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Effect of garlic extract on the luciferase, bio-luminescence, virulence factors produced by *Vibrio harveyi* with a challenge during *Penaeus monodon* larviculture

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There has been augmented curiosity in the application of herbal products against diseases causing marine pathogenic bacteria in aquaculture system. This paper describes antagonism of different types of garlic extract on the growth *of Vibrio harveyi* that produces luciferase, virulence and bio-luminescence. Aqueous filtered garlic extract (AFGE) showed zone of inhibition (12.33 ± 0.50 mm) against *V. harveyi.* But aqueous unfiltered garlic extract (AUGE) showed less zone of inhibition. *V. harveyi* treated with AFGE showed reduced growth varying from 0.32 to 0.26 OD as compared to the control. UV treated AFGE showed 9.0 ± 0.50 mm zone of inhibition against *V. harveyi.* But during heat treatment at 100 and 121°C, the extracts lost their activity. Most of the virulence factors exhibited by *V. harveyi* were found to be reduced by AFGE treatment. Crude bacteriocin values changed from 0.10 to 0.24 OD as compared to the control (1.60 OD) for the 5 days. The cellular luciferase level was reduced to 86, 98, 79 and 94 counts per second (CPS, that is, photons/second) and the different CPS values are 23, 06, 12, 12 compared to the control. AFGE treatment also reduced bio-luminescence counts (30, 29, 35 and 30 CPS) with the differences being 14, 07, 09, 06. Challenge experiment proved that AFGE used in treating post larvae of *Penaeus monodon* showed less cumulative percentage mortality. In the control, maximum mortality of 2 - 42% occurred for 30 days. But in the AFGE treatment, mortality varied from 0.5 to 5.5%. Therefore, garlic extract may be a source of herbal product for controlling bacterial diseases in aquaculture.

Key words: Garlic extracts, *Vibrio harveyi*, virulence factors, bio-luminescence, luciferase, *P. monodon* larviculture.

INTRODUCTION

The declining fish production and consumers' growing demands for fishery products are the main factors affecting expansion of aquaculture. Disease out-break, persistently causes mass mortality, which is one of the major problems faced by the aquaculture industry. *Vibrio harveyi*, a Gram-negative and bio-luminescent bacterium responsible for causing mass mortality (80-100%) among *Penaeus monodon* hatcheries (Raissy et al., 2011). The association and species composition of bio-luminous bacteria in near-shore seawater, semi-intensive and

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brackish water fed shrimp grow-out systems of Tamil Nadu was reported (Abraham et al., 2008). Five species of bio-luminous bacteria, viz., *Photobacterium leiognathi, Vibrio fischeri*, *V. harveyi*, *Vibrio orientalis* and *Vibrio splendidiis* biotype 1 were encountered in seawater fed shrimp grow-out ponds. In brackish water grow-out ponds, *Photobacterium leiognathi*, *V. harveyi* and *V. splendidus* biotype 1 were isolated from the water samples (Abraham et al., 2008). The most important habitat of bio-luminous bacteria is the digestive tract or gut contents of shrimp wherein bio-luminous bacterial counts up to 10^8 cfu/g have been reported (Abraham et al., 1998). Bio-luminous bacteria were reported in the shrimp larval rearing tanks in considerable numbers ranging from log 0.70 to log 5.41/ ml (Abraham and Palaniappan, 2004). In recent years, bio-luminous *Vibrio* spp. have been recognized as the tropical pathogens of economic importance (Owens et al., 1992). *V. harveyi* isolates from fish farms showed mortality (100%) to fish such as *Rainbow trout* and *Salmo salar* (Zhang and Austin, 2000; Haldar et al., 2011). *V. harveyi* causes fish diseases such as vasculitis, gastro-enteritis and eye lesions. Mass mortality of tiger shrimp *(P. monodon)* larvae caused by *V. harveyi* showing multiple antibiotic resistances has been reported (Karunasagar et al., 1994). Consequently, the quest for alternative methods to control infection caused by antibiotic-resistant *Vibrios* is imperative challenge for sustainable development of aquaculture. As control measures, farmers use either probiotics bacteria or antibiotics in a disorganized manner. Antibiotics and synthetic chemicals used in fish/shrimp grow-out practices may contribute optimistic effects, but cannot be recommended due to their residual side effects (Citarasu, 2010). The harmful impact of antibiotics used in shrimp grow-out practices leads to evolution of antibiotic resistant microbes that can eventually pass on resistance to other pathogenic microbes, leading to a reduced efficiency of these antibiotics for disease treatments. Few of the probiotic products that were used in Thailand for marine shrimp cultivation did not antagonize marine *V. harveyi* (Nimrat and Vuthiphandchai, 2011). Due to the increased antibiotic resistance among bacteria, WHO recommended in exploring herbs or plants as antimicrobial agents for treating various bacterial infections (Wallace, 2004). Many of the natural plant products have been used to uphold many biological functions such as anti-stress, growth enhancer, appetizer, immune-modulator, antimicrobials among aquaculture owing to their mechanistic active ingredients like alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids and essential oils (Sivaram et al., 2004). Garlic (*Allium sativum*) is one of the natural plant seeds used as food, folk medicine and as anti-tuberculosis agent for centuries (Rivlin, 2001). Garlic extract has been successfully tested against *Vibrio parahaemolyticus,* which is causative bacteria for food poisoning (Vuddhakul et al., 2007). Similarly, garlic extract was used to control multi drug-resistant *V. harveyi* isolated from shrimp (Vaseeharan et al., 2010) and against fungi (Khodavandi et al., 2011). However, application of plant-based materials treatment against aquatic pathogenic microbes as compared to antibiotics in shrimp grow-out practices is limited. So, the present study focussed on the analysis of the inhibitory activity of garlic extract against the growth of pathogenic *V. harveyi* in shrimp larviculture practice and interference with virulence factors.

MATERIALS AND METHODS

Isolation of *V. harveyi*

Water samples of less salinity (3 -17 PSU) were collected from the Muttukadu Experimental Station of CIBA, Chennai, India. The samples were pre-enriched in alkaline peptone water (APW) and serially diluted with normal saline (0.85 % NaCl w/v), 0.1 ml of each sample was surface spread on Thiosulphate citrate bile salt sucrose agar medium (TCBS), Seawater complex agar (SWC) and *V. harveyi* selective agar medium (VHSA). In SWC agar, bio-luminous colonies were observed after 20 h of incubation at 30°C. Then the colonies were repeatedly streaked on SWC agar and VHSA (Harris et al., 1996). The isolates were again confirmed by various bio-chemical tests such as arginine dihydrolase (-), lysine (+), ornithine decarboxylase (+), gelatinase (+), Voges proskauer (-) and D-glucosamine (-) etc (Abraham and Palaniappan, 2004).

