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Bacillus atrophaeus A7 Crude Chitinase: Characterization and Potential Role against *Drosophila melanogaster* Larvae

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Abstract

Microbial chitinases are important environmental biomolecules with biotechnological and medicinal applications in addition to being a source of environmental friendly biopesticides. They are considered as safe alternatives to some available chemical insecticides, especially against insects that may act as an intermediate hosts as well as vectors between manifested plant materials and humans. A crude chitinolytic enzyme was isolated from isolate A7 (*Bacillus atrophaeus* Nakamura 1989). The isolate was identified based on the morphological and biochemical characteristics as well as the sequencing of 16S rRNA. The produced enzyme had a total activity of 68.9±1.03 mU/mL; a specific activity of 2670±40.2 mU/mg protein, and was optimally active at 40°C, 4-9 pH with stability for one hour at 30-40°C and 6-7 pH. It was inhibited by Cu^{2+}, Fe^{3+}, Ni^{3+}, Zn^{2+} and Ba^{2+} metal ions and impeded the development of 50 % of *Drosophila melanogaster* larvae into adults (LD_{50}) at 17.3±1.4 mU/mL. In this study, the larvicidal activity of chitinase from *B. atrophaeus* is explored for the first time with the potential of being applied as environmental friendly biopesticide technologies.

Keywords: *Bacillus atrophaeus*, *Drosophila melanogaster*, Chitinase enzyme, Chitinolytic bacteria, Larvicidal activity, Bioinsecticides

1. Introduction

Chitin is a naturally abundant biomolecule after cellulose. It accumulates as a waste from shellfish production and processing industries in the terrestrial environment (Chakrabortty *et al*., 2012). It occurs mainly as a structural component in the exoskeleton of arthropods and to lesser extents in plants, fungi, bacteria and other animals (Zarei *et al*., 2012). It could be degraded into various chitooligomer molecules that may undergo further enzymatic breakdown generating N-acetylglucosamine (GlcNAc) monomers by sequential action of two types of chitinase enzymes: endochitinase and exochitinase. Endochitinase (EC 3.2.1.14) randomly catalyzes the cleavage of β-1,4-glycosidic bonds in chitin to release N-acetylchitooligosaccharides. On the other hand, exo-chitinase (EC 3.2.1.52, includes the formerly classified EC 3.2.1.29 and EC 3.2.1.30) catalyzes the progressive release of diacetylchitobiose starting at the non-reducing end of the chitin that is further cleaved to generate monomers of GlcNAc (Fu *et al*., 2014). Chitinases are receiving an increased attention due to their broad range of applications. They are potential biocontrol agents against plant-pathogenic fungi (Nguyen *et al*., 2015) and control insect pests (Rathore and Gupta, 2015). They have a role in the bioconversion of chitin to single cell protein from shellfish waste (Hao *et al*., 2012) and in the isolation of protoplast from fungi (Dahiya *et al*., 2005), in addition to their medical applications (Aam *et al*., 2010; Stoykov *et al*., 2015).

Drosophila melanogaster is considered an insect pest that infests various habitats where fermenting fruits are found. In fact, infestation removal is difficult as the larvae may continue to develop in fermenting fruit even if the adult population was eliminated. Moreover, *D. melanogaster* could act as an intermediate host as well as a vector between manifested plant materials (Keesey *et al*., 2017) and a source of foodborne disease outbreaks among humans (Blazar *et al*., 2011). Hence, looking for a biopesticide as an environmental friendly alternative is of primary concern, such as proteolytic and chitinase enzymes.

Chitinolytic microbes occur widely in nature. They are preferred sources of chitinase because of their low production cost and the availability of raw materials for their cultivation (Singh *et al*., 2008). Their chitinases have nutritional and morphogenetic roles in fungi (Adams, 2004), and play a role in the utilization of chitin as a carbon and energy source and in the recycling of nitrogen in nature (Chandran *et al*., 2007). Some of the best-known Gram-negative and Gram-positive bacterial genera are remarkably producers of chitinolytic enzymes including *Serratia*, *Aeromonas*, *Pseudomonas*, *Alteromonas*, *Enterobacter*, *Bacillus*, *Clostridium*, *Micronomospora*, and *Actinomyces* (Cheba *et al*., 2016). Intriguingly, some of

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them were reported to produce multiple forms of chitinases with different molecular masses (Anuradha and Revathi, 2013).

Herein, *Bacillus atrophaeus* A7 was isolated from exoskeleton of dead ground beetles (Coleoptera: Carabidae) and was selected as chitinolytic bacterium. *B. atrophaeus* is noted as a bio-agent against various fungal pathogens, in addition to having the capacity to improve crop production acting as plant growth stimulants as well as the role in other biotechnological approaches (Liu *et al*., 2012; Zhang *et al*., 2013; Sella *et al*., 2015). Recently, it was reported that *B. atrophaeus* HAB-5 exhibited a broad antifungal spectrum, particularly against *Colletotrichum* sp., *Alternaria* sp. and *Fusarium* sp. with activity under a wide pH range (pH 2-12), and heat stability between 40 °C and 100 °C and pH 5 to 10 (Rajaofera *et al*., 2017).

Nevertheless, the insecticidal activity of chitinases from *B. atrophaeus* has not been previously reported. Therefore, the produced chitinase from *B. atrophaeus* A7 was evaluated in term of its activity, stability under various conditions as well as its potential role as an insecticidal agent against *D. melanogaster.*

2. Materials and Methods

2.1. Screening and Isolation of Producing Bacteria.

Bacillus atrophaeus A7 was isolated, among other bacterial isolates, from the exoskeletons of dead beetles *Dixus* sp. (Coleoptera: Carabidae) collected from different locations in AL-Tafilah Province/South of Jordan. The beetle was identified based on the annotated checklist by Nasir and Katbeh-Bader (2017). It was in a dried out condition with decayed internal body mass (thorax and abdominal regions) and intact exoskeleton. The exoskeletons were pulverized using sterilized mortar and pestle, and were spread on top of chitin agar plates of the following composition (g/L): 0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.1 % NH_4Cl, 0.05 % NaCl, 0.005 % yeast extract, 3 % colloidal chitin and 1.8 % agar, pH 7.2. The agar plates were incubated for five days at 27ºC with continuous observation. Bacterial colonies forming clear zones due to chitin hydrolysis were selected and purified on new chitin agar plates. The purified bacterial isolates were transferred to 30 % (v/v) glycerol solution and were stored at -20ºC until the date of use.

