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Degradation of barnacle nauplii: implications to chitin regulation in the marine environment

Lidita Khandeparker*, Chetan C Gaonkar, Dattesh V Desai
CSIR - National Institute of Oceanography, Dona Paula, Goa, India

*Correspondence to: Dr. Lidita Khandeparker, CSIR-National Institute of Oceanography, Dona Paula, Goa – 403 004, India, Tel.: +91(0)832-2450432; fax: +91(0)832-2450615,
Email: klidita@nio.org

Abstract: The exoskeleton of most invertebrate larval forms is made of chitin, which is a linear polysaccharide of β (1 \rightarrow 4)-linked *N*-acetylglucosamine (GlcNAc) residues. These larval forms offer extensive body surface for bacterial attachment and colonization. In nature, degradation of chitin involves a cascade of processes brought about by chitinases produced by specific bacteria in the marine environment. Microbial decomposition of larval caracases serves as an alternate mechanism for nutrient regeneration, elemental cycling and microbial production. The present study was undertaken to assess the influence of chitinase enzyme on the degradation of the nauplii of barnacle, *Balanus amphitrite*. The survival and abundance of bacteria during the degradation process under different experimental conditions was monitored. To the best of our knowledge, no such study is conducted to understand the degradation of larval exoskeleton using chitinase and its influence on bacteria. An increase in the chitinase activity with increase in temperature was observed. Scanning electron micrographs of chitinase treated nauplii showed scars on the surface of the barnacle nauplii initially and further disruption of the exoskeleton was observed with the increase in the treatment time. Bacterial abundance of the chitinase treated nauplii increased with the increase in enzyme concentration. Pathogenic bacteria such as *Vibrio cholerae*, *V. alginolyticus*, *V. parahaemolyticus* which were initially associated with the exoskeleton were absent after chitinase treatment, however, *Bacillus* spp dominated subsequent to chitinase treatment and this might have important implications to marine ecosystem functioning.

Keywords: Chitinase; *Balanus amphitrite*; Nauplii; Zooplankton; GlcNAc; Bacteria

Introduction

Zooplankton biomass is mainly dominated by copepods, euphasiids and other crustaceans in the marine ecosystems and they make up to 90% of the total zooplankton (Kulka et al. 1982; Jeuniaux & Francoise 1991). The average annual chitin production by crustaceans in marine ecosystems presumably amount to $40 \text{ mg m}^{-2} \text{ yr}^{-1}$ (Cauchie 2002). Approximately 10^{11} metric tons of chitin is produced per year in aquatic environments, with copepods dominating the production (Keyhani & Roseman 1999). Chitin is a linear polysaccharide of β (1 \rightarrow 4)-linked *N*-acetylglucosamine (GlcNAc) residues in which the hydroxyl group of glucose is replaced by an acetamide group. In hydrosphere it is the main structural protective layer covering the outermost surface of mollusks, crustaceans, fungi, algae and many other invertebrates (Deshpande 1986; Chen & Chang 1994; Ilyina et al. 1995). Owing to its wide distribution among different phyla, chitin is one of the most abundant macromolecular biological product in the biosphere.

The degradation of chitin is mainly aided by enzymes belonging to glycosyl hydrolases called chitinases which act on β (1 \rightarrow 4) glycosidic bonds and cleaves the GlcNAc residues. The microorganisms that are able to degrade chitin are widely distributed in nature and produce chitinase in higher amount than animals and plants (Deshpande 1986). Under aerobic conditions many microorganisms enzymatically degrade chitin. On hydrolysis, the product, GlcNAc can be used as a source of carbon and nitrogen by many heterotrophic bacteria (Warnes 1988; Gooday 1990; Mudryk and Donderski 1997). The hydrolyzed products of chitin are converted to Fructose-6-phosphate in the periplasm of the bacteria, which then undergoes the glycolysis pathway leading to energy production (Bassler et al. 1991). Thus, microbial decomposition of zooplankton caracases serves for nutrient regeneration, elemental cycling and microbial production in many aquatic ecosystems (Lee & Fisher 1992). Tang et al (2009) reported that estuarine copepod, *Acartia tonsa* naturally carry diverse bacterial strains on its body. Many studies have documented association between *Vibrio cholerae* and chitinous plankton in the marine environment (Huq et al. 1995, 2005; Gil et al. 2004; Baffone et al. 2006). *Vibrio* spp are shown to possess chitinolytic enzymes and thus may be able to use crustacean exoskeleton as growth surface (Bassler et al. 1991). Huq et al. (1983, 1984) using culture technique found that *V. cholerae* and *V. parahaemolyticus* preferentially attach to planktonic copepods.

Zooplankton (meroplankton and holoplankton) offer extensive body surfaces for bacterial attachment and colonization. It was reported that most of the pathogenic bacteria introduced to Chesapeake Bay came from the bacteria associated with plankton rather than those in water column

(Ruiz et al. 2000). These microorganisms once released are well suited to be invasive, owing to their ubiquity, small size which enhances their passive dispersal and their requirements for survival are also simple (Deming 1997). Recently, ultrasonic treatment, a physical means for disruption of crustacean larvae was used to study the bacteria released upon disruption (Seth et al. 2010) and this study indicated that upon sonication of barnacle nauplii, gamut of bacteria are released in the water column. Rate of release of bacteria was dependent on power level and treatment time. In nature degradation of chitin is a slow process and involves a cascade of pathways. Chitinases are reported in the moulting fluid of arthropods and are mainly used to digest the structural polysaccharides in their gut linings and exoskeleton during moulting (Karmer et al. 1985; Karmer & Muthukrishnan 1997; Fukamizo & Kramer 1985; Fukamizo 2000). The present study aimed at understanding the influence of chitinase enzyme on the degradation of nauplii of barnacle, *B. amphitrite* at different time interval. Since zooplankton serve as bacterial microhabitats, the total bacterial abundance and also the survival and abundance of different viable bacteria after delinking from the host (barnacle nauplii) was monitored. To the best of our knowledge, no previous study has been conducted to understand the degradation of chitinous exoskeleton of barnacle nauplii using chitinases.

