

Characterization, antifungal activity, and cell immobilization of a chitinase from *Serratia marcescens* MO-1

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Abstract: Chitinases have the potential to control many pathogen species, such as fungi containing chitin on their cell walls. In the present study, chitinase from novel isolate *Serratia marcescens* MO-1, isolated from *Poecilimon tauricola* (Orthoptera: Tettigoniidae) in Turkey, was investigated. The optimum pH and temperature for the chitinase activity were found to be pH 7.0 (36.6 U/mL) and 50 °C (37.1 U/mL), respectively. Moreover, the activity was still high in acidic (23.0 U/mL at pH 3.0) and basic (17.3 U/mL at pH 11.0) conditions as well as at lower (29.5 U/mL at 20 °C) and higher (28.7 U/mL at 80 °C) temperatures. The enzyme was highly thermotolerant, keeping 63.7% (23.5 U/mL) of its activity after incubation at 90 °C for 15 min. Antifungal activity of the chitinase against *Alternaria citri*, *Fusarium oxysporum*, *Trichoderma harzianum*, *Aspergillus niger*, and *Rhizopus oryzae* was determined. In addition, chitinase production by immobilized *S. marcescens* MO-1 cells was monitored over 10 days; the enzyme activity was found to be 36–41 U/mL during this period. Our results showed that the chitinase from *S. marcescens* MO-1 seems to be valuable in terms of its biotechnological applications; it can be produced using immobilized cells and can be used against fungal pathogens as an alternative to chemical pesticides.

Key words: *Serratia marcescens*, chitinase, cell immobilization, antifungal activity

1. Introduction

Despite their involvement in environmental and human health problems, chemical pesticides have been used in the killing of pest organisms for over 55 years (1). Moreover, there is difficulty in discovering new classes of chemicals for the management of these pests because of their increasing resistance to chemical pesticides (1). Therefore, in recent years, research on alternative, biofriendly, and economically acceptable strategies such as biochemical products from plants, insects, and microorganisms for pest control has been encouraged (1,2).

Since fungal cell walls and insect exoskeletons contain chitin, a homopolymer of β -1,4-linked *N*-acetyl-*D*-glucosamine residues as a major structural component, chitinases have been reported for their potential as biopesticides for the control of the plant diseases caused by various phytopathogenic fungi (3,4) and insect pests (1,5). Chitin is degraded into disaccharides and larger oligomeric saccharides by the action of chitinases (EC 3.2.1.14) through hydrolysis of the β -(1,4)-linkages (6), and these enzymes have been divided into 2 separate

families, GH18 and GH19, based on amino acid sequences and their catalytic mechanisms (4).

Serratia marcescens is a gram-negative, facultative anaerobe soil bacterium known as one of the most efficient bacteria for degradation of chitin (7). When this bacterium is cultivated in the presence of chitin, a variety of chitinolytic enzymes and chitin-binding proteins can be detected (8). Due to its biotechnological importance, the genes encoding chitinases from different strains of *S. marcescens* have been cloned and expressed in *Escherichia coli* for characterization (9–11) and increased enzyme production (12), as well as in other bacteria such as *Bacillus thuringiensis* to increase their pesticidal activity (13,14).

In the present study, chitinase from *S. marcescens* MO-1, a novel strain isolated from a grasshopper (*Poecilimon tauricola*) in Turkey, was investigated for its antifungal potential. Effects of pH and temperature on enzyme activity, its thermal stability, and production by an immobilized biomass were also examined. To our knowledge, there is no earlier study using immobilized *S. marcescens* cells for chitinase production.

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2. Materials and methods

2.1. Isolation and identification of *S. marcescens* strain MO-1

Naturally dead grasshoppers (*Poecilimon tauricola* Ramme, 1951; Orthoptera: Tettigoniidae) were collected from fields contaminated with endosulfan, a chlorinated organochlorine pesticide, in Antalya Province, Turkey, in 2011. The insects were surface-sterilized using 70% ethanol for 1 min and crushed in a sterile mortar containing 10 mL of 0.85% (w/v) saline solution. Dilutions of 10^{-4} of each sample (0.1 mL) were inoculated on nutrient agar (NA; Merck, Germany) plates and incubated at 28 °C for 48 h. The colonies with dark red color (due to pigmentation) were isolated (15), and preliminary identification was performed using the Biolog Identification System (Biolog, Inc., USA) and Microbial Identification System (MIDI, Inc., USA). Furthermore, the isolate identification was verified by the analysis of the 16S ribosomal DNA sequence (RefGen Life Sciences, Turkey), which was compared with the National Center for Biotechnology Information database using the BLAST search on the web site (<http://www.ncbi.nlm.nih.gov/BLAST>) and submitted to GenBank.