Colloidal chitin was prepared as described in Hsu and Lockwood (1975) with minor modifications. Twenty grams of grounded crab shell chitin (Sigma-Aldrich, USA) were acid hydrolyzed by slowly adding 300 ml of concentrated HCl with continuous stirring for one hour. Thereafter, the acid hydrolyzed mixture was filtered using cheesecloths, and the partially hydrolyzed chitin was suspended in 1 L of cold distilled water and was left overnight at 4°C. The colloidal chitin suspension was collected by filtration using coffee filter paper and neutralized with 1 N of cold NaOH solution followed by washing several times with cold distilled water. The neutralized colloidal chitin was stored at 4°C until date of use.

2.2. Extracellular Crude Chitinase Production in Submerged Culture

The bacterial isolate A7 was cultivated in l L Erlenmeyer flask containing 500 mL of liquid chitin medium on an orbital shaker (150 rpm, Forma Orbital Shaker, Thermo Fisher Scientific, USA) at 27°C for eight days. During the fermentation process, 5 mL sample and thereafter daily samples were taken to monitor the bacterial growth and to estimate chitinase activity. The growth was followed by optical density (OD) measurements of 10-fold diluted samples at 580 nm (UV/Vis Spectrometer, Lambda 16, Perkin Elmer, Germany), changes in pH value (pH 523, WTW, Germany) and measurement of chitinolytic activity of the cell free culture. As the OD started to decrease or the maximum activity was attained, the cultivation process was terminated and the culture fluid was separated from the bacterial cells by centrifugation (5,000 g for fifteen minutes, Beckman GS-6, USA). Chitinase activity in the culture filtrate was estimated using the Dinitrosalicylic acid method (DNS).

2.3. Identification of B. atrophaeus A7

Bacillus atrophaeus A7 was characterized morphologically (colony form and appearance, cell shape and size) and biochemically (Gram stain, oxidase, catalase, nitrate reduction, sugar utilization, methyl red and indole tests) for 48-72 hours grown colonies on nutrient agar plates. The biochemical characteristics were determined using standard procedures (Collins *et al*., 2004; York *et al*., 2007). The production of acid from different carbohydrates was detected as described by Helmke and Weyland (1984). Moreover, the identification of the isolate was confirmed by 16S rDNA gene sequencing as described previously (Al-Zereini *et al*., 2007).

The 16S rDNA gene sequence was compared with those available in the GenBank and Ribosomal database project II (RDP). Molecular phylogenetic analysis was conducted using MEGA7 software (Kumar *et al*., 2016). Evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

2.4. Chitinase Activity Assay

The activity of the crude chitinase enzyme was determined using standard DNS method (Dixit *et al*., 2015). Briefly, 1 mL of the culture supernatant containing the crude enzyme was added to 1 mL of 1% (w/v) colloidal chitin solution prepared in 50 mM acetate buffer (pH 4.8). The assay mixture was incubated in a water bath at 37°C for thirty minutes, and the reaction was stopped by adding 1 mL of DNS reagent with boiling for ten minutes. Thereafter, the mixture was left to cool, then centrifuged (5,000 g for fifteen minutes), and the amount of the reducing sugar (N-acetyl-D-glucosamine) released was measured spectrophotometrically at 575 nm. One unit of the chitinase enzyme was defined as the amount of enzyme that produced 1 µmol of N-acetyl-D-glucosamine/min from 1 % (w/v) colloidal chitin solution prepared in 50 mM acetate buffer (pH 4.8) at 37 °C (Miller, 1959).

As a blank, 1 mL of boiled inactive enzyme was added to 1 mL of 1 % colloidal chitin under the same experimental conditions. A standard curve was constructed

by measuring the color intensity of 1 ml of N-acetyl-D-glucosamine solutions (1, 2, 5, 8 and 10 mM) dissolved in 50 mM acetate buffer (pH 4.8). The total protein content in the crude enzyme supernatant was determined using Lowry method (Lowry *et al*., 1951) with bovine serum albumin as standard.

2.5. Effect of Temperature and pH on Chitinase Activity and Stability

Determination of the optimum temperature for the chitinase activity was carried out by standard DNS assay with incubation at different temperatures (20ºC to 60ºC) with 10ºC temperature intervals. The thermostability of the chitinase enzyme was determined by pre-incubating the crude enzyme supernatant in 50 mM acetate buffer (pH 4.8) at different temperatures (30ºC to 80ºC) with 10ºC temperature intervals for one hour. The residual activity was determined using 1 % (w/v) colloidal chitin as substrate at standard assay conditions (50 mM acetate buffer, pH 4.8 at 37^{o}C and stopping the reaction by adding 1 mL of DNS reagent with boiling for ten minutes).

The optimal pH for chitinase activity was determined at 37^{o}C and different pH values (pH 3-10) with 1 pH intervals by standard DNS assay using 1 % (w/v) colloidal chitin as substrate. The solutions used were 50 mM of either citrate buffer (pH 3-6) or phosphate buffer (7-8) or Tris-base (pH 8-10). The effect of pH on the enzyme stability was evaluated by pre-incubating the crude enzyme supernatant at 37^{o}C for one hour in the same buffer ranges. The residual enzyme activity was determined under the standard assay conditions using 1 % (w/v) colloidal chitin as substrate.

2.6. Effect of Metal ions on Chitinase Activity

As several enzymes may require certain metal ions as a component of the active site to maintain its stability and for their catalytic activity, the effect of various metals on the chitinase activity was studied by performing the standard assay using 1 % (w/v) colloidal chitin with different metal ions ($CuSO_4$, $FeCl_3$, $NiCl_3$, $ZnCl_2$ and $BaCl_2$) at a final concentration of 10 mM at 37ºC. The relative inhibition in the enzyme activity was based on measuring the released N-acetyl-D-glucosamine.

2.7. Larvicidal Activity of Bacillus Atrophaeus A7 Chitinase on the Development of Drosophila Melanogaster 2nd Instar Larvae and Determination of the Lethal Dose (LD50)

Half gram of the blue colored instant *Drosophila* media (Formula 4-24®, Carolina Biological Supply Company, USA) was soaked in 2 mL of diluted crude chitinase supernatant in final concentrations ranging from 0 mU/mL up to 68.9 mU/mL. As control, 2 mL of the diluted heat-inactivated crude enzyme supernatant (at 100°C for thirty minutes) was used at final concentrations equivalent to the active crude enzyme. The media in the vials were left for few minutes to gel, and were then overlaid with 10 of *D. melanogaster* 2nd instar larvae.