Identification of *V. harveyi* hemolysin gene using PCR

In screw capped tubes, a loopful of *V. harveyi* isolates were taken from VHSA / SWC plates and suspended with 100 µl of DNA free distilled water. The suspension was heated at 98°C for 25 min in a dry bath. The lysate was used as template for DNA. The primers targeting 235 bp internal fragment of the gene were forward: 5' CTTCACGCTTGATGGCTACTG 3' and reverse: 5' GTCACCCAATGCTACGACCT 3'. Reaction was carried out for each isolate in a 30 µl reaction mixture consisting of 3.0 µl of 10 x PCR buffer (100 mM Tris-HCl pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, 0.1% (w/v) gelatin), 200 µM of each dNTPs, 25 pmol of each primer, 0.75 U of Taq polymerase (Bangalore Genei, India) and 2 µl of template DNA. PCR was carried out in thermocycler (Applied Bio-systems, USA) with reaction cycles, that is, initial denaturation at 95°C for 5 min; 30 cycles of 95°C/ 1 min, 50°C/1 min, 72°C/1 min and a final extension of 72°C/5 min. The final products were resolved on 2% agarose gels containing 0.4 µg/ ml ethidium bromide and documented using gel documentation system (BIO-RAD, USA). Reference strain of *V. harveyi* (MTCC - 3438, India) was used as positive control. A total of 26 isolates of *V. harveyi* were found positive based on hemolysin (vhh) gene (Maiti et al., 2009). The isolates were stored in sterile glycerol solution (15% w/v).

Preparation of garlic (*A.sativum*) extract

Garlic bulbs were procured from the local market (Chennai, India). The bulbs (100 g) were peeled off, washed in distilled water, homogenized by sterile pestle and mortar with 100 ml distilled water. The extract was filtered through Whatman No.1 filter paper. The filtrate was collected in a sterile vial and considered as

"aqueous filtered garlic extract" (AFGE), and the unfiltered extract considered as "aqueous unfiltered garlic extract (AUGE). Both extracts were treated against *V. harveyi* and its virulence factors. Both extracts were tested at various concentrations against *V. harveyi* (Vaseeharan et al., 2010).

Inhibitory activity of garlic extracts on *V. harveyi*

Antibacterial activities of AFGE and AUGE were performed by the "well diffusion assay" (Kannappan and Manja, 2004). 1 ml of 18 h strain of *V. harveyi* was inoculated onto 100 ml of Luria Bertani (LB) broth and shaker incubated at 28°C/150 rpm for 48 h. 50 µl (2.19 x10^7cfu/ ml) of *V. harveyi* cells (OD 1.80) was inoculated onto the Petri plates. Molten LB agar was prepared and poured in the Petri plates. Wells of 7 mm dia were made from the plates. The bottom of the wells was sealed by adding 10 µl (0.8%) of soft agar. AFGE and AUGE extracts of 200 µl were added separately into the wells. The plates were incubated at 28°C for 24 h. Antibacterial activity was observed as the diameter of the clear zone (mm) of inhibition formed on *V. harveyi* around the well.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was ascertained as the lowest concentration of garlic extract against the highest dilution which inhibited the growth of *V. harveyi* in LB agar medium using "disk diffusion assay". Various concentrations of extracts attempted were 10 to 70 µl. The plates were incubated at 28°C for 24 h.

Effect of garlic extracts on the growth *of V. harveyi* and its virulence factors

Two millilitres of *V. harveyi* (1.80 OD) was inoculated into 250 ml LB broth medium. Twenty five ml of freshly prepared AFGE and AUGE extracts were separately added into the LB broth, called "Garlic *Vibrio* broth" (GVB) and shaker incubated at 28°C/ 150 rpm for 5 days. Control was done without adding garlic extracts. The growth of *V. harveyi* was monitored for 5 days by estimating OD at 600 nm using spectrophotometer. Suitable quantity of broth was taken every day from GVB and various virulence factors such as thermonuclease activity, proteolytic activity, lipolytic activity, phospholipase activity, extracellular crude protein (Bacteriocin), cell wall bound protein, exopolysaccharide (EPS) production were estimated. Protease enzyme produced by *V. harveyi* was analysed separately after treating with AFGE and AUGE extracts (Manilal et al., 2010).

Disruption of virulence protease activity from *V. harveyi treated with* garlic extracts

Three millilitres of GVB was taken separately for three days. V. harveyi cells were harvested by refrigerated centrifuge (10,000 rpm at 4°C for 10 min) and spotted on the surface of skimmed milk agar (25 ml of nutrient agar with 5% (w/v) skimmed milk powder). The plates were allowed to remain at 28°C for 30 min and then incubated at 28°C for 12 h. The formation of clear zone around the bacterial spot was confirmed for protease production. For quantification of protease from V. harveyi treated with AFGE, the fermentation growth medium (150 ml) was prepared separately (w/v 0.75% glucose, 0.75% peptone, 0.5% Magnesium sulphate, 0.5% Potassium dihydrogen phosphate, 0.01% Ferrus sulphate). One ml of V. harveyi (OD 1.80) was inoculated into the fermentation broth (150 ml) with 1.5 ml of AFGE and incubated at 28°C for 72 h in shaker incubator at 150 rpm. Cells were harvested by refrigerated centrifuge (10,000 rpm at 4°C) from the fermentation broth and supernatant was used as crude protease enzyme and analyzed for protease activity with azocasein as substrate (Olajuyigbe and Ajele, 2005). The protease enzyme activity was assessed using 500 µl of 0.5% azocasein (w/v) (Sigma) in Tris HCl buffer with 100 µl enzyme solution and incubated for 60 min at 37°C. The reaction was stopped by the adding of 500 µl of 15% Trichloro acetic acid (TCA, w/v). This solution was incubated at 30°C/ 15 min and then centrifuged at 4°C/15 min at 3000 rpm. One mlliliter of supernatant was added to 1.0 ml of NaOH and the absorbance was measured at 440 nm (One unit of protease activity is defined to be the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvettee under the condition of the assay).

Lipolytic and phospholipase activities

Nutrient agar plates were supplemented with 2% tributyrin (w/v). Five ml of GVB was centrifuged at 7000 rpm for 10 min. The cell pellet was spotted on the surface of agar plates and incubated at 28°C for 48h. The non-forming zone of clearance around the spot was considered negative for lipolytic activity (Sindhu and Surendran, 2008). Nutrient agar plates were supplemented with 5% egg yolk emulsion (w/v). Five ml of GVB was centrifuged at 7000 rpm/ 10 min. The cell pellet was spotted on the surface of the agar plates. The plates were incubated at 28°C for 48 h and observed for lecithinase activity. Absence of distinct grey to white precipitate around the colony indicates that no free fatty acid liberation took place upon lecithinase activity on phospholipids.

Crude extracellular protein (bacteriocin)

Five milliliters of GVB was centrifuged for 7000 rpm/10min. The supernatant was quantified for crude extracellular protein (Lowry et al., 1951). Untreated *V. harveyi* was taken as control. For cell wall bound protein, 5 ml of GVB was centrifuged for 7000 rpm/10 min. The cells were suspended in 3 ml of sterile saline [0.85 % (w/v)] to which 0.5 ml of Tris HCl buffer was added (0.01M Tris HCl, 0.01 M EDTA, 0.01 M NaCl and 2% SDS, pH at 8.0). Then the cells were kept at 100°C for 5 min. Again, the cells were centrifuged at 7000 rpm for 10 min. The supernatant was collected and estimated for crude extracellular protein.