Materials and methods

Rearing of *B. amphitrite* nauplii

The life cycle of *B. amphitrite* includes six planktotropic naupliar instars and a non-feeding cypris instar. The first instar nauplii do not feed and moult into the second instar within 1-2 h. Instars II to VI are phytoplanktotrophic. Instar II nauplii were mass reared in 2L glass beakers using filtered seawater (salinity 35) on a diet of *Chaetoceros calcitrans*, a unicellular diatom, at a cell concentration of 2×10^5 cells ml^{-1} . The feed was replenished everyday while changing the seawater medium. Instar IV nauplii were used to carry out all the experiments.

Evaluation of the chitinase activity using MBTH method

Crab shell chitin (Sigma) and Chitinase from *Streptomyces griseus* (Sigma) were used for the assay of enzyme activity. Chitinase assay was carried out following a standard protocol using 3-methyl-2-benzothiazolinone hydrazone (MBTH) method which was also modified using seawater instead of buffer in order to mimic the conditions in nature (Anthon & Barrett 2002). 200 μl of the *N*-Acetyl-D-glucosamine (GlcNAc) was mixed with 200 μl of 0.5 N sodium hydroxide and 200 μl of MBTH solution. The reaction mixture was mixed vigorously and incubated at 85°C for 15 min. Subsequently, 400 μl of 0.5% ferrous ammonium sulphate, 0.5% sulfamic acid and 0.25N

hydrochloric acid was added to the reaction mixture. The color formed was measured at an absorbance of 610 nm (Anthon & Barrett 2002; Horn & Eijsink 2004).

Standardization of chitinase assay

Chitinase activity was assessed using colloidal chitin as a substrate. Substrate concentration was optimized using 1U of chitinase with varying amount of chitin (5, 10, 25 and 50 $\mu\text{g ml}^{-1}$). 10 $\mu\text{g ml}^{-1}$ of chitin was incubated with varying concentration of chitinase enzyme. 1-5 Unit (U) of chitinase was used in chitinase assay with 50 mM acetate buffer (pH 5). The reaction tubes were mixed properly and then incubated for 1 h at 37°C. Immediately after incubation, 200 μl of the reaction mixture was taken; 200 μl of 0.5N NaOH was added to stop the enzyme reaction. The reducing sugars were estimated using MBTH method (Horn & Eijsink 2004). One unit of enzyme activity is defined as the amount of enzyme required to catalyze the release of 1 μmol of GlcNAc per ml in 60 min at 37°C.

Estimation of GlcNAc from *B. amphitrite* nauplii

Approximately 1000 nauplii (Instar IV) were pooled together and filtered through 0.22 μm filter paper. Subsequently, the nauplii were weighed to determine the wet weight. The nauplii were then suspended in 50 mM Acetate buffer (pH 5) containing 4U of chitinase, which was optimized for chitinase assay. This reaction mixture was mixed thoroughly and incubated at 37°C for 60 min in order to complete the reaction. Reaction was stopped by adding 200 μl of 0.5N NaOH. The total GlcNAc residues liberated from the chitinase activity were determined. The reducing sugars were determined using MBTH method as described above. Similarly, chitinase activity was evaluated with the pooled larvae in 0.22 μm filtered sea water in order to enumerate the release of GlcNAc and the end product was determined using MBTH method.

Effect of temperature and treatment time on *B. amphitrite* nauplii exposed to chitinase

B. amphitrite nauplii were exposed to chitinase enzyme at a concentration of 1U at different temperatures ranging from 21 to 37°C and measured the enzyme activity. *B. amphitrite* nauplii were also exposed to chitinase enzyme at a concentration of 1U prepared in 50 mM of acetate buffer (pH 5) for different time interval of 30, 60, 90 and 120 min respectively at 37°C (enzyme activity was maximum at 37°C). The barnacle nauplii were also treated with different enzyme concentrations (1U, 2U and 5U) in autoclaved 0.22 μm filtered sea water for 60, 120, 180, 240, 300 and 360 min at 30 (± 2) °C, in order to mimic the effect of chitinase in nature. Chitinase treated nauplii were electron

micro-graphed using a standard protocol (Truby 1997) described below, to evaluate the effect of enzyme on the exoskeleton of nauplii.

Scanning Electron Microscopy (SEM) of *B. amphitrite* nauplii

Visual observations on the effect of chitinase on naupliar exoskeleton were done by using scanning electron microscopy (SEM) of naupliar surface. *B. amphitrite* nauplii for SEM were preserved in 2% glutaraldehyde. These samples were first fixed in 2% osmium tetroxide for 30 min followed by dehydration using ethanol series. Subsequently, single nauplii (n=10) were exposed to tertiary butyl alcohol for an hour and mounted on brass stubs (Truby 1997). The samples were then freeze dried for 20 min (Seth et al. 2010), sputter coated and viewed using SEM (JEOL 5800LV).

Quantitative assessment of total and viable (cultivable) bacteria from *B. amphitrite* nauplii on chitinase treatment

Total bacterial abundance was determined by evaluating the Total Bacterial Count (TBC) using acridine orange direct count (AODC) method, under epifluorescence microscope (Daley & Hobbie 1975). The abundance was expressed as no. ml⁻¹.

For enumeration of viable bacteria, the nauplii were rinsed with 5 ml of autoclaved filtered sea water (0.22 µm) in order to enumerate the loosely adhered surface bacteria (epibiotic) (Control). Subsequently, this rinsed sea water (5ml) was serially diluted to 10 folds with autoclaved filtered sea water (0.22 µm) and spread plated (0.1 ml) on Zobell Marine Agar (ZMA) 2216 (HiMedia). The plates were then incubated at room temperature for 24 h before they were analyzed for viable bacterial count.

In the treatment condition, the nauplii were subjected to 5U of chitinase in 5ml of autoclaved sea water for 5 h. This chitinase treated larval suspension was serially diluted as described above, to evaluate the bacterial abundance. Total Viable Counts (TVC) on ZMA plates were expressed as Colony Forming Units (CFU) ml⁻¹.

The isolated colonies obtained were routinely streaked on ZMA, in order to check purity of the culture. Colonies were grouped according to morphology, pigmentation, shape and surface properties. To further characterize the isolates, viable (cultivable) bacteria were subjected to different biochemical tests and identified using Bergey's manual of systematic bacteriology (Krieg 1984).