The required concentration of the chitinolytic crude supernatants to cause death of 50 % (LD_{50}) of *D. melanogaster* larvae was determined under controlled laboratory conditions (21-22^{o}C, germ-free medium throughout the test) as recommended in the working sheet of Formula 4-24® medium by Carolina Biological Supply Company. The effect of different concentrations of the chitinolytic crude extract of *B. atrophaeus* A7 on the development of the larvae into pupal and adult stages was observed daily (loss of larval intensive activity as voracious feeders and foraging ability) for a period of eight days in both the control and experimental treatments. The dose-response relation was appraised using PROBIT regression analysis with 95 % confidence limit (Finney, 1978).

To confirm that the larvicidal effect is mainly attributed to the activity of the crude chitinase of *B. atrophaeus* A7, the chitinase inhibitor pentoxifylline (Sigma-Aldrich, USA) was added to 10 ml of the crude enzyme supernatants to a final concentration of 250 and 500 µg/mL. The preparations were left for thirty minutes at 37^{o}C, after which 2 mL were withdrawn and added to the insect medium; the medium was overlaid with 10 *D. melanogaster* 2nd instar larvae and was monitored daily for larval development.

2.8. Statistical Analysis

Data were represented as mean averages of three independent assays with standard deviations. Mean values and standard deviations as well as PROBIT analysis were deduced using *Microsoft Excel software*. Results were analyzed by one-way ANOVA and Tukey HSD post hoc test using the Statistical Package for the Social Sciences software (SPSS, version 16). Data were considered significant at $p<0.05$ (one-way ANOVA). Tukey HSD post hoc test was carried out on data where $p<0.01$ to identify which of tested factor pairs are significantly different from each other.

3. Results

3.1. Bacterial Isolate Identification and Crude Chitinase Activity Measurement.

Bacterial isolate A7 is a Gram-positive spore forming bacillus, forms circular colonies of 1.5-2 mm in diameter and of a white opaque colour with undulating margins. It is oxidase-negative, catalase-positive bacterium, able to utilize glucose, sucrose, and mannitol, but not maltose, xylose, or lactose as a carbon source, reducing nitrate and hydrolyzes gelatin. It gave positive results in indole and methyl red tests, and was identified morphologically and biochemically as a member of the genus *Bacillus*. 16S rRNA sequencing indicated that isolate A7 is related to *B. atrophaeus* (Accession no. KU 955655.1) with a similarity level of 99 % (Figure 1). A photograph for the bacterial isolate A7 colonies grown on chitin agar and showing its ability to produce extracellular chitinase is presented (Figure 2).

The production of chitinase was detected after twenty-four hours of *B. atrophaeus* cultivation in a liquid chitin medium with approximating the maximum activity at the 8th day of growth. The total activity measured by DNS assay was 68.9±1.03 mU/mL with a specific activity of 2670±40.2 mU/mg. The increase in the daily production and subsequently the chitinolytic activity of the exochitinase is illustrated (Figure 3). However, prolonged cultivation time resulted in a reduction and even loss of the chitinase activity.

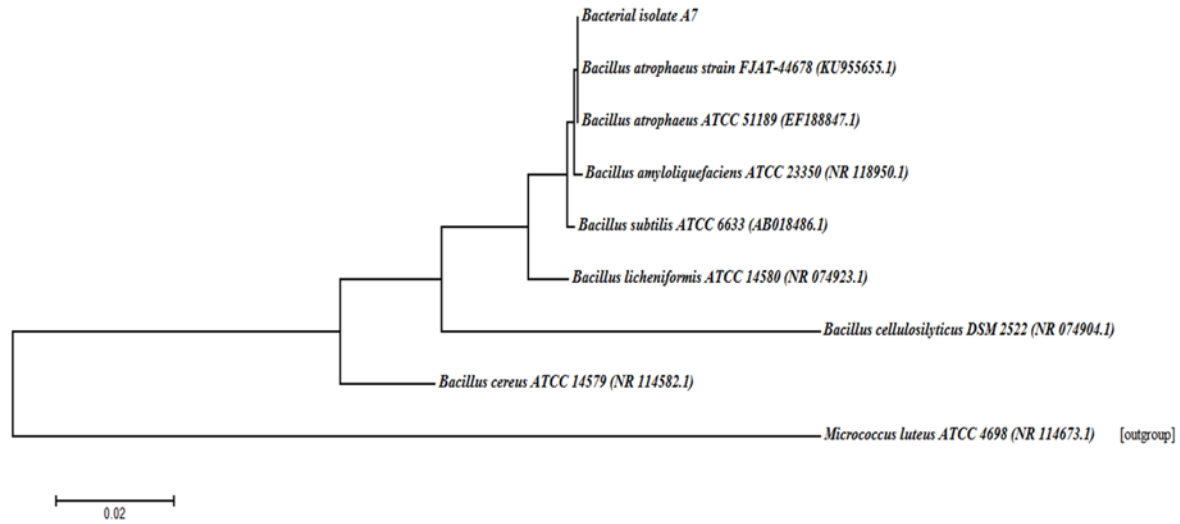

Figure 1. Phylogenetic tree of bacterial isolate A7, constructed by Maximum Likelihood Algorithm using MEGA7 software. The scale bar indicates 2 % estimated base substitution per nucleotide position.

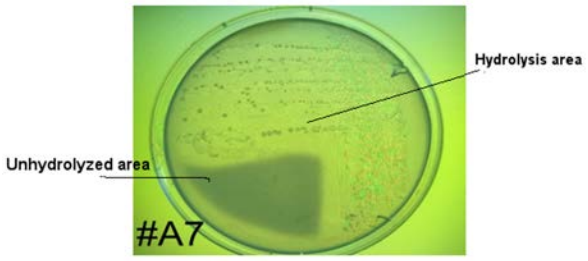

Figure 2. Hydrolysis zone formed during the growth of bacterial isolate A7 on a chitin agar plate incubated at 37°C for seven days. The wide clear areas around the gray colonies grown on the plate indicate the hydrolysis of chitin.