2.2. Media and culture conditions

S. marcescens MO-1 was grown and maintained on NA (Merck) plates at 28 °C and 4 °C, respectively. For chitinase production, the bacteria were grown in nutrient broth (NB; Merck) containing 0.2% (w/v) colloidal chitin (16) at 28 °C for 24 h at 200 rpm. Chitinase activity of *S. marcescens* MO-1 was also shown on NA plates containing 0.2% colloidal chitin, which were incubated at 28 °C for 10 days. The immobilized cells were incubated in 25 mM Tris buffer (pH 7.2) at 28 °C.

2.3. Immobilization of *S. marcescens* MO-1 cells

S. marcescens MO-1 cells were immobilized in calcium alginate gel beads as described previously (17). *S. marcescens* MO-1 was grown in NB (Merck) at 28 °C for 24 h. The culture was centrifuged at 5000 rpm for 25 min and the biomass was washed twice with sterile 0.85% (w/v) saline solution (SSS). Wet cells (4 g) were thoroughly resuspended in 40 mL of SSS and the total volume was completed to 50 mL with SSS. Sodium alginate solution (3%, w/v) was prepared by dissolving sodium alginate in SSS at 70 °C. The cell suspension (50 mL) was mixed with an equal volume of sodium alginate solution and stirred for 5 min. The mixture was dropped into a well-stirred sterile CaCl_2 solution (3.5%, w/v) using a syringe. Each alginate drop solidified upon contact with CaCl_2 and formed beads that encapsulated the *S. marcescens* cells. The beads were left to harden for 30 min at room temperature after they were washed with SSS to remove excess calcium ions and unencapsulated cells. The average bead diameter was approximately 2–3 mm.

2.4. Measurement of chitinase activity

The chitinase activity of *S. marcescens* MO-1 was measured by the determination of reducing sugar (18). The assay was carried out according to the method of Babashpour et al. (4) with minor modifications. Briefly, 1 mL of cell-free culture supernatant as crude enzyme source was mixed with 1 mL of substrate containing 1% colloidal chitin prepared in 20 mM phosphate buffer (pH 7.5). The reaction mixture was incubated at 50 °C for 60 min, and 2 mL of 1% 3,5-dinitrosalicylic acid (Sigma, Germany) was added to stop the reaction. The mixture was boiled for 15 min, cooled to room temperature, and centrifuged at 5000 rpm for 5 min. The absorbance was measured at 530 nm. N-acetyl-D-glucosamine (Sigma) was used as the standard and 1 unit of enzyme activity (U) was defined as the amount of enzyme that liberated 1 μmol reducing sugar per minute.

For investigation of the effect of pH on chitinase activity, the pH of the substrates was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0. For the effect of temperature, the reaction mixtures were incubated at 20, 30, 40, 50, 60, 70, and 80 °C. To determine the thermal stability of the chitinase, 10 mL of the crude extract was placed in a water bath and incubated at 40 °C for 15 min. Later, the temperature of the water bath was increased up to 50 °C and the enzyme source was incubated for 15 min. This was repeated for 60, 70, 80, and 90 °C, respectively. After incubations, 1 mL of sample was used for measurement of enzyme activity. The enzyme assays were performed in triplicate.

2.5. Antifungal activity

Antifungal activity of the chitinase from *S. marcescens* MO-1 was determined using the agar-disk diffusion method. *Aspergillus niger* (saprophyte), *Rhizopus oryzae* (saprophyte), *Fusarium oxysporum* (phytopathogen), *Trichoderma harzianum* (antagonistic activity against phytopathogens), and *Alternaria citri* (phytopathogen) were obtained from our laboratory collection (Microbiology Laboratory, Department of Biology, Atatürk University). The fungi were first grown on potato dextrose agar (PDA; Merck) plates at 28 °C for 7 days. Spore suspension of each fungal strain was prepared by agitation of PDA cultures in 5 mL of SSS including 0.1% Tween-80 and diluted to a concentration of 10^6 spores/mL. Next, 100 μL of spore suspension was spread on PDA plates. A sterile filter disk with a diameter of 5 mm (Whatman paper No. 3) was placed in the plate and 30 μL of crude enzyme was applied to the disk. Negative control disks were applied with 30 μL of SSS. The plates were incubated at 28 °C for 48 h and the diameters of inhibition zones around the disks were measured. All the tests were conducted in triplicate.