For the quantification of pathogenic bacteria, the suspensions obtained from control as well as chitinase treated nauplii were spread plated on Thiosulphate Citrate Bile Salts Agar (TCBS), Macconkey Agar and Enterococcus Confirmatory Agar for assessing CFU ml⁻¹. TCBS medium is recommended for the selective isolation of *V. cholerae* and other enteropathogenic *Vibrio* spp. Macconkey Agar was used for selective isolation of coliforms and Enterococcus Confirmatory Agar for *Streptococcus* spp. The plates were incubated at 37°C for 24 h before they were analyzed for quantification of viable pathogenic bacteria. Although the pathogenic bacteria were counted using specific media, in order to reduce the uncertainties associated with counting the pathogenic bacteria, *V. cholerae*, *S. faecalis* and *Escherichia coli* were randomly picked from the selective agar and were confirmed by using a series of appropriate biochemical tests like gram staining, string test, oxidase, catalase, motility, indole, gas from glucose, Methyl Red, Voges Proskauer and citrate utilization. The bacterial abundance is expressed as CFU ml⁻¹.

Quantification of *Vibrio* spp after degradation of molt

Barnacle nauplii molts collected from nature were brought to the laboratory and rinsed with 0.22 µm filtered autoclaved sea water. Subsequently, these molts were transferred individually in 24 well multiwells (n=48) with 2 ml of natural sea water and 0.22 µm filtered autoclaved sea water. After 1, 5 and 9 days of incubation at room temperature (27 ± 2 °C), the natural sea water and 0.22 µm filtered autoclaved sea water containing molts were assessed for the enumeration of different *Vibrio* spp. The methodology for enumeration of the bacteria was followed as explained above.

Results

Standardization of MBTH Method

MBTH method is mainly used to determine reducing sugars due to its high sensitivity, and was used in the present investigation. The method is based on the quantification of the reducing ends in a mixture of chito-oligosaccharides based on the standard curve of GlcNAc (Fig. 1).

Determination of chitinase activity using MBTH method

The degradation of chitin by chitinase was monitored using MBTH method as shown in Fig. 2. In order to test the applicability of the MBTH method for the quantitative determination of chitinase activity, the initial rate of chitin degradation was determined using different enzyme and substrate concentrations. Since the chitin content of barnacle nauplii was very less, low concentration of

substrate was used to evaluate the product formation. The product formed by $5 \mu\text{g ml}^{-1}$ of chitin was below detectable level and a concentration of $10 \mu\text{g ml}^{-1}$ of chitin was adequate to release minimal concentration of end products which was easily detected by MBTH method.

Influence of temperature and chitinase on *B. amphitrite* nauplii

Chitinase activity varied with the enzyme concentration as well as the incubation temperature. The enzyme activity gradually increased from 1U to 2U with a substrate concentration of $10 \mu\text{g ml}^{-1}$, and subsequently increased from 2U to 3U with optimum activity at 4U. A decrease in the enzyme activity was evident when the enzyme concentration was increased to 5U and more (Fig 2). Chitinase activity increased with the increase in temperature and it almost doubled when the temperature was increased from $21 (\pm 2) ^\circ\text{C}$ to $37 (\pm 2) ^\circ\text{C}$ (Fig. 3). At room temperature ($28 (\pm 2) ^\circ\text{C}$), the enzyme activity was moderate.

Estimation of GlcNAc released from *B. amphitrite* nauplii using chitinase

Approximately 1000 nauplii released $1.78 \mu\text{M}$ ($0.4 \mu\text{g ml}^{-1}$) of GlcNAc when 4U of chitinase was used, whereas the GlcNAc released from 1U of chitinase was estimated to be $0.72 \mu\text{M}$ ($0.16 \mu\text{g ml}^{-1}$) in 50 mM of Acetate buffer (pH 5). The pooled nauplii were also subjected to chitinase treatment in filtered sea water containing 4U of enzyme. 4U of chitinase in filtered sea water was adequate enough to release $0.92 \mu\text{M}$ ($0.20 \mu\text{g ml}^{-1}$) of GlcNAc from the pooled nauplii.

Effect of chitinase on *B. amphitrite* nauplii

Chitinase treatment was carried out on individual *B. amphitrite* nauplii to assess the effect of chitinase on their exoskeleton. From the scanning electron micrograph images, it was revealed that 1U of Chitinase enzyme disrupted the cell wall of individual nauplii within 90 min in 50 mM Acetate buffer (pH 5) at 37°C (Plate. 1). The enzyme took a longer duration when used in sea water and was effective at 5U. A concentration of 5U of chitinase in $0.22 \mu\text{m}$ filtered sea water was sufficient to disrupt the exoskeleton of barnacle nauplii in 360 min in natural sea water at $30 \pm 2^\circ\text{C}$ (Plate. 2). Nauplii of *B. amphitrite* which were not treated (Control) with chitinase had a smooth and undisturbed surface. After treatment with chitinase, the naupliar exoskeleton showed scars on the surface initially (200 min onwards) and disruption of the exoskeleton was observed after 300 min and above (Plate. 2). Treating the nauplii for increased duration in chitinase showed higher disruption on the surface of naupliar exoskeleton.

Bacterial diversity from *B. amphitrite* nauplii

Among the isolates that were obtained from control and chitinase treated *B. amphitrite* nauplii, 18 dominant bacterial strains were selected for morphological and biochemical analysis based on their distinctive colony characteristics (Table 1 and 2). Diverse bacteria were obtained after chitinase treatment and *Bacillus* spp were dominant after the nauplii were treated with chitinase (Table 2). Total and viable bacterial abundance of the chitinase treated nauplii increased with the increase in enzyme concentration (Fig. 4a and 4b).

Abundance of pathogenic bacteria from barnacle nauplii before and after chitinase treatment *Vibrio* spp and *Streptococcus* spp were dominant pathogenic forms associated with barnacle nauplii. *Streptococcal* forms were present in higher numbers (1555 CFUml⁻¹) compared to all other pathogens. The bacterial density of the *Vibrio alginolyticus* (1033 CFUml⁻¹) was high compared to other *Vibrio* spp (*V. cholerae* - 597 CFUml⁻¹ and *V. parahaemolyticus* - 40 CFUml⁻¹) followed by *Shigella* spp and *Salmonella* spp which were less abundant (713 CFUml⁻¹). *E. coli* and *S. faecalis* were not reported. No viable pathogenic forms were observed after treatment with chitinase (Fig. 5).