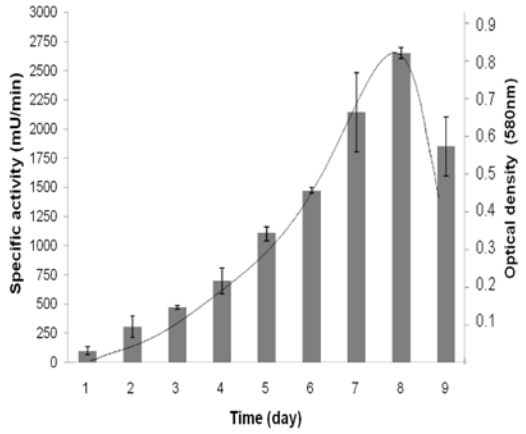

Figure 3. Chitinolytic activity of daily samples collected during the growth of *B. atrophaeus* A7 in a chitin liquid medium at pH 7.2 and 27 °C.

3.2. Effect of Temperature and pH on Crude Chitinase Enzyme Activity and Stability

Under the experimental conditions, the optimum enzyme activity was achieved at 40°C with a decrease in the relative activity to 81±11.1% (55.8±7.6 mU/mL) at 60°C. Nevertheless, data analysis indicated that carrying out the enzyme assays at different tested temperatures did not significantly affect the enzyme activity (p>0.05). The crude enzyme was stable, so the optimum activity was maintained for one hour at 30-40°C; however, this activity was significantly altered at temperatures above 40°C (p<0.05). In fact, a maximum loss of the enzyme activity was noticed after one hour at 80°C (42±4.2%, 40±2.9 mU/mL) (Figure 4 A and B).

The chitinolytic enzyme produced by *B. atrophaeues* was active with insignificant differences in its relative activity at a broad pH of 4-9 (p>0.05). Intriguingly, significant reduction in the total activity to 80 % (55.1±2.76 mU/mL) and 85 % deactivation (10.34±2.55 mU/mL) was detected at 10 and 3 pH, respectively (p<0.01). However, the enzyme was stable at 6-7 pH with a significant reduction in its activity to 40 %-50 % at 8-10 or 4-5 pH and 95 % at pH 3 (Figure 4 C and D).

3.3. Effect of Different Metal Ions on Crude Chitinase Enzyme Activity

The activity of chitinase from *B. atrophaeus* A7 was highly inhibited with 10 mM of all used metal ions (p < 0.01). In fact, the order of potency of the tested metal ions to inhibit the chitinase activity was Fe^{3+} > Ni^{3+} > Cu^{2+} > Zn^{2+} > Ba^{2+}. Iron ions (Fe^{3+}) caused 90 % inhibition in the enzyme activity under the assay conditions; meanwhile 60-75 % inhibition was obtained by other metal ions (Figure 5).

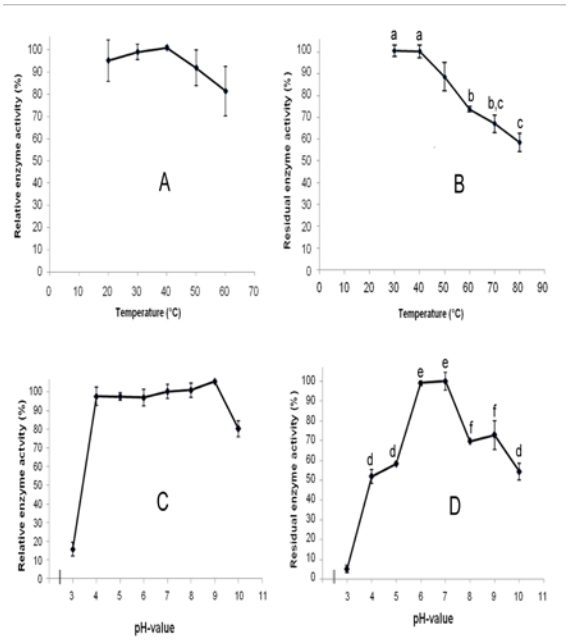

Figure 4. Effect of different temperatures and pH values on activity and stability of crude chitinase from *B. atrophaeus* A7. A) relative enzyme activity at different temperatures for thirty minutes, pH 4.8; B) residual activity as a measure of enzyme stability at different temperatures for one hour, pH 4.8; C) relative enzyme activity at different pH values for thirty minutes, 37°C; and D) residual activity as a measure of enzyme stability at different pH values for one hour, 37°C. Means with similar letters are not significantly different from each other based on post hoc Tukey HSD test at $p<0.01$.

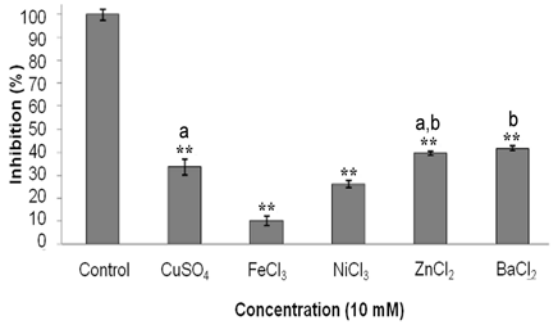

Figure 5. Effect of different metal ions on the activity of the chitinase enzyme from *B. atrophaeues* A7 at 10 mM. Asterisks indicated statistically significant values from control (** p<0.01). Means with similar letters are not significantly different from each other based on post hoc Tukey HSD test at $p<0.01$.

3.4. Larvicidal Activity of Crude Chitinase Enzyme from B. atrophaeus A7 on D. Melanogaster and Determination of LD50

The Chitinolytic enzyme produced by *B. atrophaeus* A7 interfered with the development of *D. melanogaster* larvae. More than 80 % of these larvae were unable to metamorphose into the 3rd instar stage, pupae, or emerge as adult flies at 27.56 mU/mL. Plotting the dose-larval response to the crude chitinase enzyme (Figure 6) and the PROBIT analysis calculated from the bioassay 8th day post-treatment revealed that LD_{50} is 17.3±0.9 mU/mL

. The lethal effect of the chitinase was concentration-dependent, and the larvae development was significantly retarded at most applied concentrations as compared to control ($p<0.01$).

The development of 60-70 % of larvae to adults was delayed to the 6th day under the experimental condition at 13.8 mU/mL and the addition of pentoxifylline caused 1.4 (24.7±1.2 mU/mL) and 1.8 (31.7±1.5 mU/mL) fold increase in LD_{50} at 250 and 500 µg/ml, respectively.