Exopolysaccharide (EPS) production

One milliliter of overnight culture of *V. harveyi* was grown in mineral salts medium (100 ml) containing (w/v) 0.2% of glucose, 12.6% of K_2HPO_4, 18.2% of KH_2PO_4, 10% of NH_4NO_3, 1% of $MgSO_4$ 7 H_2O, 0.6% of $MnSO_4$, 1% of sodium molybdate, 1% of Ca Cl_2 $2H_2O$, 0.06% of $FeSO_4$ $2H_2O$ and 1.5% of NaCl in 1 L of distilled water. The cells were grown till they got to 1.80 OD. AFGE and AUGE (10 ml of 100 mg/L) were added separately in shaker and incubated at 150 rpm for 48 h for 5 days. Five milliliters of this broth was centrifuged at 7000 rpm/10min. The cells were decanted and the filtrate was measured at 520 nm using spectrophotometer for EPS (Bramhachari and Dubey, 2006).

Adherence ability of *V. harveyi* against the garlic extracts (bacterial adhesion to hydrocarbons test– BATH)

One milliliter of overnight culture of *V. harveyi* (OD 1.80) was inoculated into 100 ml of LB broth. Then 10 ml of AFGE and AUGE were added into the LB broth and shaker incubated at 28°C/150 rpm for 5 days. Five ml of this broth was centrifuged at 7000 rpm/10 min. The cell pellet was suspended in saline phosphate buffer

(pH 7.2) and the suspension was adjusted to an absorbency of 0.16 at 600 nm with saline phosphate buffer. The suspension was overlaid with 5 different volumes of n-octane (Sigma) in 10 mm glass tubes (Santos et al., 1990). After 2 min of constant agitation, the mixtures were allowed to separate for 15 min. Finally, the absorbance for the aqueous phase was read at 600 nm. The percentage of partition in the hydrocarbon phase was calculated using the following formula:

$$\frac{A_{600}\text{ (Original bacterial suspension)} - A_{600}\text{ (Aqueous phase)}}{A_{600}\text{ (Original bacterial suspension)}} \times 100$$

Adherence ability of *V. harveyi* against the garlic extract (salt aggregation test- SAT)

One ml of overnight culture of *V. harveyi* (OD 1.80) was inoculated into 100 ml of LB broth. Then 10 ml of AFGE and AUGE was added into the LB broth and shaker incubated at 28°C/150 rpm for 5 days. 5 ml of this broth was centrifuged at 7000 rpm/10 min. The cell pellet was washed with 2.5% NaCl sterile saline solution and suspended in 0.002 M sodium phosphate buffer (pH 6.8). The optical density of each bacterial suspension was adjusted to 1.0 at 420 nm. An aliquot of 30 µl of this suspension (in duplicate) was mixed with an equal volume of each concentration of $(NH_4)_2SO_4$ from 0.05 to 4.0 M in 96-well micro-titer plates. The plates were kept at room temperature for 3 h. The SAT value was defined as the lowest molarity of ammonium sulfate that caused visible agglutination of a test organism. The cells are precipitated by increasing salt concentrations. The more hydrophobic the surface of the cells, the lower the salt concentration required to aggregate the cells. So, the most hydrophobic cells precipitate first, at low salt concentrations (Lee and Yii, 1996). Interpretations are as shown for the SAT test (0.0 to 1.0 M = strongly hydrophobic (S); 1.0 to 2.0 M = moderately hydrophobic (M); 2.0 to 4.0 M = weakly hydrophobic (W); and > 4.0 M = neither hydrophobic nor hydrophilic).

Effect of UV and heat treated AFGE and AUGE on *V. harveyi*

Five milliliters each of AFGE, AUFG extracts were treated at 100, 121°C and UV (@260 nm) for 10 min, respectively. The extracts thereafter were tested for antimicrobial activity against *V. harveyi* through “Well diffusion assay” as described earlier. The treated AFGE and AUGE extracts were (250 µl) added into the wells and incubated at 28°C/ 48 h. Sterile distilled water was used as control in other wells (Vaseeharan et al., 2010). The zone of inhibition was observed as antimicrobial activity against *V. harveyi*.

Effect of AFGE on the production of luciferase and luminescence by *V. harveyi*

AFGE (10 ml) was added into 90 ml LB broth. One ml of *V. harveyi* (1.80 OD) was inoculated into this broth and shaker and incubated at 28°C/150 rpm for 4 days. 5 ml of AFGE treated broth was centrifuged at 7000 rpm/10min. The cells were tested for luciferase production using a kit (LUC1 Sigma, USA), measured by Luminometer (Victor™ X3, Perkin Elmer, USA) and expressed as counts per second (CPS or photons per second). The supernatant was checked for bio-luminescence using kit and Luminometer. AUGE treated *V. harveyi* was not exercised for the estimation of luciferase and bio-luminescence.

Scanning electron microscopy (SEM) picture of *V. harveyi* after AFGE treatment

One ml of *V. harveyi* (1.8 OD) was serially diluted to 10^{-4}. A sterile micro centrifuge tube containing 0.5 ml of 10^{-4} dilution (5×10^3 cfu/ml) was incubated with 0.5 ml of AFGE at 28°C for 24 h in a shaker. The cells after incubation were centrifuged at 6000 rpm for 15 min and washed twice with 0.01M potassium phosphate buffer (pH 7.0). The pellets obtained after centrifugation were fixed with 2% (v/v) glutaraldehyde for 2 h at 4°C and dehydrated in a gradient ethanol (10 to 100%). Control specimens were prepared parallel with an exception that it did not contain AFGE. The slides containing the cells were dried in desiccator. Specimens were coated with gold and placed inside the scanning electron microscope (Leo 435 VP, UK) at 20 KV attached to Mitsubishi Video copy processor. The amplified image was obtained by a 35 mm Ricoh camera, connected to a monitor optically through fiber optics (McDougall et al., 1994).

Prophylatic treatment of AFGE against *V. harveyi* in the experimental conditions of *P. monodon* larviculture

Three glass tanks (50 L) were washed with 1% $KMnO_4$ solution. Tanks were filled with 20 l (20 PSU) low saline seawater. Before starting the experiment, healthy post larvae (PL 18 days) of *P. monodon*, procured from commercial shrimp hatchery were acclimatized in low saline seawater for 4 days at 29° C with aeration. The PL had an average weight of 0.1 to 0.2 g and 2000 numbers were stocked separately in each experimental tank (20 L). Two hundred ml of AFGE was inoculated into the treatment tank (Tank 1) with 5.0 ml of *V.harveyi* (OD 1.8). The tanks were provided aeration continuously not more than 4 mg/L. The PL were fed with powdered pellet feed and the experiment was conducted for 30 days without exchanging water. A control tank was kept in this way without adding AFGE (Tank 2). AFGE was inoculated alone in another tank with PL without *V. harveyi* (Tank 3) for estimating stress on PL, if any due to the garlic extract. The mortality of PL was observed every day by collecting dead larvae from all the tanks.

Cumulative percentage of mortality (CPM) was calculated. All the tanks were top closed during the experiment, to avoid contamination and they were opened only for sampling. Water quality parameters like salinity, temperature, pH were measured using salinometer, thermometer and pH meter respectively. The values are average of triplicate determinations. Total heterogeneous bacteria and total *V. harveyi* counts were determined in both treatment and control tanks in every five days interval (Defoirdt et al., 2006).

Growth evaluation of the total heterogenous bacteria (THB) during larviculture experimental conditions

Two milliliters of water samples were taken in sterile tubes from the treatment and control tanks and 10 fold serial dilutions were made. One ml of each dilution was taken in separate Petri plate. Zobel marine agar was added and mixed thoroughly. The plates were allowed to solidify and incubated at 37°C/24 h; and counts were determined.