Abundance of pathogenic bacteria from the molts collected from the field

The abundance of *Vibrio* spp (*V. cholerae*, *V. parahaemolyticus* and *V. alginolyticus*) released from the molt collected from field is shown in Fig. 6. When the molts were incubated in 0.22µm autoclaved seawater, *V. cholerae* numbers decreased from day 1 to 9. *V. alginolyticus* showed slight increase from day 1 to day 5 and further decrease when incubated for 9 days. Interestingly, *V. parahaemolyticus* which were absent on day1 appeared on day 5 and 9 but their abundance was low when compared to other *Vibrio* spp (Fig. 6a). However, when the molts were incubated in natural seawater, *V. cholerae* and *V. alginolyticus* increased from day 1 to day 5 and further decreased on day 9. Although *V. parahaemolyticus* were observed after 1 and 5 day incubation they were absent on day 9, and their abundance was low when compared to other *Vibrio* spp (Fig. 6b).

Discussion

Chitin produced by chitinous organisms is decomposed by chitinoclastic bacteria, mainly detritivorous or decomposers which obtain their energy from the chemical breakdown of dead organisms and their propagules. Once decomposition of chitinous zooplankton or larvae is initiated by the chitinase producing organisms, the organic matter synthesized by these organisms can be used by other microorganisms inhabiting these ecosystems creating a secondary source of nutrition. Thus,

chitin plays a key role in maintaining the biogeochemical cycles of Carbon (C) and Nitrogen (N), shift in the rate of C and N production and degradation plays an important role in its availability (Poulicek et al. 1998). This highlights the importance of understanding the fate of chitin degradation in the marine environment.

The chitin content of many marine crustaceans (Copepoda, Amphipoda and Branchiopoda) range from 2.5 to 8.5% of the total dry weight of the body mass (Cauchie 2002) and the chitin content of the total freeze dried cypris larvae of *Balanus balanoides* is 3.2% (Holland & Walker 1975). Since the chitin content of the individual barnacle larva is comparatively low, which would make the detection of end products a difficult task by using MBTH method, the barnacle nauplii (~1000) were pooled and GlcNAc released during the chitin degradation by chitinase was measured. In the present study, natural sea water instead of buffer was used to mimic the conditions in nature thus modifying the chitinase assay. It was found that the chitinolytic effect of chitinase on the chitin molecule in sea water was less when compared to acetate buffer (50mM, pH 5.0). After treatment with chitinase, the exoskeleton of nauplii which was smooth and undisturbed, showed scars on the surface initially (200 min onwards) and clear disruption of the exoskeleton was observed after 300 min and above. Higher degree of disruption on the surface of naupliar exoskeleton was observed when the treatment time with chitinase was increased. Also, an increase in the chitinase activity with the increase in temperature (from 21 to 37 °C) was observed. The temperature of the coastal water is variable until 500 m and below this region and in sediments the temperature is low and constantly varying between -1 to 5°C (Hedges & Oades 1997). Due to continuous mixing of nutrients and gases in oceanic water the temperature and pH of the water permutes often, which leads to variable change in the enzyme activity. The degradation of the chitin content is up to 90% within 150 h in open water, where a longer duration of time is needed in sediments in marine ecosystems (Poulicek & Jeuniaux 1991). The higher rate of degradation of chitin in open water is attributed to the presence of high rate of chitinolytic bacteria in aquatic environment, 13% in water column with that of 5% in sediment (Brzezinska et al. 2009). Seki (1965) also reported similar pattern of chitin degradation, wherein chitin in open water is mineralized within 50 and 140 days at 25 and 15°C respectively. Their studies revealed that maximum degradation of chitin is in water column when compared to sediment. The present study helps in understanding the fate of chitin degradation of crustacean exoskeleton under different conditions where temperature plays a major role in chitin degradation via enzyme activity. As the conditions of pH and temperature differ in different water bodies or ecosystems, future studies should focus on conducting experiments using these variables and different crustacean

forms and this data would be extremely useful as it will provide the details on the *in situ* chitin degradation.

It is also interesting to understand why chitinoclastic bacteria associated with marine zooplankton do not degrade the chitinous exoskeleton. One means of avoiding chitinase degradation in marine zooplankton by chitinase producing bacteria may be moulting of these organisms within a short period (e.g. nauplii of *B. amphitrite* moult 6 times within 5-6 day interval). Due to moulting the bacterial load on the chitinous surface of crustaceans are purged and if any damage has been done by any chitinases it would be eliminated during moulting. It is also possible that the proteinaceous epicuticle (procuticle) which act as a diffusion barrier, present on the surface of the marine crustaceans may protect the exoskeletal damage by relatively small quantity of chitinase produced from the surface associated chitinoclastic bacteria (Getchell 1989; Stewart 1993).

Bacterial colonization of the crustacean exoskeleton has been reported by several researchers (Carman & Dobbs 1997; Heidelberg et al. 2002; Holland & Hergenrader 1981; Kaneko & Colwell 1975; Sochard et al. 1979). Bacterial adherence to chitinous surface is due to bacterial interactions colonizing the exoskeleton of marine organisms, via specific interaction between bacteria and chitin containing surfaces mediated by lectin binding, chitin binding and other surface proteins (Tarsi & Pruzzo 1999; Pruzzo et al. 2008). In addition, chemotaxis to chitin hydrolysis products has also been suggested (Bassler et al. 1991). Many marine bacteria, particularly chitin-degrading *Vibrio* spp possess complex signal transduction systems for finding and adhering to chitinous substrates, mediated by chemical attractants or growth promoting compounds specific to *Vibrio* spp (Goffredi et al. 2008; Keyhani & Roseman 1999; Yu et al. 1993). *Vibrio* spp densely colonizes the surface as well as the oral regions and egg sacs of most copepods. The presence of these *Vibrio* spp on the copepod surfaces is significant, since it showed inhibitory effect on the strains of *Pseudomonas* spp and *E. coli* which try to compete for space on surface of both live and dead copepods (Huq et al. 1984). In this study we observed that the numbers of *Vibrio* spp released from the molts of barnacle larvae increased with time. The *Vibrio* numbers were highest during day 5 and decreased later. Studies by Bassler et al. (1991) indicated that *Vibrio* spp may be able to use crustacean exoskeleton as a growth substrate as they possess chitinolytic enzymes suggesting that *Vibrio* spp could have decreased due to unavailability of the chitinous substrate, which act as a nutrient source and needs further validation. Hood et al. (1984) also noted that the preferred habitat of *V. cholerae* is the epibiotic one in which it lives attached to the substrate.