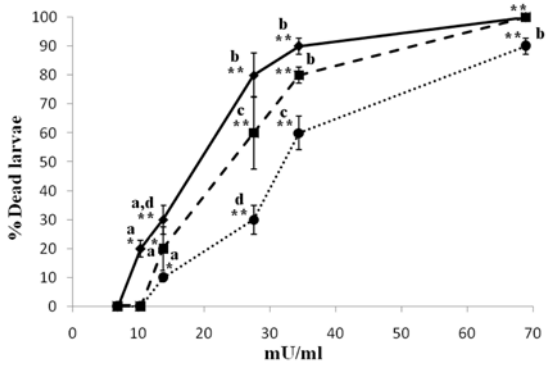

Figure 6. Effect of the crude chitinase enzyme from *B. atrophaeues* A7 on survival and development of the 2nd instar *D. melanogaster* larvae to adults. Asterisks indicated statistically significant values from control treated with heat inactivated-crude chitinase (*: 0.01<p<0.05, ** $p<0.01$). The percentage of the dead larvae means with similar letters are not significantly different from each other based on post hoc Tukey HSD test at $p<0.01$. Solid line: chitinase without pentoxifylline; dash line: chitinase with 250 µg/mL pentoxifylline; and dots: chitinase with 500 µg/mL pentoxifylline.

4. Discussion

Despite the fact that chitinases play a role in the arthropod insects' molting process (edysis), since their exoskeleton and gut lining consist largely of chitin, it was postulated that the bacterial chitinase could be involved in biocontrol regimes aiming to select potential insecticidal agents. Bacterial chitinase plays a role not only in recycling chitin in nature to provide carbon and nitrogen as a nutrient, but also a role in plant defense systems against chitin-containing pathogens and biocontrol (Zhu *et al*., 2007). The production of more than one type as well as multiple forms of chitinases was reported in different *Bacillus* spp. such as *B. amyloliquefaciens* and *B. megatrium* (Sabry, 1992), *B. cereus* (Chang *et al*., 2007), *B. licheniformis* (Waldeck *et al*., 2006), and *B. subtilis* (Wang *et al*., 2006). Nevertheless, the chitinase production and activity from *B. atrophaeus* were not utterly contemplated.

In the current study, the production of the chitinolytic enzyme from *B. atrophaeus* A7 was observed since the first day following the bacterial cultivation under experimental conditions; however, the decrease in activity with a prolonged growth regime after the 8th day might be due to the reduced level of nutrients in the culture medium and/or denaturation of chitinase by proteases (Zarei *et al*., 2010). The optimum enzyme activity and stability of the isolated chitinase from *B. atrophaeus* A7 at different temperatures and pH values coincided with previously reported bacterial chitinases. Previous studies showed that the chitinase produced by *B. atrophaeus* has an optimum working temperature of 50ºC and optimum pH of 8, while

that from *B. subtilis* was active at 40ºC and pH 5. Moreover, chitinase from both strains was still active up to pH 8 (Anuradha and Revathi, 2013). The chitinolytic enzyme from *Bacillus* sp. WY22 was optimally active at 37ºC and pH 5.5, and was stable at 30-40ºC and pH 4.5 (Woo and Park, 2003). *B. laterosporus* MML2270 produced chitinase with the highest activity at 35ºC and pH 8.0, and interestingly, it was inactive at pH 4 (Shanmugaiah *et al*., 2008).

Several bacterial chitinases have broad pH stability, and they are thermotolerant. Chitinases from *B. licheniformis* have optimum temperature at 60-65ºC and optimum pH 6, and were stable up to 60ºC and broad pH 4-11 (Sandalli *et al*., 2008; Songsiriritthigul *et al*., 2010). *B. cereus* TKU 028 produced chitinase with an optimum temperature of 60ºC and pH 5, and was stable for sixty minutes at temperatures below 60ºC and over a broad pH range from 4 to 9 (Liang *et al*., 2014). The chitinase from *Bacillus* sp. 7079 was optimally active at pH 7.5 and 45ºC; stable under wide pH range between pH 5 and 9, and more than 80% of the original activity was retained at 40ºC (Kyung *et al*., 2003). Other bacterial chitinases were maximally-active at 50ºC and pH 8 with a stability at 50ºC and 6-9 pH in *Pseudomonas aerogenosa* (Hiraga *et al*., 1997); at 40ºC and 5-8 pH in *Aeromonas hydrophila* H-2330 (Wang and Chang, 1997); at 40ºC and pH 7.5 in *Pseudoalteromonas* sp. DXK012 (Liu *et al*., 2015); and at 30ºC and pH 5 with stability at 40ºC and pH 8 in *Streptomyces* sp. M-20 (Kim *et al*., 2003).

The stability of bacterial chitinase at different conditions, including broad pH ranges or temperatures, refers to the their production of more than one isoform of the enzyme (Kuzu *et al*., 2012), or this is because of the deletion or insertion of additional domains at both N- and C-terminals that keep its structure more stable, and thus avoiding its disturbance in these conditions (Chen *et al*., 2015; Thimoteo *et al*., 2017). Two chitinase isoforms with a molecular mass 43 and 18.5 KDa were reported from fluorescent pseudomonads with antifungal activity (Ajit *et al*., 2006). Six isoforms (39-92 KDa) were produced by *Streptomyces* S242 (Saadoun *et al*., 2009). Six chitinases were reported from *B. circulans* and *Bacillus* sp. with the molecular mass 38-73 KDa and 22–77 KDa, respectively (Watanabe *et al*., 1990; Sakai *et al*., 1998).

The activities of most enzymes, if not all, require and are affected by the presence of certain metal ions. Herein, all examined metals significantly inhibited the activity of the chitinolytic enzyme produced by *B. atrophaeus*, and the extent of inhibition was metal ion type dependent. The findings of the current study agreed with previous reports on bacterial chitinases, wherein they were inhibited at 1 mM of different divalent metals (Sri *et al*., 2004; Lee *et al*., 2007; Annamalai *et al*., 2010; Zarei *et al*., 2011; Cheba *et al*., 2016). At 1 mM concentration, Cu^{2+}, Fe^{3+}, Ni^{3+}, Zn^{2+} and Ba^{2+} offered partial inhibition in the chitinase activity of *Serratia marcescens* B4A (Cheba *et al*., 2016) and *Bacillus* sp. R2 (Zarei *et al*., 2011). In contrary to the results of this study, it was reported that chitinase was activated by 10 mM of $ZnSO_4$ in *B. atrophaeus* (Anuradha and Revathi, 2013); by 5 mM of $FeSO_4$ and $NiCl_2$ in *Micrococcus* sp. AG84 (Annamalai *et al*., 2010), while it was not affected by 1 mM of $BaCl_2$ in *Alcaligenes xylosoxydans* or *Serratia marscesens* B4A (Vaidya *et al*., 2003; Zarei *et al*., 2011) indicating that the bioactive site of the herein isolated chitinase most probably structurally different from those tested in the above studies. The role of such metal ions in the enzyme inhibition may be attributed to the ability of these ions to attach the catalytically active subunits of the enzyme by forming complexes with carboxylic groups of aspartic and glutamic acid residues at the active site, changing the conformational shape of the enzyme and competing with cation activators bound with the formation of a substrate-enzyme complex (Donderski and Brzezinska, 2005; Thimoteo *et al*., 2017).

