

Production of Chitinase by *Serratia marcescens* from Soil and Its Antifungal Activity

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Abstract

36 [90%] *Serratia marcescens* isolates were obtained out of 150 samples of soil, taken from different locations in a farm in Babylon city. The isolate that produced chitinase in higher level was chosen to purify chitinase through: ammonium sulfate precipitation. The purified chitinase has molecular weight 54000 Daltons by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Chitinase showed stronger inhibitory activity to *Trichophyton rubrum*, *Alternaria alternate*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*.

Keywords: chitinase, *Serratia marcescens*, antifungal activity

1.Introduction

Serratia sp. are gram negative bacteria, classified in the large family of Enterobacteriaceae (Caprette, 2009). The *Serratia* genus includes different species, the best characterized species, and the one most frequently recovered from human, is *Serratia marcescens* (Hamid, *et al.*, 2013) This common microbe is found as a saprophyte in soil, water and plants (Rebecca, *et al.*, 2013). In addition, *Serratia* species frequently have been recovered from diseased or dead insects (Orunsi and Trinci, 1985). *Serratia* sp. have a good set of exoenzymes that may be harmful to insects such as proteases, gelatinase and chitinase (Suginta, *et al.*, 2005).

Chitin a homopolymer of N-acetyl-D-glucosamine (GlcNAc) residues linked by β 1, 4 bonds is widely distributed in nature as a constituent of insects exo skeleton, shells of crustaceans and fungal and algae cell walls (Grimont and Grimont, 2006). Chitinase plays an important role in the virulence of *Serratia* for insects and fungi, since this enzyme is active in lysing the cell walls of many insects and fungi. Also chitinase plays an important role in the medical field as antitumor as well as the direct medical use of chitinase enzymes in the treatment of fungal diseases by introducing it within the medical ointments as antifungal drugs (Hejazi & Falkiner, 1997). This enzyme has also antimicrobial and cell lysis activities against many kinds of bacteria (Khanafari, *et al.*, 2006). Therefore, chitinase has wide range of biotechnological and medical applications (Zhu, *et al.*, 2007). For these reasons, the goal of our research was to production chitinase, to purify this enzyme, to study the antifungal activity.

2. Materials and Methods

2.1 Collection of Samples

Soil samples were collected from various regions in Babylon city through April to July (2012). The soil samples were collected from a depth of two inches using a spatula and stored in clean bags and transported to the lab. (Mahmood, 2007).

2.2 Isolation and Identification of *Serratia marcescens*

One gram of each sample was added to 10 ml of sterilized normal saline solution in test tubes and mixed thoroughly (Zbar, 2011). Serial dilutions for each sample were achieved, and then 0.1 ml aliquots from the appropriate dilution were spread on nutrient agar plates. After incubation at 28°C for 18-24 hrs, red pigmented colonies were selected for further identifications. For identification of *Serratia marcescens*, several biochemical tests were done. As well as using Microgen™ GnA+B-ID System (Microgen Bioproducts Ltd/ UK) to differentiate *Serratia marcescens* from the other microorganisms.

2.3 Detection of Chitinolytic Activity on Plates

A single colony of *Serratia marcescens* culture was placed on the chitin agar medium that contained 2.0g colloidal chitin, 0.07 g K_2HPO_4 , 0.03 g KH_2PO_4 , 0.05g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, (pH=7.0) and 2.0 g agar in 100 ml of distilled water and incubated at 30°C for 6 days. After 18-24 h, the chitinolytic activity was indicated by the formation of clear halos around each colony (Murthy and Bleakley, 2012).

2.4 Enzyme extraction

The extraction of enzyme was made as per the procedure described by (Boller & Mauch, 1988). Chitin broth culture medium having 2 % colloidal chitin was used for extraction of the enzyme. The freshly prepared medium was inoculated with 5% seed culture. The inoculum was incubated at temperature controlled rotary shaker under shaking conditions (150 rpm) at 30°C for the period of 6 days. The culture was centrifuged at 6,000 g for 30 min at 4°C.

2.5 Enzyme Assay

For the measurement of chitinase activity, colloidal chitin was selected as the substrate. The reaction mixture containing 1 ml of 1% w/v colloidal chitin in 0.02 M phosphate buffer pH 7.5 and 1 ml enzyme solution was incubated in shaker water bath at 30 °C for one hour. Then stop the enzymatic reaction by placing them in boiling water bath for 3 minutes (Boller & Mauch, 1988). After centrifugation, determination of reducing sugar in supernatant was accomplished by the modified method of (Reissig, *et al.*, 1955). Absorbance was measured at 585 nm using UV spectrophotometer along with substrate and blank. For determination of enzyme unit, serial dilutions of *N*-acetylglucosamine (from 0 to 50 mM) were prepared. One unit (U) of the chitinase activity was defined as amount of enzyme required to release 1 μ mol of *N*-acetyl D-glucosamine (as a standard) from chitin / min.