In this study, chitinase activity increased with an increase in enzyme concentration. The enzyme activity was optimum at 4U of chitinase at $10 \mu\text{g ml}^{-1}$ of chitin. 5U of chitinase showed a decrease in the enzyme activity with same concentration of substrate. End product inhibition may be a reason for this decrease in the enzyme activity. Similar results were reported by Pinto et al. (1997) and Rattanakit et al. (2007) from chitinase of *Metarhizium anisopliae*, wherein they reported 21% of inhibition in the presence of GlcNAc.

Total viable bacterial numbers in case of chitinase treated nauplii progressively increased when compared to that of the control. The disruption of the larval exoskeleton after chitinase treatment may be a possible reason for release of such a large number of bacteria. Earlier studies have reported that gamut of bacteria are released on disruption of the exoskeleton of the nauplii (Seth et al. 2010). The composition of the bacteria isolated from chitinase treated nauplii showed a diverse group of bacterial community. The majority of the bacteria isolated in the study were Gram positive (12 out of 13 genera). *Bacillus* spp was most abundant after chitinase treatment. Approximately 56% of the bacteria obtained after chitinase treatment belonged to family Bacillaceae (Table. 2), which were similar to the results obtained in the nematode, *Steinernema* spp wherein the population of *Bacillus* spp was dominant (46%) (Georgieva et al. 2003).

Vibrio spp and *Streptococcus* spp were most dominant pathogenic forms associated with barnacle nauplii. Pathogenic bacteria were studied due to the concern for human health, which are extensively studied for their survival in marine environment (Huq et al. 1984; Byrd and Colwell 1990; Oliver et al. 1995). Members of the family Enterobacteraceae are common inhabitants of gut of humans to insects (Georgieva et al. 2003). Chitinivorous organisms includes many bacteria (Xiao et al. 2005) and fungi, which are either pathogenic or detritivorous. Chitinases are widely distributed in bacteria such as *Serratia*, *Chromobacterium*, *Klebsiella*, *Pseudomonas*, *Clostridium*, *Vibrio*, *Arthrobacter*, *Beneckea*, *Aeromonas* and *Streptomyces* (Hunt et al. 2008). They are also found in fungi like *Trichoderma*, *Penicillium*, *Lecanicillium*, *Neurospora*, *Mucor*, *Beauveria*, *Lycoperdon*, *Aspergillus*, *Myrothecium*, *Conidiobolus*, *Metarhizium*, *Stachybotrys* and *Agaricus* (Deshpande 1986). In the present study, *Vibrio alginolyticus* is reported as dominant *Vibrio* spp associated with the larvae. These results were similar to those of Hameed (1993), wherein the *Vibrio* spp were dominant during the developmental stages in the larval forms of *P. japonicas*. Vandenberghe et al. (1999) also reported the presence of *Vibrio* spp associated with environment of healthy nauplii of *P. chinensis*. *V. parahaemolyticus* was the least abundant in the larval surface next to *V. cholerae*.

The absence of pathogenic bacteria after treatment with chitinase is noteworthy and may be due to the antagonistic effect of the *Bacillus* spp which was dominant after treatment. The presence of *Bacillus* spp has been reported to be inhibitory to the pathogenic bacteria such as *Vibrio* spp (Jöborn et al. 1997; Sugita et al. 1998; Rengipat et al. 1998; Moriarty 1998). *Bacillus* spp has been showing antagonistic activity to the pathogenic bacteria in aquaculture (Devaraja et al. 2002; Vaseeharan & Ramasamy 2003; Gatesoupe 1999; Rengipat et al. 2000). *Bacillus* spp might produce some metabolites, for instance antibiotics (Williams & Vickers, 1986) or enzymes for inhibition and/or digestion (Bruno & Montville 1993). *Bacillus* spp contributes a large part in the microflora of the gills, skin and intestinal tracts of shrimps (Sharmila et al. 1996) and competes for nutrients and thereby inhibits other bacterial proliferation and grows rapidly in surrounding habitat. They compete for the space on surface lining of the organisms, e.g. the gut wall, and displace other bacteria if they are present in high numbers (Moriarty 1998). Thus, any resistant bacteria cannot multiply or transfer resistance genes nor colonize on the zooplankton surface.

In a previous study by Lidita and Anil (personal observation) inputs of pathogenic bacteria associated with zooplankton, subsequent to pulverization was evaluated and they indicated that the number of viable pathogenic bacteria such as *V. cholerae*, *S. faecalis* and *E. coli* increased enormously subsequent to zooplankton pulverization. Plankton associated microbes are also important in marine bio-invasion and ship's ballast water treatment technology. In the present study we observed that these pathogenic bacteria including *V. alginolyticus*, *V. parahaemolyticus* were absent subsequent to chitinase treatment. The dominance of *Bacillus* spp subsequent to chitinase treatment could have important implications on the ecosystem functioning as the water column after treatment with chitinase had a different bacterial community. This can also find implication in treatment technology as discharge of pathogenic bacteria while large planktonic organisms are killed or destroyed is a cause of concern to the environment and human health.

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Table 1. Culturable bacterial isolates before chitinase treatment

Isolate number	Name of the bacteria
1	<i>Pseudomonas maltophila</i>
2	<i>Corynebacterium</i> sp.
3	<i>Nocardia camae</i>
4	<i>Staphylococcus lantus</i>
5	<i>Kurtia</i> sp.