In developing countries, land cultivation represents part of the main individual's income and living needs. Consequently, the control of plant pests and diseases-related insect vectors is crucial to the reliable production of food and healthcare management. However, the living and breeding of *D. melanogaster* in high-density, and within microbe-rich food substrates such as rotten and decaying fruit endowed its evolutionary adaptation to interact naturally with pathogenic bacteria (Keesey *et al*., 2017). In fact, it could be exploited by these bacteria as a potential vector between plants accounting, even more, for a possible threat to human health. Therefore, the exploring of an easily-handled natural source, such as these microorganisms, for environmental-friendly insecticides against fruit fly larvae was of high priority.

To date, chitinases with the insecticidal action were known from *B. thuringiensis*, *B. cereus*, *B. subtilis* (Liu *et al*., 2002; Chandrasekaran *et al*., 2012) and several actinomycete species (Gadelhak *et al*., 2005). *Bacillus thuringiensis* is a well-known biocontrol agent that has been in use for decades for pest control in agriculture and for the control of disease-related insect vectors (Veliz *et al*., 2017). Its toxins had been demonstrated against many typical insect orders, such as Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes) and Coleoptera (beetles and weevils) (Xu, 2014) that encompass major invertebrate plant pests from agricultural as well as some insect vectors of mammalian pathogens (Nester *et al*., 2002). The activities of these toxins were attributed to the parasporal crystals (Cry proteins), which are produced and assembled during *B thuringiensis* sporulation. After ingestion by larvae, the crystals are solubilized and converted into active toxins by proteases in the larval midgut causing the death of the larvae. Meanwhile, chitinase from different actinomycetes played an important role against the house fly *Musca domestica* and is used against *Culex quinquefasciatus* (Gadelhak, 2005).

Herein, the larvicidal activity of *B. atrophaeus* A7 is reported against the development of the *D. melanogaster* 2nd instar larvae into adult fruit fly. Although other potential effectors, such as proteases and toxins, could be one of the fundamental causes of the larvicidal activity. However, the inability of the larvae to ecdyse and develop to 3rd instar larval and hereafter to pupal stages hint for cuticle defect due to the chitinolytic attack. Moreover, the increase in crude enzyme concentration that cause 50 % inhibition in the larval development (LD_{50}) in presence of the chitinase inhibitor pentoxifylline indicated that such larvicidal activity is attributed, mostly if not entirely, to the effect of the tested crude chitinase supernatant.

The current findings coincided with the reported activity of chitinases from both *Actinomyces phlippinensis* and *A. missouriensis* that had significantly reduced *D. melanogaster* pupal formation when applied to the rearing medium individually (Gadelhak 2005). Chitinolytic enzymes may inhibit chitin formation either by affecting the catalytic site of the synthase, by interfering at the sulfhydril-sensitive sites of the synthase during polymerization of the β-1,4-N-acetyl-D-glucosamine residues, or by the ability to interfere with chitin deposition (Gadelhak *et al*., 2005). Besides, chitinase applied in an insect's rearing medium induces damage to the peritrophic membrane in the insect gut causing a significant reduction in the nutrient utilization, and consequently affecting insect development and growth (Deepthi, 2014).

Interestingly, it was documented that the chitinase-producing *B. thuringiensis* enhanced the insecticidal toxicity of the active *B. thuringiensis* against newly-hatched beet armyworm *Spodoptera exigua* larvae with an enhancing ratio of 2.35-fold (Liu *et al.,* 2002). Hence, *B. atrophaeus* A7 might be a source of insecticidal chitinase that could act individually as a biopesticide agent or in synergistic with other bioinsicticides by enhancing their effectiveness.

5. Conclusion

This study represents the first report that verifies the ability of *B. atrophaeus* to produce chitinolytic enzyme with a potential insecticidal activity. The produced chitinase was active at broad pH ranges, and was thermotolerant suggesting that it could be useful for biotechnological and agricultural applications. Further work is in plan for the molecular characterization of chitinase genes, to purify and determine the type and molecular weight of chitinase produced by *B. atrophaeus* A7.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

Aam BB, Heggset EB, Norberg AL, Sørlie M, Vårum KM and Eijsink VG. 2010. Production of chitooligosaccharides and their potential applications in medicine. *Mar Drugs*, **8**: 1482-1517.

Adams DJ. 2004. Fungal cell wall chitinases and glucanases. *Microbiol.*,**150**: 2029-2035.

Ajit NS, Verma R and Shanmugam V. 2006. Extracellular chitinases of fluorescent pseudomonads antifungal to Fusarium oxysporum f. sp. dianthi causing carnation wilt. *Curr Microbiol*, **52**: 310-316.

Al-Zereini WA, Schuhmann I, Laatsch H, Helmke E and Anke H. 2007. New aromatic nitrocompounds from *Salegentibacter* sp. T436, an arctic sea ice bacterium: taxonomy, fermentation, isolation and biological activities. *J Antibiot*, **60**: 301-308.

Annamalai N, Giji S, Arumugam M and Balasubramanian T. 2010. Purification and characterization of chitinase from *Micrococcus* sp. AG84 isolated from marine environment. *Afr J Microbiol Res*, **4**: 2822-2827.

Anuradha V and Revathi K. 2013. Purification and characterization of chitinase from two *Bacillus* sp. isolated from crustacean shells. *J Microbiol Biotech Res*, **3**: 160-167.

Blazar JM, Lienau EK and Allard MW. 2011. Insects as vectors of foodborne pathogenic bacteria. *Terr Arthropod Rev*, **4**: 5-16.

Chakrabortty S, Bhattacharya S and Das A. 2012. Optimization of process parameters for chitinase production by a marine isolate of *Serratia marcescens. Int J Pharm Biol Sci*, **2**: 8-20.

Chandran S, Parameswaran B and Pandey A. 2007. Microbial chitinases: effective biocontrol agents. In: Chincholkar SB and Mukerji KG (Eds.), **Biological Control of Plant Diseases**, The Haworth Press, Inc., New York, pp. 381-382.