2.6. Statistical analysis

An analysis of variance (ANOVA) and Tukey's test were used for mean comparison of antifungal activity zones between groups using GraphPad Prism version 5.00 for Windows (Graph-Pad Software, US). Standard deviations were calculated using Microsoft Office Excel 2010.

3. Results and discussion

Chitin is the second most abundant naturally occurring polysaccharide after cellulose (19); therefore, chitinases have great biotechnological importance in terms of converting chitin-containing biomass into useful (depolymerized) components, as well as for control of fungal pathogens and insect pests of plants (8). *S. marcescens* is one of the most effective bacteria for degradation of chitin by a variety of chitinolytic enzymes and chitin-binding proteins (8). In the present study, we report characterization and production of a chitinase from *S. marcescens* strain MO-1.

3.1. Strain identification

Among the bacteria isolated from a grasshopper (*Poecilimon tauricola*, Orthoptera: Tettigoniidae) collected in Antalya Province in Turkey, primary identification of the *Serratia* colonies was conducted according to pigment production. Several of the species of bacteria contained identical pigments (20); *S. marcescens* produces a dark red pigment called prodigiosin (15). Afterwards, pigmented colonies were verified as *S. marcescens* by the Biolog Identification System and Microbial Identification System according to metabolic enzyme products and fatty acids, respectively. Finally, an 837-bp 16S ribosomal DNA sequence of the strain was BLAST-searched (<http://www.ncbi.nlm.nih.gov/BLAST>) and aligned with *S. marcescens* sequences. The sequence was deposited in GenBank with the accession number of JX315621. The isolate was named as *S. marcescens* strain MO-1.



Figure 1. Chitinase production by *S. marcescens* MO-1 on the chitin plate. The clear zone shows the degradation of chitin.

3.2. Chitinase activity of *S. marcescens* MO-1

S. marcescens strain MO-1 was grown on NA plates containing chitin and its chitinase activity was shown via formation of a clear zone due to chitin degradation (Figure 1). Chitinase activity of *S. marcescens* MO-1 was monitored for 40 h in relation to its growth (Figure 2). Chitinase activity increased sharply after 8 h of cultivation and peaked at 24 h, being similar to *S. marcescens* Bn10 (14). The average activity of chitinase produced by *S. marcescens* MO-1 was found to be 35 U/mL, being 59 times higher than that produced by *S. marcescens* IMR-1E1, a chitinase over-producer mutant (21), as well as 16 times higher than that produced by *S. marcescens* Nima (7), reported to be 43 times higher than those produced by other *S. marcescens* strains.

To determine the effects of pH and temperature on chitinase activity, the reaction mixtures were incubated at a pH range from 3.0 to 11.0 and a temperature range from 20 to 80 °C. The optimum pH and temperature for the enzyme activity were determined to be pH 7.0 (36.6 U/mL) and 50 °C (37.1 U/mL), which were reported as pH 9.0 and 45 °C for that from *S. marcescens* Bn10 (14), pH 7.0 and 45 °C for that from *S. marcescens* ATCC 990 (12), and pH 6.0 and 55 °C for that from *S. marcescens* B4A (4).

Chitinase from *S. marcescens* MO-1 has high activity and stability in stringent conditions such as high acidity (23.0 U/mL at pH 3.0, 62.8% remaining activity) and alkalinity (17.3 U/mL at pH 11.0, 47.3% remaining activity), as well as at lower (29.5 U/mL at 20 °C, 79.5% remaining activity) and higher (28.7 U/mL at 80 °C, 77.4% remaining activity) temperatures (Figure 3). Chitinases from *S. marcescens* strains Bn10 (14) and B4A (4) lost almost all of their activity at these pH values. At lower and higher temperatures, activity of chitinase from *S.*

