residual chitinase activity (%) was calculated compared with the normal enzymatic reaction applying unheated crude

Figure 6



pH stability of the crude *Streptomyces halstedii* H2 chitinase activity.

enzyme sample. The data in Fig. 6 clarify that the enzyme exhibited the highest stability and retained all its activity (100%) after 30 min heating at 30°C and pH 7.2. The enzyme at the aforementioned pH 7.2 was still highly stable and retained most of its original activity (92.6 and 92.6%) for 45 and 60 min, respectively. It is noteworthy that the enzyme still retained 90.5 and 95.7% of the original after 30 min heating at pH 6.5 and 8, respectively. Conclusively, these findings confirmed the enzyme excellent pH stability and indicated its superiority to other streptomycetal chitinases, which were thermostable at temperatures below 40°C [59,60]. On the other hand, pH treatment of the enzyme at 5.7 was more harmful than that at the alkaline pH 8.0, so that the enzyme lost more than 61.2% of the original after 45 and 60 min at 30°C and pH 5.7, respectively and this was more or less accorded with those reported by Wang *et al.* [61], who reported that the chitinase CH1 from *Pseudomonas* sp. TKU015 had good stability at pH 5–7 and temperatures less than 50°C.

2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants. The data in Fig. 7 disclose the DPPH radical scavenging activity of the crude chitinase enzyme, which ranged from 14.43 to 94.55% at varying concentrations from 0.25 to 10 mg enzyme preparation wt/ml in ethanol solution, whereas ascorbic acid displayed more than 90% at the concentration 5 mg/ml. The DPPH radical scavenging activity increased in an enzyme dose-dependent manner. In this respect, the antioxidant activity of *Alcaligenes faecalis* AU02 culture supernatant was determined through scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) as 84%, and the antioxidant compound was characterized by TLC [62].

According to Fig. 7, the IC_{50} value was calculated for the applied crude chitinase to be at 0.95 mg, which contains 160 μg enzyme protein. It could be decided here that the *S. halstedii* H2 chitinase in its crude form exhibited a considerable scavenging activity comparing with the other related studies [62].

Concerning the antifungal activity, unfortunately the crude enzyme afforded low activity, and this will be followed up with the extended studies on the partially purified enzyme form, which is expected to exhibit good antimicrobial activity.

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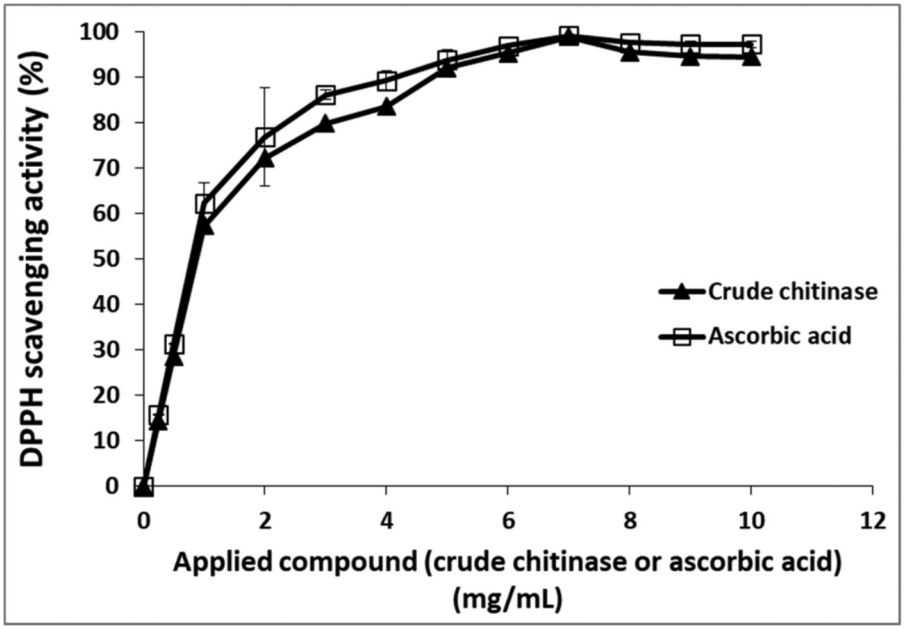
Figure 7



2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity of the crude chitinase enzyme and ascorbic acid.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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