Chandrasekaran R, Revathi K, Nisha S, Kirubakaran SA, Sathish-Narayanan S and Senthil-Nathan S. 2012. Physiological effect of chitinase purified from *Bacillus subtilis* against the tobacco cutworm *Spodoptera litura* Fab. *Pestic Biochem Physiol*, **104**: 65-71.

Chang WT, Chen YC and Jao CL. 2007. Antifungal activity and enhancement of plant growth by *Bacillus cereus* grown on shellfish chitin wastes. *Bioresour Technol*, **98**: 1224-1230.

Cheba BA, Zaghloul TI, EL-Massry MH and EL-Mahdy AR. 2016. Effect of metal ions, chemical agents, and organic solvent on *Bacillus* sp. R2 chitinase activity. *Procedia Technol*, **22**: 465-470.

Chen L, Chen J, Kumar A and Liu Z. 2015. Effects of domains modification on the catalytic potential of chitinase from *Pseudomonas aeruginosa. Int J Biol Macromol*, **78**: 266-272.

Collins CH, Lyne PM and Grange JM. 2004. **Collins and Lyne's Microbiological Methods**, 8th ed. Butterworth-Heinemann, London.

Dahiya N, Tewari R, Tiwari RP and Hoondal GS. 2005. Production of an antifungal chitinase from *Enterobacter* sp. NRG4 and its application in protoplast production. *World J Microbiol Biotechnol*, **21**: 1611-1616.

Deepthi A. 2014. Actinomycete isolates from Arabian Sea and Bay of Bengal: biochemical, molecular and functional characterization. PhD. Thesis, Cochin University of Science and Technology, Kuchi, India.

Dixit R, Singh RB and Singh HB. 2015. Screening of antagonistic potential and plant growth promotion activities of *Trichoderma* spp. and fluorescent *Pseudomonas* spp. isolates against *Sclerotinia sclerotiorum* causing stem rot of French bean. *Legume Res*, **38**: 375-381.

Donderski W and Brzezinska MS. 2005. The influence of heavy metals on the activity of chitinases produced by planktonic, benthic and epiphytic bacteria. *Pol J Environ Stud*, **14**: 851-859.

Finney DJ. 1978. **Statistical Method in Biological Assay**. Charles Griffin and Co., London.

Fu X, Yan Q, Yang S, Yang X, Guo Y and Jiang Z. 2014. An acidic, thermostable exochitinase with β-N acetylglucosaminidase activity from *Paenibacillus barengoltzii* converting chitin to N-acetyl glucosamine. *Biotechnol Biofuels*, **7**: 174.

Gadelhak GG, El-Tarabily KA and Al-Kaabi FK. 2005. Insect control using chitinolytic soil actinomycetes as biocontrol agents. *Int J Agric Biol*, **7**: 627-633.

Hao Z, Cai Y, Liao X, Zhang X, Fang Z and Zhang D. 2012. Optimization of nutrition factors on chitinase production from a newly isolated *Chitiolyticbacter meiyuanensis* SYBC-H1. *Braz J Microbiol*, **43**: 177-186.

Helmke E and Weyland H. 1984. *Rhodococcus marinonascens* sp. nov., an actinomycete from the sea. *Int J Syst Bacteriol*, **34**: 127-138.

Hiraga K, Shou L, Kitazawa M, Takahashi S, Shimada M, Sato R and Oda K. 1997. Isolation and characterization of chitinase from a flake-chitin degrading marine bacterium, *Aeromonas hydrophila* H-2330. *Biosci Biotechnol Biochem*, **61**: 174-176.

Hsu SC and Lockwood JL. 1975. Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Appl Microbiol*, **29**: 422-426.

Keesey IW, Koerte S, Khallaf MS, Retzke T, Guillou A, Grosse-Wilde E, Buchon N, Knaden M and Hansson BS. 2017. Pathogenic bacteria enhance dispersal through alteration of Drosophila social communication. *Nat Commun*, **8**: 265.

Kim KJ, Yang YJ and Kim JG. 2003. Purification and characterization of chitinase from *Streptomyces* sp. M-20. *J Biochem Mol Biol*, **36**: 185-189.

Kumar S, Stecher G and Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol*, **33**: 1870-1874.

Kuzu SB, Güvenmez HK and Denizci AA. 2012. Production of a thermostable and alkaline chitinase by *Bacillus thuringiensis* subsp. kurstaki strain HBK-51. *Biotechnol Res Int*, **2012**: 1-6.

Kyung HO, Lee ET, Lee YS and Kim SD. 2003. Purification of chitinase from an antagonistic bacterium *Bacillus* sp. 7079 and pro-inflammatory cytokine gene expression by PCTC. *J Microbiol Biotechnol*, **13**: 77-84.

Lee YS, Park IH, Yoo JS, Chung SY, Lee YC, Cho YS, Ahn SC, Kim CM and Choi YL. 2007. Cloning, purification, and characterization of chitinase from *Bacillus* sp. DAU101. *Bioresour Technol*, **98**: 2734-2741.

Liang T, Hsieh T and Wang S. 2014. Purification of a thermostable chitinase from *Bacillus cereus* by chitin affinity and its application in microbial community changes in soil. *Bioprocess Biosyst Eng*, **37**: 1201-1209.

Liu F, Sun W, Su F, Zhou K and Li Z. 2012. Draft genome sequence of the sponge-associated strain *Bacillus atrophaeus* C89, a potential producer of marine drugs. *J Bacteriol*, **194**: 4454.

Liu M, Cai QX, Liu HZ, Zhang BH, Yan JP and Yuan ZM. 2002. Chitinolytic activities in *Bacillus thuringiensis* and their synergistic effects on larvicidal activity. *J Appl Microbiol*, **93**: 374-379.

Liu Y, Chan Z and Zeng R. 2015. Purification and characterization of chitinase secreted by *Pseudoalteromonas* sp. DXK012 isolated from deepsea sediment. *J Nat Sci*, **1**: e37.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem*, **193**: 265-275.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem*, **31**: 426-428.

Nasir WM and Katbeh-Bader A. 2017. Annotated checklist of ground beetles (Coleoptera: Carabidae) of Jordan. *Zool Ecol.* **27**: 269-291.

Nester EW, Thomashow LS and Metz M. 2002. 100 years of *Bacillus thuringiensis*: a critical scientific assessment. Colloquium Reports, American Society for Microbiology (ASM), Washington, DC, USA.