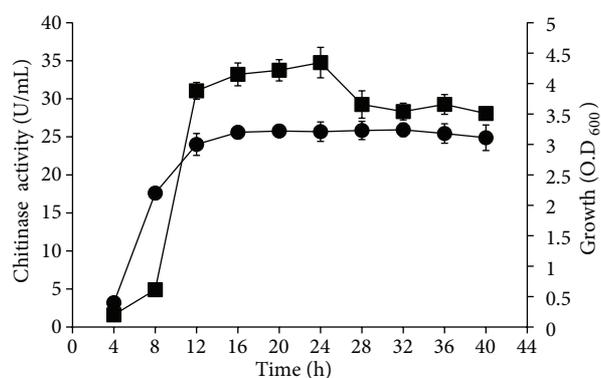


Figure 2. Chitinase activity (squares) in relation to growth (circles) of *S. marcescens* MO-1.

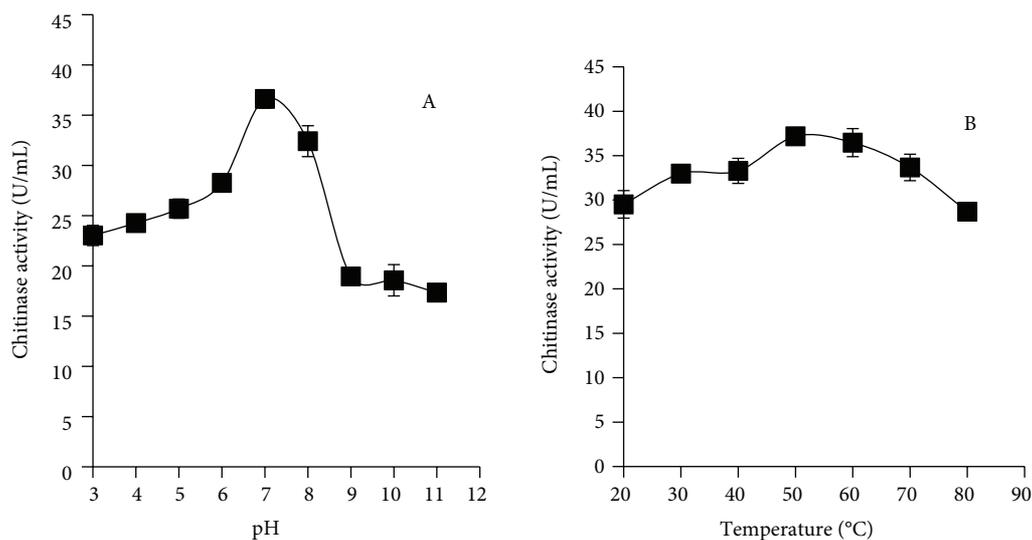


Figure 3. Effects of pH (A) and temperature (B) on the activity of chitinase from *S. marcescens* MO-1.

marcescens MO-1 was similar to that from strain Bn10 (14), being higher than that from strain B4A (4).

The thermal stability of the chitinase was determined by incubating the enzyme at temperatures that ranged from 40 to 90 °C for 15 min prior to measurement of the activity. The preincubation chitinase activity was 36.9 U/mL, and it was found to be 32.4 U/mL (87.8% remaining activity) and 23.5 U/mL (63.7% remaining activity) after incubation at 40 °C and 90 °C, respectively (Figure 4). Stability of chitinase from *S. marcescens* ATCC 990 (12) and strain B4A (4) was significantly decreased at temperatures higher than 50 °C.

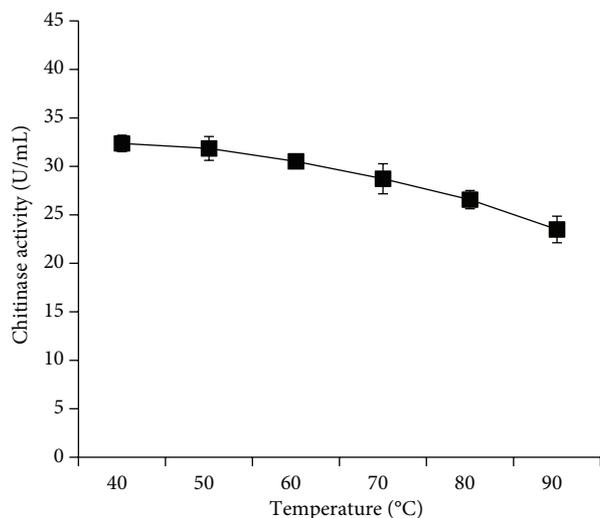


Figure 4. Thermal stability of the chitinase from *S. marcescens* MO-1. The enzyme was incubated at different temperature ranges for 15 min prior to measurement of activity at standard conditions.