Table 2. Culturable bacterial isolates after chitinase treatment

Isolate number	Name of the bacteria
1	<i>Bacillus coagulans</i>
2	<i>Flavobacterium meningosepticum</i>
3	<i>Saccharococcus</i> sp.
4	<i>Bacillus anthracis</i>
5	<i>Renibacterium</i> sp.
6	<i>Brevibacterium</i> sp.
7	<i>Bacillus brevis</i>
8	<i>Staphylococcus haemolyticus</i>
9	<i>Bacillus endophyticus</i>
10	<i>Marinococcus albus</i>
11	<i>Bacillus subtilis</i>
12	<i>Cristispira pectins</i>

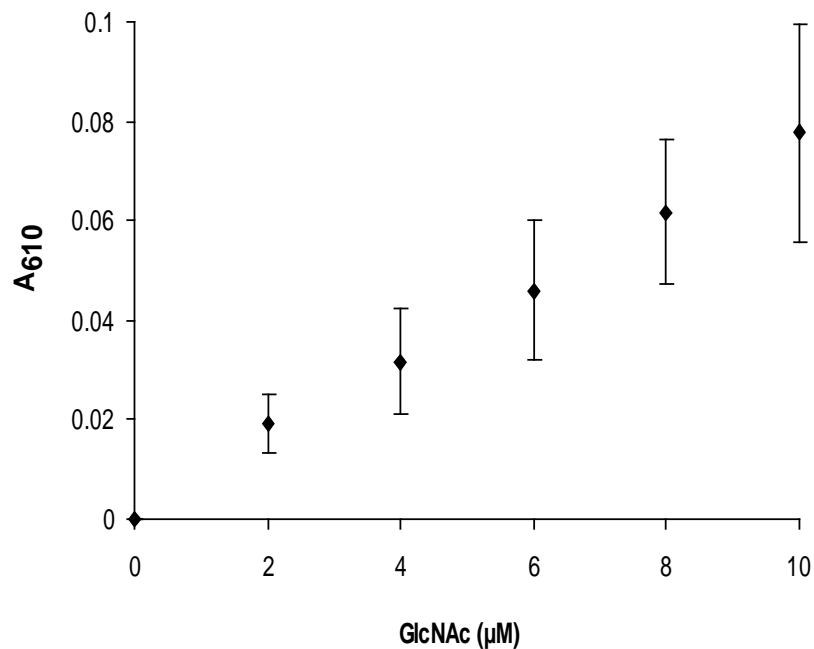


Fig 1. Calibration of MBTH method using GlcNAc. The figure shows the absorbance vs concentration curve at 610 nm. The values are represented as mean numbers. Vertical lines indicate standard deviation from the mean.

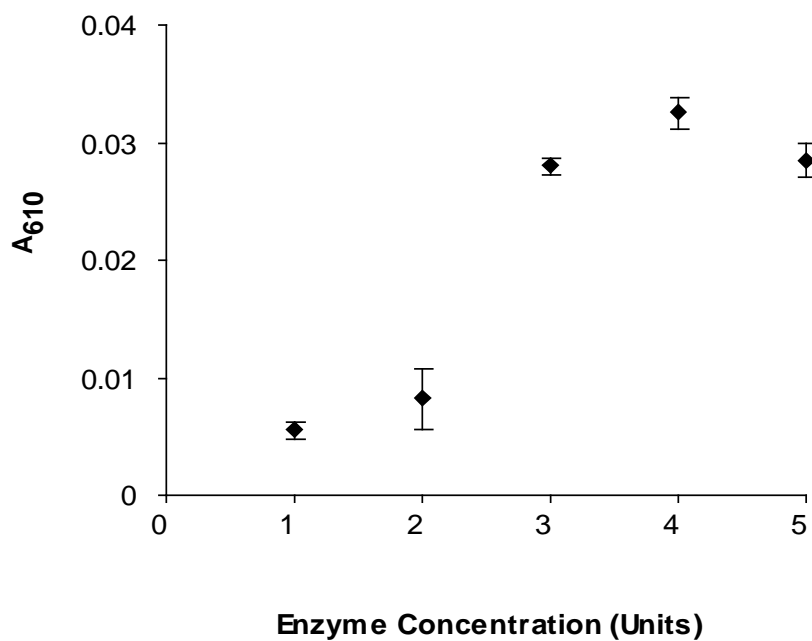


Fig. 2. Degradation of Chitin using chitinase enzyme from *Streptomyces griseus*. Reducing ends were estimated using 10 µg of chitin with different concentrations of enzyme (Units). Vertical lines indicate standard deviation from the mean.

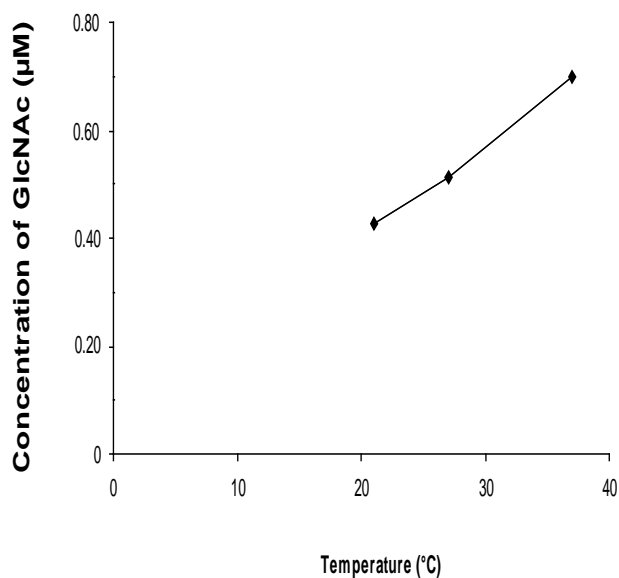


Fig. 3. Effect of temperature on the chitinase activity of *B. amphitrite* nauplii exposed to chitinase