2.6 Protein assay: The protein concentration was determined by Bradford Method (1976) using bovine serum albumin as a standard solution and the absorbance was measured at 595 nm.

2.7 Partial Purification

Serial concentrations of ammonium sulfate from 20 to 80% was added slowly to the enzyme solution and left at 4 °C for at least 3 h with vigorous stirring. Then the solution was centrifuged at 6,000 rpm for 20 min. Next, sediment was dissolved in minimum phosphate buffer 0.02 M in pH 7.5. After that, the concentrated solution dialyzed in the same buffer twice and was checked for maximum chitinolytic activity (Ward and Swiatek, 2009).

2.8 Determination of Molecular Weight

The partially purified enzyme was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE 16%) to determine its molecular weight and its purity by Laemmli method (Laemmli, 1970). Enzyme solutions obtained from culture medium. Then the proteins were separated from each other at constant voltage (165v). Next the electrophoresized gel bands were colorized with Coomassie brilliant blue R-250 and destained using acetic acid and methanol. Finally the relative molecular weight of the protein was estimated using standard molecular weight (Prestained protein ladder; Geneaid).

2.9 Determination of Antifungal Activity of Chitinase

2.9.1 Isolation of fungi

Trichophyton rubrum, *Alternaria alternate*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus* were obtained from the Life Sciences laboratory of Science College in Babylon University. This isolates were isolated from different regions of human body activated on Potato dextrose agar at 28°C for (7-10) days.

2.9.2 Antifungal Activity

For the detection of chitinase antifungal activity, the zone of inhibition assay (Zarei, *et al.*, 2011) was carried out. The mycelium of fungi was inseminated in the center of the Petri plates containing potato dextrose agar (PDA). When the diameter of the colony was almost 2 cm, sterile blank paper disks were located around with equal distance from the center of the plates.

Equal aliquots (50 μ l) of phosphate buffer 0.02 M pH 7.5 (as control) and partially purified chitinase were presented onto disks respectively and then incubated at 25°C until the colony started growing.

3.Results and Discussion

A total of 150 soil samples were collected from different regions in Babylon city, 40 of them showed positive result. Results also showed that among the total 40 isolates, only 36 (90%) were able to produce prodigiosin red pigment, which gives an indicator that these isolates belong to *Serratia spp.*, while the other 4 isolates may belong to other pathogenic or nonpathogenic bacteria from different genera. These 36 isolates were further characterized and identified according to their cultural, morphological characteristics and biochemical tests, as shown in (Table 1 and 2).

3.1 Chitinolytic activity on plates

Bacterial isolates were tested for chitinase production by measuring the diameter of clear zone of lysis in colloidal chitin medium agar. Only eighteen (18) isolates produced chitinase enzyme and isolate no. 22 (figure 1) produced chitinase in higher level among the producer isolates, hence this isolate was selected for further study. Green *et al.*, 2004 reported that the optimum conditions (pH 7.0, 32.5°C and 1.0 % (w/v) substrate induced a higher level of enzyme activity. Also (Ordentlich and Chet, 1988) showed that the maximum production of *Serratia marcescens* chitinase was between pH 6 and 7 at 30 °C and the optimal shaking speed at 150 rpm. Chitinase production was reduced by 50% at pH 8.5 of production medium. In a study done by (Hyun-soo, *et al.*, 2007) found that among 102 *Serratia marcescens* strains screened, 57 strains showed chitinase activity.

Table 1. Biochemical tests for the bacterial isolates

No.	Biochemical Tests	Results
1.	Gram stain	Gram-negative rod
2.	Catalase Test	+
3.	Motility test	+
4.	DNase	+
5.	Oxidase test	-
6.	Methyl Red Test	-
7.	Gelatin hydrolysis	+
8.	Growth at 40 °C	+

3.2 Molecular Weight and Protein Concentration

An enzyme solution partially purified by ammonium sulfate 75 % with maximum activity was examined for purity and molecular weight determination by SDS-PAGE. Molecular weight of the enzyme was estimated to be almost 54 kDa (Figure 2). Molecular weights of chitinases obtained from different strains of *Serratia* were 47-60 kDa (Wang, *et al.*, 2008). The molecular weight of chitinases from *Serratia marcescens* QM13 1466 (Roberts and Cabib, 1982) and *Serratia plymuthica* HRO_C 48 (Frankowski, *et al.*, 2001) were 58 kDa and 60.5 kDa respectively. The molecular weight of chitinases obtained from *Enterobacter* sp. was about 60 kDa. Molecular weight of chitinase from *Enterobacter* sp. NRG4 (Dahiya, *et al.*, 2005) *Enterobacter* sp. G-1 (Park, *et al.*, 1997) and *Enterobacter aerogenes* (Vogelsang and Barz, 1993) were 60 kDa, 60 kDa and 42.5 kDa respectively that were almost similar to our results.