3.3. Chitinase production by immobilized cells

Immobilized cells have many important advantages in terms of industrial application since they have their original biological functions with increased stability and cell productivity, and they can be reused for repeated or continuous processes without cell wash-out and with easy separation of the cells from the reaction system (22). Chitinase production by immobilized cells of *Penicillium janthinellum* (22), *Trichoderma* spp. (23), and *Bacillus* sp. R2 (24) has been shown previously. Chen and Chang (25) immobilized the chitinase from *S. marcescens* and reported a 30% loss in enzyme activity during the first cycle. In our study, *S. marcescens* MO-1 cells were immobilized in 3% alginate gel and incubated in Tris buffer at 28 °C for 10 days, the buffer being changed every 24 h. The chitinase production was monitored daily measuring the enzyme activity, and the activity was found to be 36–41 U/mL during this period (Figure 5). The lowest and the highest activities were measured at days 8 and 6, respectively.

3.4. Antifungal activity

An important property of chitinases is their use as biocontrol agents as an alternative to chemical pesticides. Antifungal and insecticidal activities of chitinases from different sources have been reported (1,3–5). Antifungal activity of the chitinase from *S. marcescens* MO-1 was investigated on *Aspergillus niger*, *Rhizopus oryzae*, *Fusarium oxysporum*, *Trichoderma harzianum*, and *Alternaria citri* (Figure 6). The inhibition zones for *A. citri*, which causes Alternaria black rot, a postharvest fruit disease, on a broad range of citrus cultivars (26) and *T. harzianum* were not significantly different ($P > 0.05$), albeit higher ($P < 0.001$) than those for *F. oxysporum*, *A. niger*, and *R. oryzae*. The

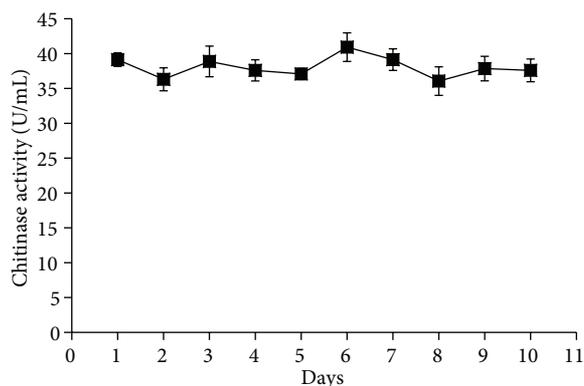


Figure 5. Cell immobilization for chitinase production. *S. marcescens* MO-1 cells were immobilized in 3% alginate gel and incubated in 25 mM Tris buffer at 28 °C for 10 days, the buffer being changed daily. The enzyme activity at each day was measured using the culture supernatant as crude enzyme source.

inhibition zone for *F. oxysporum* was also significantly higher ($P < 0.05$) than that for *R. oryzae*.

In conclusion, *S. marcescens* MO-1, a novel strain isolated from a grasshopper (*Poecilimon tauricola*) in Turkey, has high chitinolytic activity. Its chitinase is very stable in stringent conditions. The enzyme produced by immobilized cells seems to be valuable in biotechnological

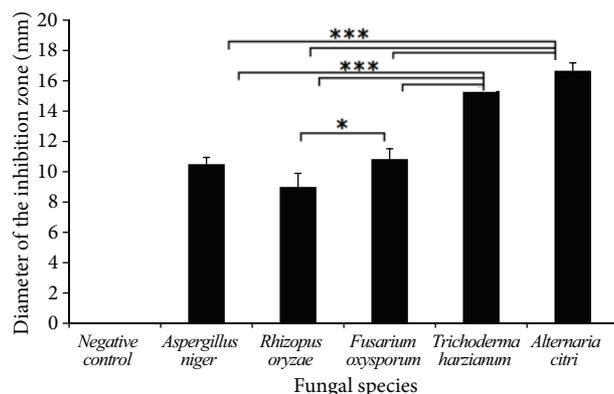


Figure 6. Antifungal activity of the chitinase from *S. marcescens* MO-1 against different fungal species (*: $P < 0.05$, ***: $P < 0.001$).

applications and can be used against fungal pathogens as an alternative to chemical pesticides.

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