Nguyen HQ, Quyen DT, Nguyen SLT and Van Hanh VU. 2015. An extracellular antifungal chitinase from *Lecanicillium lecanii*: purification, properties, and application in biocontrol against plant pathogenic fungi. *Turk J Biol*, **39**: 6-14.

Rajaofera MN, Jin PF, Fan YM, Sun QQ, Huang WK, Wang WB, Shen HY, Zhang S, Lin CH, Liu WB and Zheng FC. 2017. Antifungal activity of the bioactive substance from *Bacillus atrophaeus* strain HAB-5 and its toxicity assessment on Danio rerio. *Pestic Biochem Physiol*, in press.

Rathore AS and Gupta RD. 2015. Chitinases from bacteria to human: properties, applications, and future perspectives. *Enzyme Res*, **2015**: 1-8.

Saadoun I, Al-Omari R, Jaradat Z and Ababneh Q. 2009. Influence of culture conditions of *Streptomyces* sp. (strain S242) on chitinase production. *Pol J Microbiol*, **58**: 339-345.

Sabry SA. 1992. Microbial degradation of shrimp-shell waste. *J Basic Microbiol*, **32**: 107-111.

Sakai K, Yokota A, Kurokawa H, Wakayama M and Moriguchi M. 1998. Purification and characterization of three thermostable endochitinases of a noble *Bacillus* Strain, MH-1, isolated from chitin-containing compost. *Appl Environ Microbiol*, **64:** 3397-3402.

Sandalli C, Kacagan M, Canakci S and Belduz AO. 2008. Cloning, expression, purification and characterization of a thermostable chitinase from *Bacillus licheniformis* A1. *Ann Microbiol*, **58**: 245-251.

Sella SR, Vandenberghe LP and Soccol CR. 2015. *Bacillus atrophaeus*: main characteristics and biotechnological applications–a review. *Crit Rev Biotechnol*, **35**: 533-545.

Shanmugaiah V, Mathivanan N, Balasubramanian N and Manoharan PT. 2008. Optimization of cultural conditions for production of chitinase by *Bacillus laterosporous* MML2270 isolated from rice rhizosphere soil. *Afr J Biotechnol*, **7**: 2562-2568.

Singh G, Sharma JR and Hoondal GS. 2008. Chitinase production by *Serratia marcescens* GG5. *Turk J Biol*, **32**: 231-236.

Songsiriritthigul C, Lapboonrueng S, Pechsrichuang P, Pesatcha P, and Yamabhai, M. 2010. Expression and characterization of *Bacillus licheniformis* chitinase (ChiA), suitable for bioconversion of chitin waste. *Bioresour Technol*, **101**: 4096-4103.

Sri R, Tanuwidjaja F, Rukayadi Y, Suwanto A, Thenawidjaja SM, Hwang JK and Pyun YR. 2004. Study of thermostable chitinase enzymes from Indonesian *Bacillus* K29-14. *J Microbiol Biotechnol*, **14**: 647-652.

Stoykov YM, Pavlov AI and Krastanov AI. 2015. Chitinase biotechnology: production, purification, and application. *Eng Life Sci*, **15**: 30-38.

Tamura K and Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, **10**: 512-526.

Thimoteo SS, Glogauer A, Faoro H, de Souza EM, Huergo LF, Moerschbacher BM and Pedrosa FO. 2017. A broad pH range and processive chitinase from a metagenome library. *Braz J Med Biol Res*, **50**: e5658. Vaidya R, Roy S, Macmil S, Gandhi S, Vyas P and Chhatpar HS. 2003. Purification and characterization of chitinase from *Alcaligenes xylosoxydans*. *Biotechnol Lett*, **25**: 715-717.

Veliz EA, Martinez-Hidalgo P and Hirsch AM. 2017. Chitinase producing bacteria and their role in biocontrol. *AIMS Microbiol*, **3**: 689–705.

Waldeck J, Daum G, Bisping B and Meinhardt F. 2006. Isolation and molecular characterization of chitinase deficient *Bacillus licheniformis* strains capable of deproteinization of shrimp shell waste to obtain highly viscous chitin. *Appl Environ Microbiol*, **72**: 7879-7885.

Wang SL and Chang WT. 1997. Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced

by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Appl Environ Microbiol*, **63**: 380-386.

Wang SL, Lin TY, Yen YH, Liao HF and Chen YJ. 2006. Bioconversion of shellfish chitin wastes for the production of *Bacillus subtilis* W-118 chitinase. *Carbohydr Res*, **6:** 2507-2515.

Watanabe TA, Oyanagi W, Suzuki K and Tanaka H. 1990. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J Bacteriol*, **172**: 4017-4022.

Woo CJ and Park HD. 2003. An extracellular *Bacillus* sp. chitinase for the production of chitotriose as a major chitinolytic product. *Biotechnol Lett*, **25**: 409-412.

Xu C, Wang BC, Yu Z and Sun M. 2014. Structural insights into Bacillus thuringiensis Cry, Cyt and parasporin toxins. *Toxins*, **6:** 2732-2770.

York MK, Taylor MM, Hardy J and Henry M. 2007. Biochemical tests for the identification of aerobic bacteria. In: Garcia LS and Isenberg HD (Eds.), **Clinical Microbiology Procedures Handbook**, 2nd ed. ASM Press, Washington, DC, Section 3.17.

Zarei M, Aminzadeh S, Ghoroghi A, Motalebi AA, Alikhajeh J and Daliri M. 2012. Chitinase isolated from water and soil bacteria in shrimp farming ponds. *Iran J Fish Sci*, **11**: 911-925.

Zarei M, Aminzadeh S, Zolgharnein H, Safahieh A, Daliri M, Noghabi KA, Ghoroghi A and Motallebi A. 2011. Characterization of a chitinase with antifungal activity from a native *Serratia marcescens* B4A. *Braz J Microbiol*, **42**: 1017-1029.

Zarei M, Aminzadeh S, Zolgharnein H, Safahieh A, Ghoroghi A, Motallebi A, Daliri M and Lotfi AS. 2010. *Serratia marcescens* B4A chitinase product optimization using Taguchi approach. *Iran J Biotechnol*, **8**: 252-262.

Zhang X, Li B, Wang Y, Guo Q, Lu X, Li S and Ma P. 2013. Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. *Appl Microbiol Biotechnol*, **97**: 9525-9534.

Zhu XF, Zhou Y and Feng JL. 2007. Analysis of both chitinase and chitosanase produced by *Sphingomonas* sp. CJ-5. *Zhejiang Univ Sci B*, **8**: 831-838.