Estimation of growth pattern of *V. harveyi* during larviculture

Two ml of water samples were taken in sterile tubes from the larviculture tanks. One ml of each was inoculated into 25 ml of alkaline peptone water and shaker incubated at 250 rpm and 28°C for 12 h for enrichment. After 10 fold dilution, one ml was inoculated into the Petri plates. *V. harveyi* selective agar medium was added for growth. The plates were allowed to solidify and were incubated at 28°C for 24 h. Growth of blue azure colonies was confirmed as the presence of *V. harveyi.*

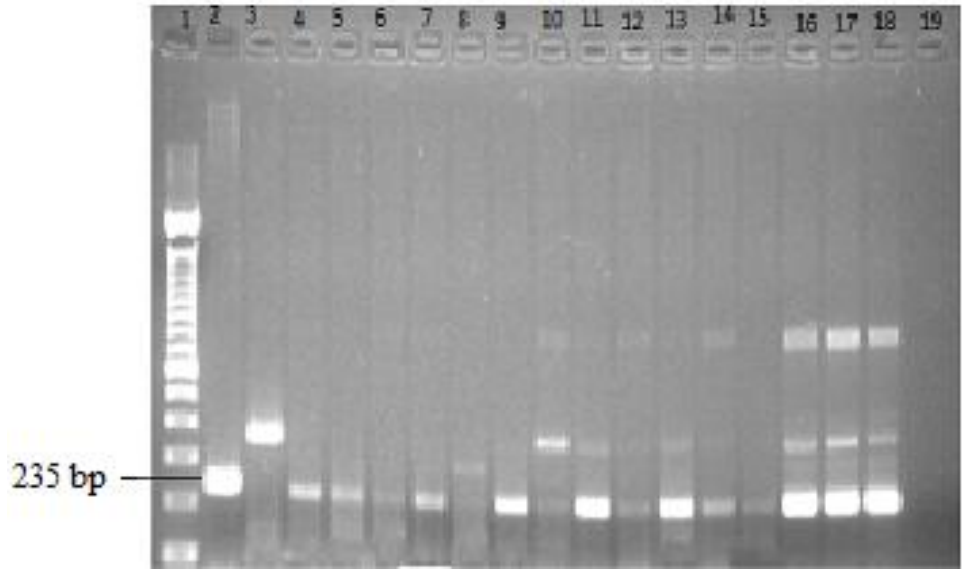

Figure 1. Detection of *vhh* gene from *V. harveyi* using PCR (1: 100 bp DNA Ladder, 2: *V. harveyi* MTCC control, 4 to 7 and 9 to 18: *V. harveyi* isolates).

Statistical analysis

The relative efficiency of AFGE with *V. harveyi* and AFGE alone was compared with control. The survival of PL was analyzed using Analysis of Variance (ANOVA) to ascertain the difference, if any, between the survivals of PL treated AFGE with *V. harveyi*, AFGE alone and control. All the experimental values were average of three replications expressed with SD. Cumulative percentage mortality (CPM) is calculated by dividing the cumulative frequency by the total number of observations (n), then multiplying it by 100 (the last value will always be equal to 100%). Thus, cumulative percentage = (cumulative frequency ÷ n) x 100.

RESULTS AND DISCUSSION

Out of 50 wild isolates of *Vibrio* species tested for bio-chemical reactions, 26 strains were found to be positive for *V. harveyi*. These strains again further confirmed positive for the presence of *V. harveyi* hemolysin gene (Figure 1). SWC and VHSA (2 to 5 mm light green with dark green centered colonies) were found to be the best selective medium for detection of *V. harveyi.* Both the extracts of AFGE and AUGE were tested for inhibition because unfiltered garlic extract would make less production cost with more yield than the filtered extract. AFGE (250 µl) showed higher zone of inhibition (12.33 ± 0.5 mm) against *V. harveyi*. But AUGE only showed less zone of inhibition (6.0 mm). The zone of inhibition of the garlic extracts may be due to the crude substances present (Table 1). This is in line with the findings of Vaseeharan et al. (2010). A similar study was reported by Vuddhakul et al. (2007) using fresh garlic extract against *V. parahaemolyticus* and reported zone of inhibition (11.6 ± 0.5 mm). Here, it was found that 60 µl of extract was found as the MIC for inhibiting 50 µl of *V. harveyi* at 1.80 OD (2.19 x 10^7 cfu/ml). For all the AFGE treatments, the level of *V. harveyi* used was 1.80 OD because the process of bio-luminescence starts at 1.80 OD and above. It was also reported that the MIC of garlic extract for *V. harveyi* was 0.156 mg/ml (Kasornchandra et al., 2005). The garlic extracts were prepared in water and not with any organic solvents during the extraction process, so the inhibitory zones formed may be due to the presence of active ingredients like allicin in the garlic extract. Grela and Klebaniuk (2007) have reported 11.12 g/ kg of alliin and 4.91 g/ kg of allicin can be obtained from garlic bulbs. Allicin was the chief active pharmaceutical molecule reported in crushed garlic; nevertheless it had a short half-life as it interacts with many surrounding proteins. Allicin is derived from an amino acid called alliin which is a stable precursor that will be converted to allicin by the action of an enzyme allinase presented in the garlic (Kasornchandra et al., 2005).

Effect of AFGE and AUGE on the growth of *V. harveyi*

Both types of garlic extracts were used to assess the inhibitory potential against *V. harveyi.* AFGE and AUGE treatments inhibited the growth of *V. harveyi* in LB medium under aerobic conditions. The growth of *V. harveyi* varied from 0.32 to 0.26 OD from 1st to 5th day under AFGE treatment. The growth reduction values are 0.54, 1.13, 1.37, 0.93, 0.91 ODs from day 1 to 5 as compared to control (1.17 OD) in the 5th day (Table 1). However, AUGE showed little variation in the OD values

Table 1. Effect of AFGE and AUGE against virulence factors and growth on *V. harveyi.*

Days	AFGE treated against *V.harveyi*	AUGE treated against *V. harveyi*	*V. harveyi* control
Thermo nuclease activity			
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	+
5	-	-	+
Proteolytic activity			
1	-	-	5.66 ± 0.21
2	-	-	7.11 ± 0.31
3	-	-	6.54 ± 0.30
4	-	-	6.66 ± 0.31
5	-	-	5.92 ± 0.21
Lipolysis activity			
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	+
5	-	-	+
Phospholipase activity			
1	-	-	+
2	-	-	+
3	-	-	+
4	-	-	+
5	-	-	+
Crude bacteriocin (OD@ 660 nm)	**OD values**	**OD values**	
1	1.352 ± 0.03	1.344 ± 0.06	1.601 ± 0.07
2	1.447 ± 0.03	1.461 ± 0.06	1.694 ± 0.06
3	1.502 ± 0.04	1.502 ± 0.03	1.694 ± 0.06
4	1.505 ± 0.04	1.515 ± 0.05	1.637 ± 0.05
5	1.447 ± 0.03	1.458 ± 0.06	1.577 ± 0.05
Cell wall bound protein (OD @ 660 nm)			
1	0.620 ± 0.02	0.710 ± 0.03	0.681 ± 0.02
2	0.687 ± 0.03	0.693 ± 0.03	1.327 ± 0.02
3	0.702 ± 0.03	0.700 ± 0.03	1.402 ± 0.02
4	0.724 ± 0.03	0.720 ± 0.04	1.344 ± 0.03
5	0.712 ± 0.03	0.710 ± 0.03	1.161 ± 0.03
Protease (OD @ 440 nm)			
1	0.004 ± 0.00	0.003 ± 0.00	0.224 ± 0.01
2	0.047 ± 0.00	0.037 ± 0.00	0.364 ± 0.01
3	0.039 ± 0.00	0.025 ± 0.00	0.189 ± 0.00
4	0.021 ± 0.00	0.019 ± 0.00	0.141 ± 0.00
5	0.018 ± 0.00	0.009 ± 0.00	0.138 ± 0.00
Exopolysaccharide (OD @ 520 nm)			
0	0.391 ± 0.01	0.414 ± 0.01	0.656 ± 0.00

Table 1. Continued.