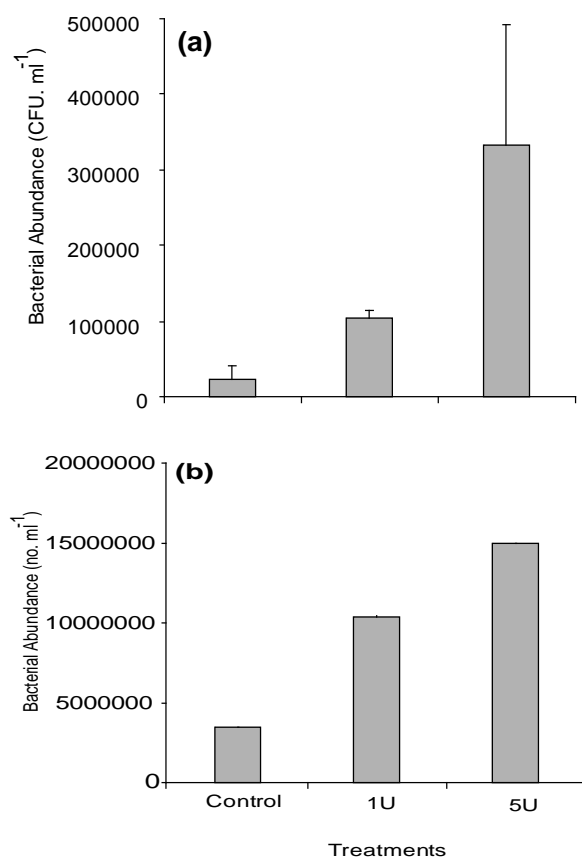


Fig 4 Bacterial abundance from chitinase treated *B. amphitrite* nauplii
 (a) Total Viable (Cultivable) Bacterial Abundance expressed as CFU ml⁻¹
 (b) Total Bacterial Abundance expressed as no. ml⁻¹

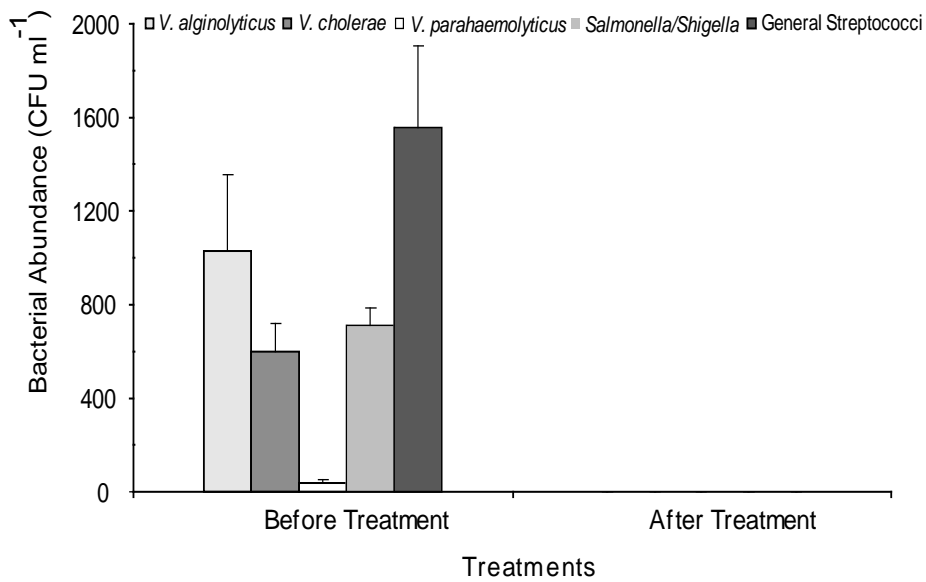


Fig 5. Abundance of total viable pathogenic bacteria from *B. amphitrite* nauplii before and after chitinase treatment

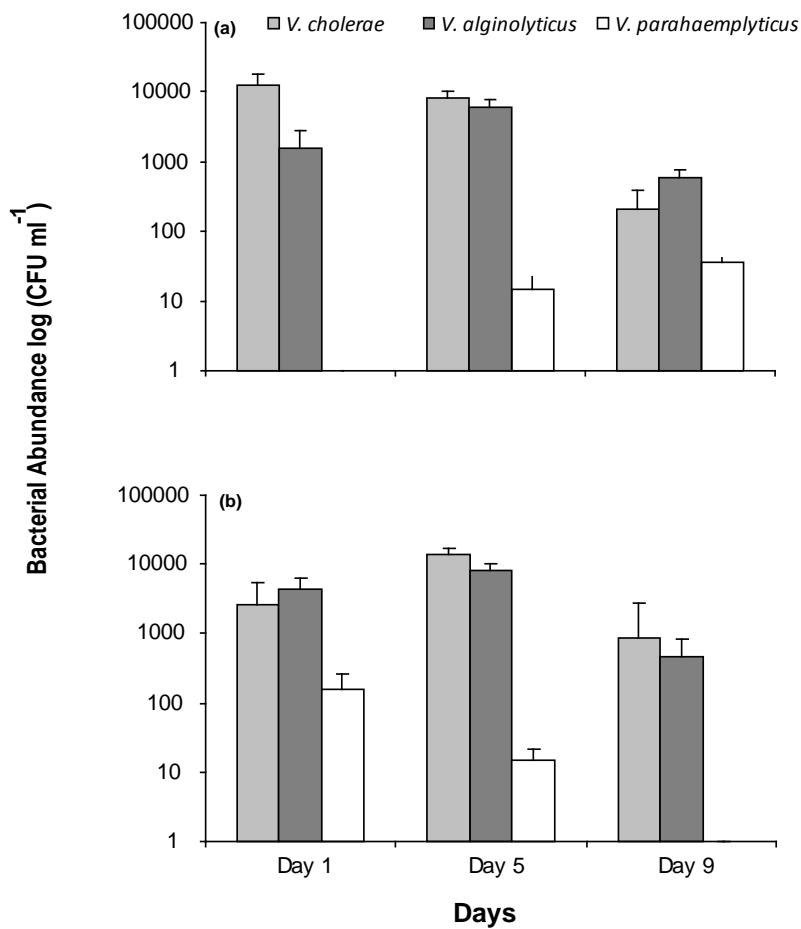
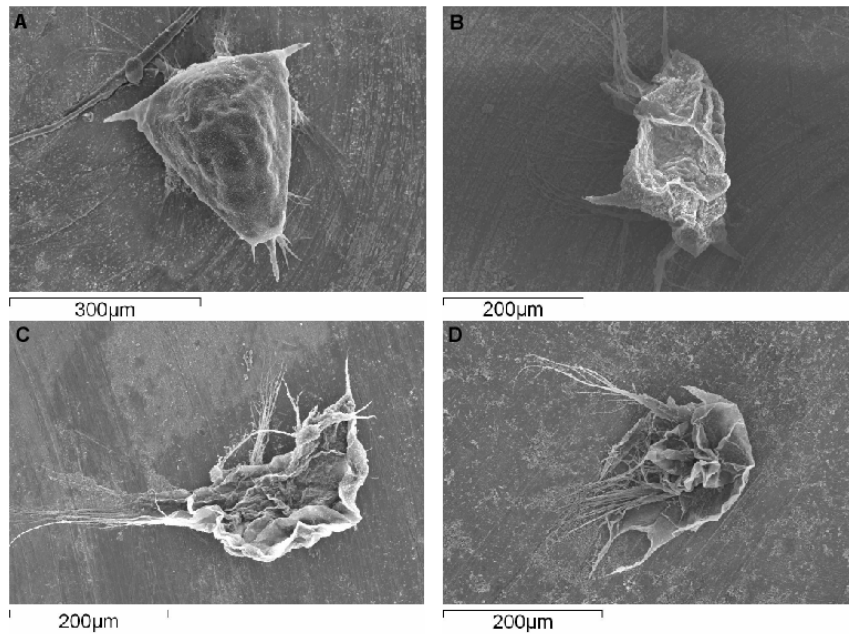