3.3 Antifungal Activity of Chitinase

Figure 3 presents antifungal activity of chitinase against a wide range of fungi. (Dahiya, *et al.*, 2005) identified antifungal chitinase against *R. solani* from *Enterobacter* sp. NRG4 with molecular weight 64 kDa. Antifungal chitinase also isolated from *Bacillus subtilis* CH426 (Yang, *et al.*, 2009), *Bacillus cereus* J1-1 (Wang and Hwang, 2001) *Bacillus* sp. DAU101 (Lee, *et al.*, 2007) *Cellulosimicrobium cellulans* 191 (Fleuri, *et al.*, 2009) with molecular weight 64 kDa, 65 kDa 62 kDa and 61 kDa respectively.

Table 2. Microgen™ GnA+B-ID identification system results

No.	Reaction	result
1.	lysine	+
2.	Ornithine	+
3.	H ₂ S	-
4.	Glucose	+
5.	Mannitol	+
6.	Xylose	-
7.	ONPG	+
8.	Indole	-
9.	Urease	-
10.	V.P.	+
11.	Citrate	+
12.	TDA*	-

* TDA: tryptophan deaminase



Figure 1. Zone of chitinolysis

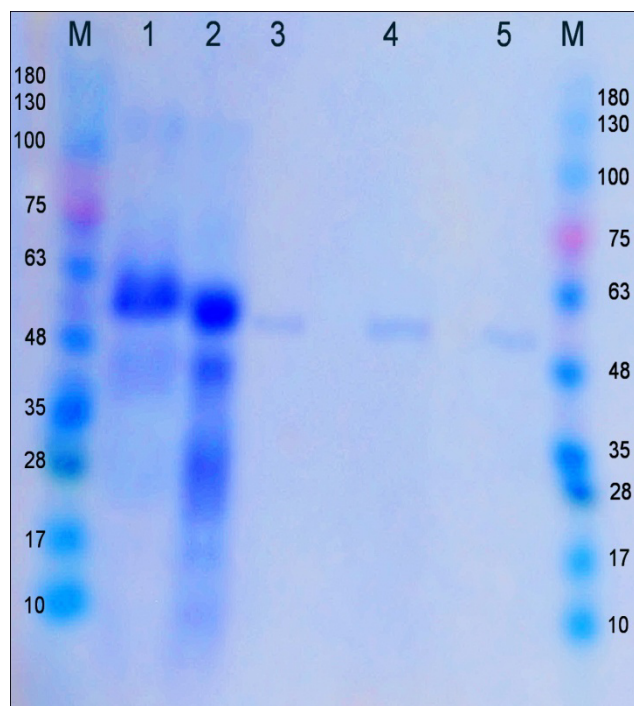
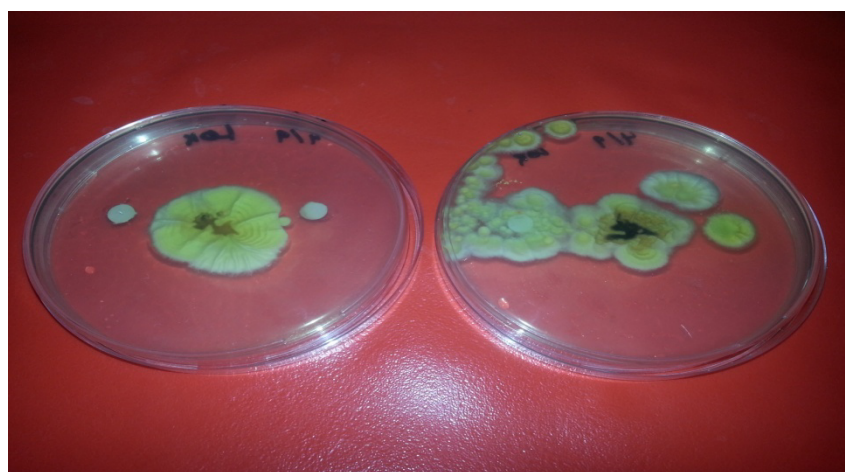


Figure 2. SDS-PAGE analysis of chitinase. Lane 1 and 2 crude enzyme; Lane 3,4 and 5 , partial purified chitinase by ammonium sulfate 75%.



(A)

(B)

Figure 3. Antifungal activity of chitinase. (A) Partial purified chitinase
(B) Control, phosphate buffer 0.02 M pH 7.5.

The chitin has an intrinsic variability due to its natural origin and it exists in several forms with their specific properties each. This polymer of fungi possesses principally the same structure as the chitin occurring in other organisms. However, a major difference results from the fact that fungal chitin is associated with other polysaccharides which do not occur in the exoskeleton of arthropods. Difference in chitinolytic ability must result from the subsite structure in the binding cleft. This implies that why the enzyme didn't show significant antifungal activity against each Fungus (Sasaki, *et al.*, 2002).

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