1	0.789 ± 0.03	0.727 ± 0.03	1.405 ± 0.05
2	1.016 ± 0.04	1.195 ± 0.03	1.911 ± 0.06
3	1.482 ± 0.06	1.443 ± 0.05	2.163 ± 0.09
4	1.408 ± 0.05	1.450 ± 0.05	2.050 ± 0.09
5	1.408 ± 0.05	1.451 ± 0.05	2. 069 ± 0.09
Growth (OD @ 600 nm)			
0	0.326 ± 0.01	0.331 ± 0.01	0.341 ± 0.00
1	0.648 ± 0.03	0.578 ± 0.03	1.192 ± 0.03
2	0.843 ± 0.03	0.994 ± 0.03	1.981 ± 0.03
3	0.970 ± 0.01	0.880 ± 0.04	2. 346 ± 0.03
4	0.260 ± 0.01	0.225 ± 0.05	1.199 ± 0.05
5	0.260 ± 0.01	0.231 ± 0.05	1.173 ± 0.06

Values are means ± standard deviations of three independent determinations., + : positive and – negative.

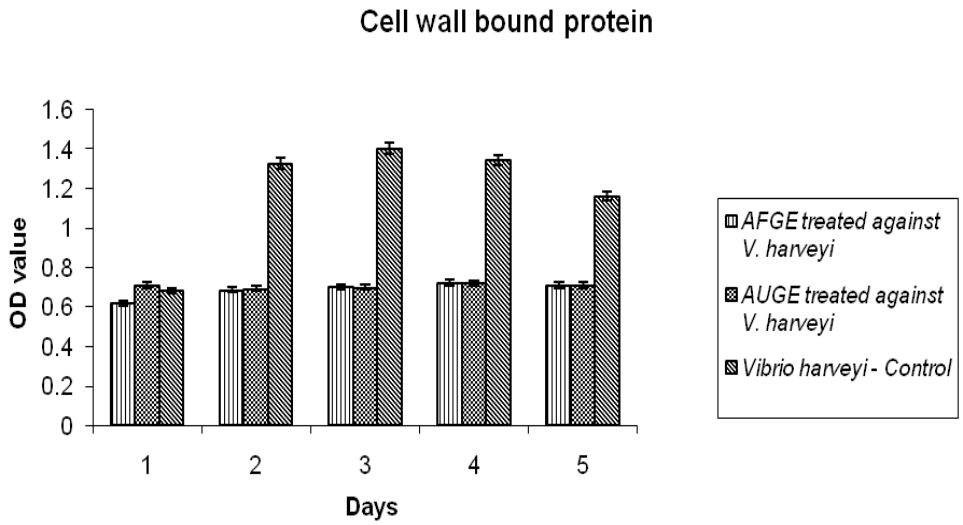

Figure 2. The cell wall bound protein of *V. harveyi* against AFGE and AUGE treatments.

as compared to AFGE. Vaseeharan et al. (2010) observed the total growth inhibition of *V. harveyi* using 12 µl freshly squeezed garlic extract in LB broth.

Effect of AFGE and AUGE on the virulence factors of *V. harveyi*

Proteolysis, lipolysis, phospholipase and thermo nuclease levels of *V. harveyi* were not observed when treated with AFGE and AUGE. But, in the control, a zone of 6.2 mm was observed for proteolytic activity in the "Agar spot assay" for all the 5 days. Crude bacteriocin values were changed from 0.10 to 0.24 OD as compared to control (1.60 OD) for the 5 days. On an average, 0.19 OD reductions in terms of crude bacteriocin were observed for the 5 days against AFGE. AUGE treatment showed little variation than AFGE; not much dissimilarity was noticed. Bodini et al. (2009) reported that garlic extract interferes with the quorum sensing receptors on marine *Vibrios*. There was much reduction (0.44 to 0.64 OD) in the cell wall bound protein of *V. harveyi* when treated with AFGE as compared to the control (1.16 to 1.32 OD). On an average, 0.68 OD variations were observed in terms of cell wall bound protein (Figure 2). AUGE treatment showed little variation than AFGE (Table 1). AFGE treatment reduced the protease enzyme and the differences are 0.22, 0.31, 0.15, 0.12, 0.12 ODs respectively from the 1st to 5th day compared to the control (Figure 3). AUGE treatment as well showed little variation than AFGE. Not many reports are available to support the effect of herbal extract on virulence factors. The

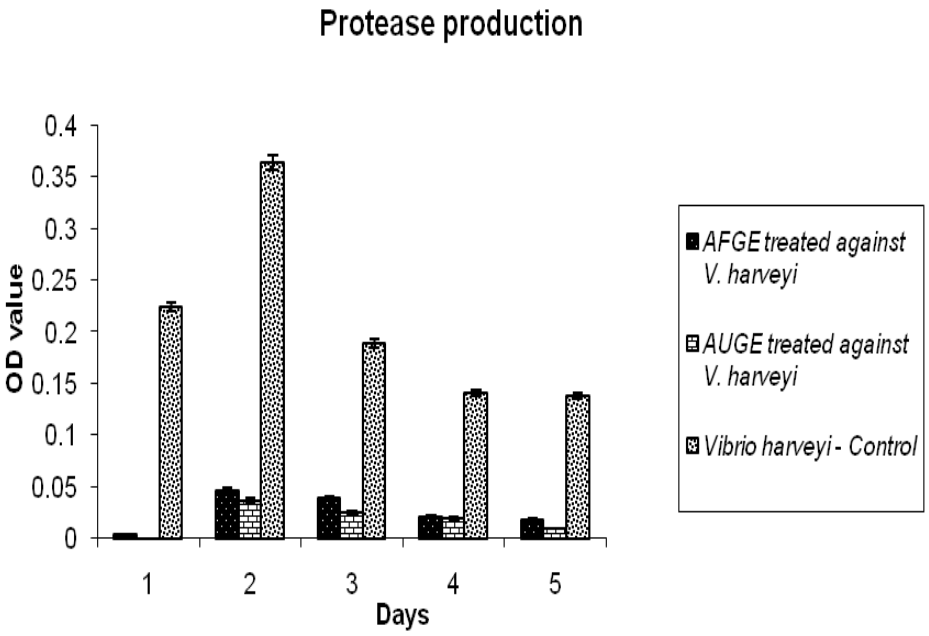

Figure 3. Protease production of *V.harveyi* against AFGE and AUGE treatments.