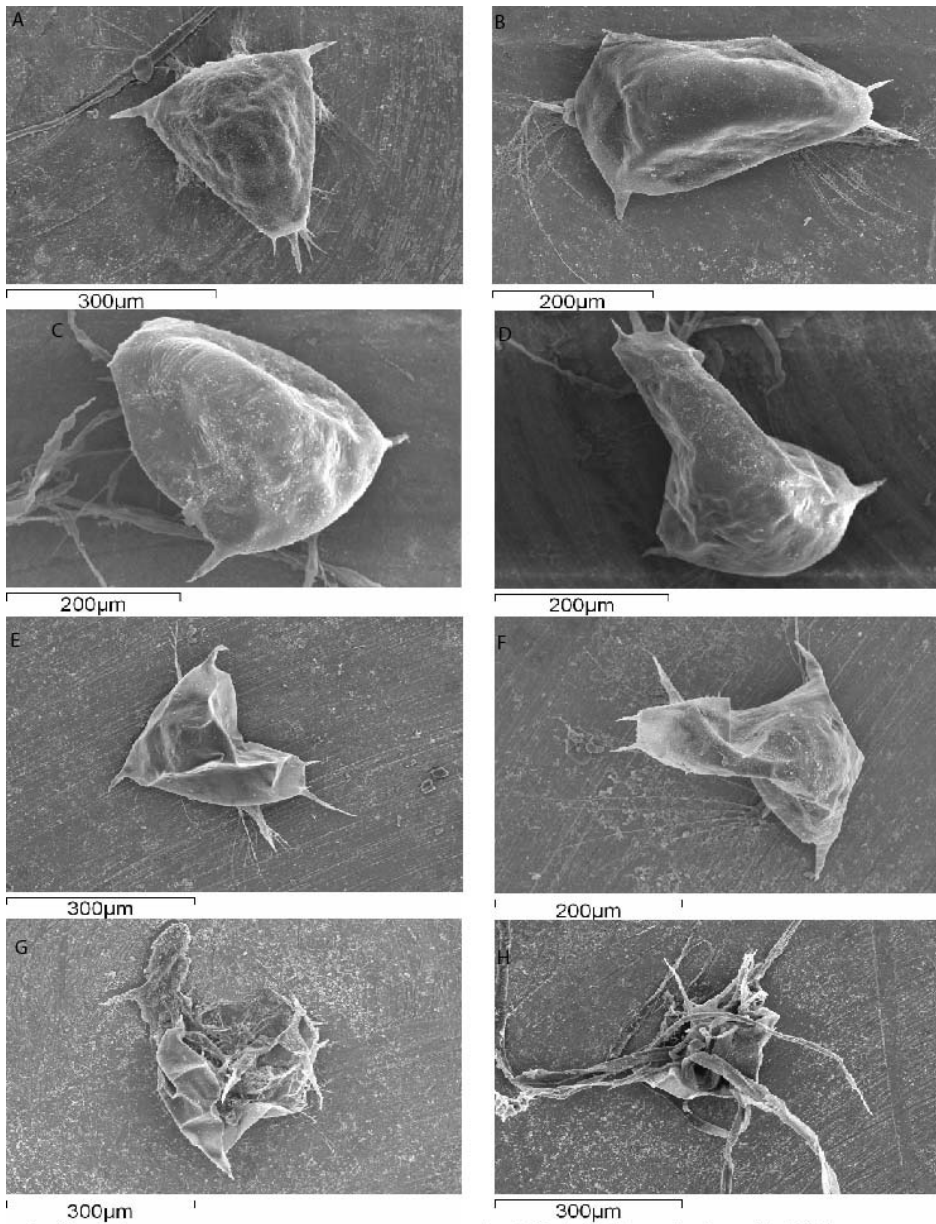
Fig. 6. Abundance of total viable *Vibrio* spp during molt degradation in (a) 0.22 μ m Autoclaved sea water (b) Natural sea water

Plate. 1. Barnacle nauplii treated with 1U of chitinase in 50 mM of acetate buffer (pH 5) at 37 °C at different time intervals.



- A** Control
- B** 30min Chitinase Treatment in 50mM Acetate buffer
- C** 60min Chitinase Treatment in 50mM Acetate buffer
- D** 90min Chitinase Treatment in 50mM Acetate buffer

Plate. 2. Barnacle nauplii treated with 5U of chitinase in 0.22 μm filtered sea water at 30 ± 2 $^{\circ}\text{C}$ at different time intervals.



A Control
 B 30min Chitinase treatment in FSW
 C 60min Chitinase treatment in FSW
 D 90min Chitinase treatment in FSW

E 150min Chitinase treatment in FSW
 F 200min Chitinase treatment in FSW
 G 340min Chitinase treatment in FSW
 H More than 360min Chitinase treatment in FSW

Plate 2