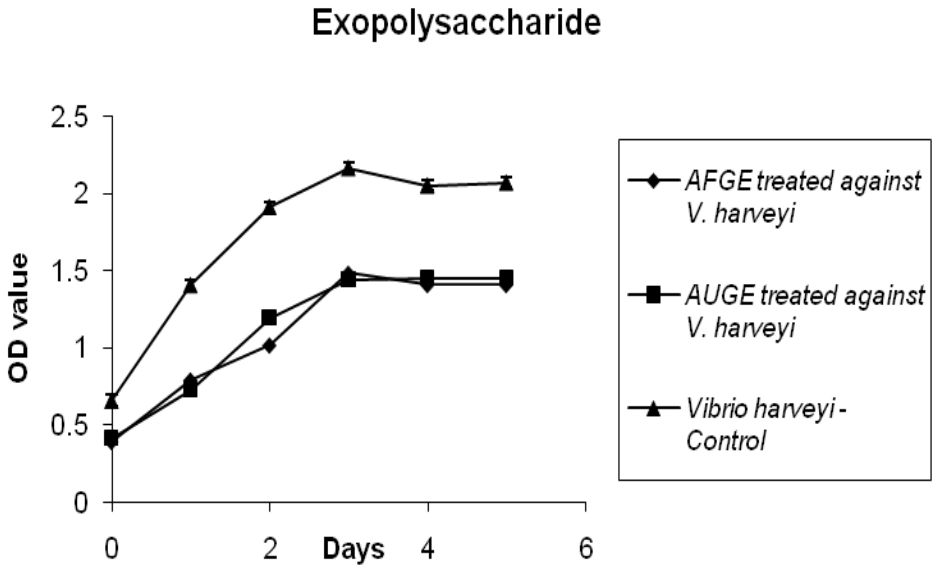

Figure 4. Exopolysaccharide level of *V. harveyi* against AFGE and AUGE treatments.

production of EPS values reduced when treated with AFGE from the first two days and subsequently maintained the same level. The reduction values ranged from 0.60 - 0.80 OD in both treatments in the first two days compared to control (Figure 4). In the control, EPS values increased to 2.10 OD as average from the 3 rd to 5th day. But in both treatments, the OD was 1.44 during 3rd to 5th day (Table 1). Methanolic extract of *Cuminum cyminum* at 2 mg/ml could control the functions of bio film formation such as flagellar motility and EPS production (Packiavathy et al., 2012).

Changes of cellular bio-luminescence and luciferase against AFGE

The production of luciferase enzyme is intracellular whereas luminescence is extracellular. AFGE treatment

Table 2. Intra cellular luciferase and extra cellular bio-luminescence produced by *V. harveyi* against AFGE treatment.

Treatments	Luminescence (counts per second)			
	1st day	2nd day	3rd day	4th day
AFGE @ 100 mg/ml in LB broth	86 ± 0.01	98 ± 0.05	79 ± 0.01	94 ±0.01
V. harveyi without AFGE extract- (control)	107± 0.02	104 ± 0.01	91 ± 0.02	116 ± 0.03
	Luciferase (counts per second)			
AFGE @ 100 mg/ml in LB broth	30 ± 0.30	29 ± 0.02	35 ± 0.01	30 ± 0.01
V. harveyi without AFGE extract-(control)	44 ± 0.20	36 ± 0.10	44 ± 0.02	36 ± 0.02

Values are means ± standard deviations of three independent determinations (n =3).

Table 3. The SAT and BATH results of *V. harveyi* treated with AFGE @ 100 mg/ ml for 5 days.

Days	SAT (in molarity)	BATH (in % partition)	Control values for SAT	Control values for BATH
1	1.23 ± 0.05	48.42 ± 1.21	0.10 ± 0.004	95.34 ± 2.97
2	1.40 ± 0.06	45.15 ± 2.01	0.10 ± 0.004	94.37 ± 2.00
3	1.53 ± 0.06	40.51 ± 1.26	0.10 ± 0.004	93.48 ± 2.77
4	1.70 ± 0.07	36.49 ± 1.70	0.13 ± 0.005	93.14 ± 2.17
5	1.83 ± 0.08	29.98 ± 1.30	0.13 ± 0.005	93.11 ± 2.03

Values are means ± standard deviations of three independent determinations. (n=3), Control SAT and BATH values are indicated that *V.harveyi* cells are more hydrophobic with out garlic extract treatment. But treated *V.harveyi* cells shows the values of moderately hydrophobic.

reduced the luminescence values such as 86, 98, 79, 94 CPS from day 1 - 4. Garlic extract proved to have antagonistic compounds against quorum sensing AHL compounds (Rasmussen et al., 2005). The different luminescence values in CPS are 21,6,12, 8 compared to control (107,104, 91,102 CPS). AFGE treatment also reduced luciferase level in 30, 29, 35, 30 CPS respectively. The differences of luciferase values are 14, 07, 09, 06 as compared to control (44, 36, 44, 36). Hence, it was evident from this study that AFGE would be effective agent for controlling both the cellular processes produced by *V. harveyi* (Table 2).

Effect of AFGE on the hydrophobicity of *V.harveyi* (SAT and BATH)

SAT and BATH values showed that *V. harveyi* cells are more hydrophobic in nature. But *V. harveyi* cells treated with AFGE showed the values between 1.20 and 1.83 (Table 3). As per the observation, AFGE treatment made the cells as moderately hydrophobic (0.0 to 1.0 M = strongly hydrophobic (S), 1.0 to 2.0 M = moderately hydrophobic (M), 2.0 to 4.0 M = weakly hydrophobic (W), and > 4.0 M = neither hydrophobic nor hydrophilic), possibly more quantity of garlic extract would have changed to weakly hydrophobic. In BATH test, in almost all the five days, AFGE treatment showed values that ranged from 29 to 48% partitioning on *V. harveyi* cells as compared to control (93 to 95%). Therefore, the cells have become moderately hydrophobic (50% partitioning = strongly hydrophobic; 20 to 50% partitioning = moderately hydrophobic and < 20% partitioning = neither hydrophobic nor hydrophilic), since the control values showed more than 50% partitioning and the cells are considered as strongly hydrophobic before the garlic extract treatment.

Effect of UV and heat treated AFGE and AUGE on *V. harveyi*

At 100 to 121°C treatment, the AFGE extract lost its inhibition against *V. harveyi*, but UV treatment did not (Table 4), (Figures 5 and 6). Garlic heated to 121°C was found to strongly inhibit the growth of yeasts, but not that of bacteria (Kim and Kyung, 2003). But garlic extract stored at room temperature showed inhibition against *Escherichia coli, Salmonella typhi* and *Bacillus subtilis* (Durairaj et al., 2009). Similar result was obtained with AUGE. Without heat and UV treatments, both the extracts showed zone of inhibition of 12 and 6 mm respectively. SEM picture showed that, *V. harveyi* treated with AFGE lost its structure (Figures 7 and 8).

Prophylatic treatment of AFGE against *V. harveyi* during *P. monodon* larviculture

The challenge experiment confirmed that PL treated with AFGE had low mortality. But in the case of control tanks,

Table 4. Effect of UV and heat treated AFGE and AUGE against *V. harveyi.*

Treatment	AFGE (µl)	Zone of inhibition	AUGE (µl)	Zone of inhibition
UV	280	9.0 ± 0.50	280	5.10 ± 0.50
100°C	280	Nil	280	Nil
121.1°C	280	Nil	280	Nil
Untreated *V. harveyi*	250	12.33 ± 0.50	280	6.10 ± 0.50

NB: Values are means ± standard deviations of three independent determinations. (n=3).

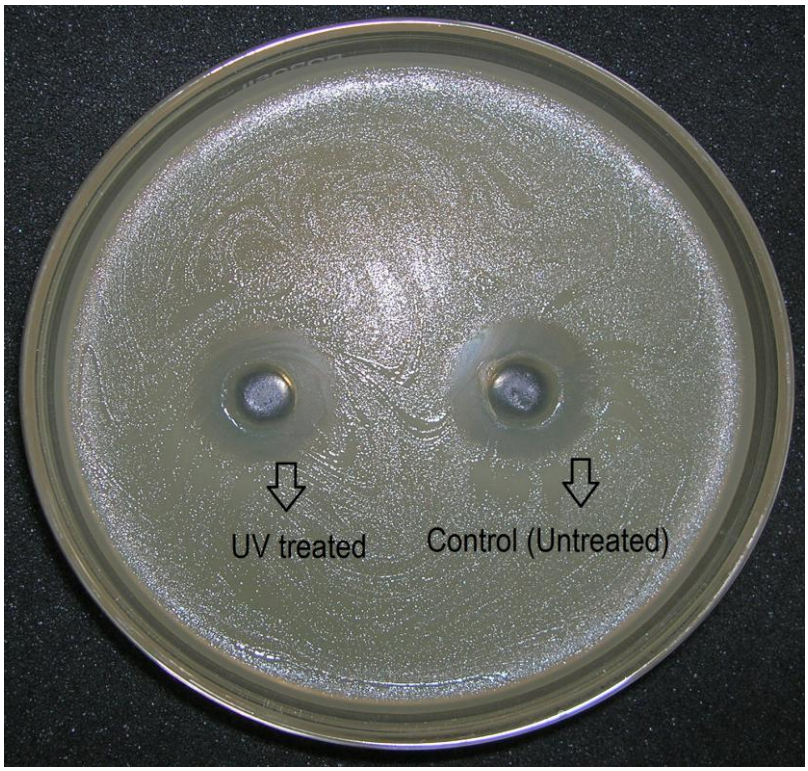

Figure 5. Zone of inhibition produced by AFGE against *V. harveyi* after UV treatment.

initially there was two-fold mortality on the 5th day followed by 3 folds on 10th, 15th and 20th days of experiment. In the control, the mortality ranged from 2 to 42 % in the 30 days of challenge. But in the AFGE treatment, the mortality ranged from 0.5 to 5.5 % for the 30 days. Almost 35% reduction in the mortality among PL was found by the AFGE treatment during the challenge experiment (Table 5). But Vaseeharan et al. (2010) showed 75% reduction in the cumulative mortality caused by *V. harveyi* among the *Fenneropenaeus indicus* juveniles (11 g) by treating with 1% garlic extract. Antibacterial activity of six medicinal herb oils used in cooking was reported to inhibit *V. parahaemolyticus* and *V. harveyi* isolated from infected fish. The highest level of antibacterial activity against *V. harveyi* was shown by the essential oil of *Satureja achtiarica* (15 µg/ml) (Pirbalouti et al., 2011). The rate of growth inhibition on *V. harveyi* was based on the concentration of the herbal extract treatment with increased time duration (Penduka et al., 2011). Effects of dietary garlic extracts on growth, feed utilization and fatty acid profile and blood plasma changes were studied on starlet of sturgeon fish (*Acipenser ruthenus*) by Lee and Kim (2010). AFGE alone without *V. harveyi* showed no abnormal symptoms or mortality among PL. The water quality values were at normal range. The growth of *V. harveyi* was 1.98 ± 0.02 OD during the first day of both the treatment and control tanks. Then the OD was reduced to 1.0 ± 0.01 in the treatment tank during the 3rd day and remained 1.0 ± 0.01 OD throughout the period. Whereas in the control tank,

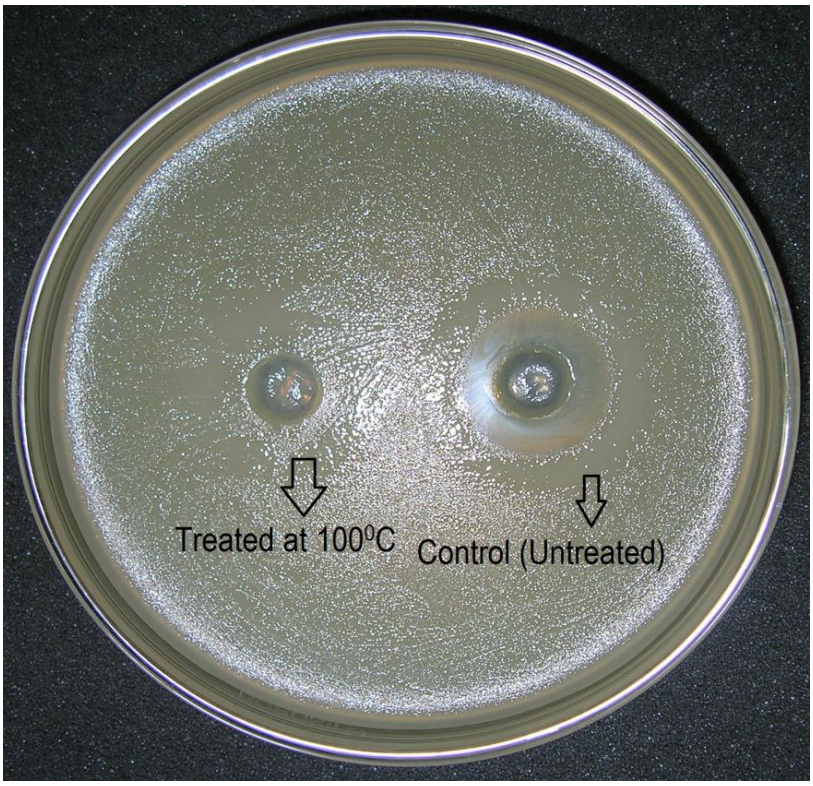

Figure 6. Heat treated AFGE at 100°C does not show inhibition on *V. harveyi* but showed inhibition without heat treatment.

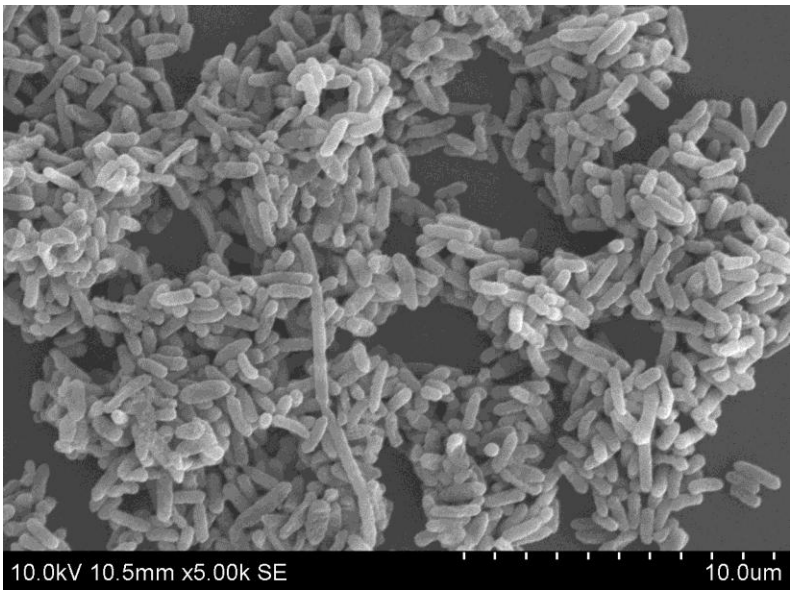

Figure 7. SEM picture for *V. harveyi* as control.

OD for *V. harveyi* was 2.0 on the second day and remained the same. Relative performance of AFGE was done against survival of post larvae. Statistically there was no significant difference ($p > 0.01$) observed between the mean values of AFGE with *V. harveyi* treatment and AFGE alone. However, the treatments of

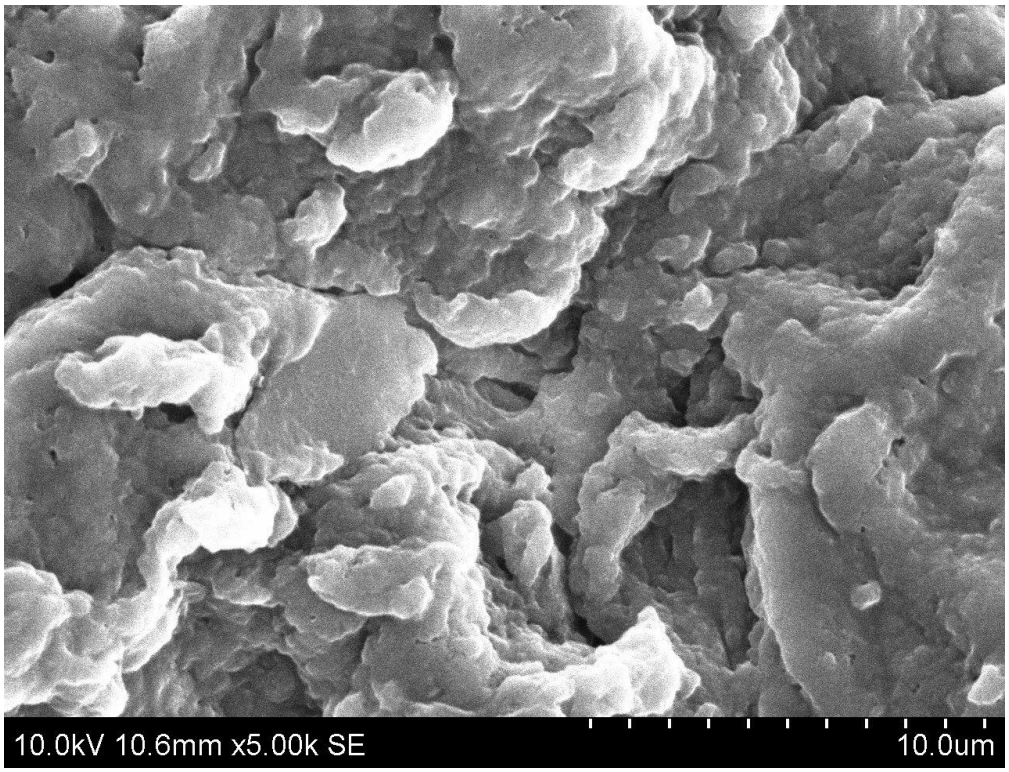

Figure 8. SEM picture for AFGE treated *V. harveyi.*

AFGE with *V. harveyi* and AFGE alone was highly significant ($p < 0.01$) when compared with the control. Coefficient of variation = 94.286, treatments were significant at 1% and 5% level. Critical difference (CD) (0.01) = 72.951; CD (0.05) = 52.033.

Kasornchandra, et al. (2005) reported that garlic has bactericidal property against the *Vibrio* spp with the mechanistic principle to control the protozoa; gregarines infection in grow-out shrimps. They also proved that garlic could stimulate the shrimp hemocytes as determined by the cellular immune responses such as phagocytic activity, superoxide anion production and phenoloxidase activity.

The total heterotrophic bacterial counts, from the first day were increased from 2.0 ± 0.02 OD to 4.0 ± 0.01 OD and always fluctuating. Fermented garlic liquid was used as one of the feed ingredients with phytoncide oil and oregano oil for the white leg shrimp *Litopenaeus vannamei* (Kim et al., 2011). Herbal diets prepared from five different herbs effectively suppressed the pathogens such as *Aeromonas hydrophila, V. harveyi* and *V. parahaemolyticus* in the *P. monodon* immune system (Velmurugan and Citarasu, 2010). Indian herbs were used to replace antibiotics applied on PL of *P. monodon* (Velmurugan et al., 2010). Garlic was used to control bacterial and fungal diseases of fish in China. They administered one kg of garlic with 5 kg feed, 1 kg of sodium chloride and 1 kg of binder (Bai, 1994). In Thailand, few shrimp farmers use garlic in their farm to clean up the intestinal tract of shrimp. They believe that garlic facilitate shrimps have better feeding after including the garlic paste in shrimp feed. Nevertheless, the scientific reason for this effect has not been postulated yet.

Conclusion

It was evident from this study that garlic had antagonistic activity against marine *V. harveyi* and its virulence factors. Based on the other literatures cited here, garlic was also controlling marine *Vibrios* and pathogenic non-vibrios. Further, garlic extract has added advantage as growth enhancer, maturation agent for shrimps and as immuno-modulator (since it activates the function of shrimp hemocytes). It also controls protozoa, gregarines infection in cultured shrimps. Garlic extract was used as one of the ingredients with chitosan, herbs, marine probiotic bacteria and being used as a product of organic agent (AV14) against controlling 15 pathogenic marine *Vibrios* during aquaculture. Therefore, garlic extract may be a suitable plant based bio therapeutic agent and as alternative in place of chemical preservatives used in the aquaculture system. But garlic extract might antagonize little with the native bacteria present in the aquaculture system but not completely. The cost of this herbal agent is less compared with various chemical agents used in aquaculture.

Table 5. The cumulative percentage mortality (CPM) pattern of shrimp post - larvae against *V. harveyi* with abiotic factors during AFGE treatment (mean ± SD).

Days of larviculture	AFGE + *V. harveyi* (CPM)	*V. harveyi* as control (%)	AFGE alone (%)	Temperature (°C)	pH	Salinity (PSU)	THB (CFU/ml)	Growth of *V. harveyi* (CFU/ml)
1st	Nil	Nil	Nil	28 ± 1.0	8.0 ± 0.1	20 ± 0.2	1.9×10^6	2.1×10^7
5th	10 (0.5% mortality)	40 (2% mortality)	5 (0.25 % mortality)	29 ± 1.0	8.0 ± 0.1	20 ± 0.1	2.7×10^7	2.0×10^6
10th	15 (0.75% + 0.5 = 1.25)	100 (5 +2 = 7%)	5 (0.25 + 0.25 = 0.50)	26 ± 0.6	7.9 ± 0.2	20 ± 0.2	2.1×10^6	1.4×10^6
15th	20 (1 + 0.75 = 2.25)	200 (10 + 7 = 17%)	8 (0.40 + 0.50 = 0.90)	27 ± 0.3	8.1 ± 0.2	20 ± 0.1	1.7×10^5	1.0×10^4
20th	25 (1.25 + 2.25 = 3.50)	200 (10 +17 = 27%)	10 (0.50 + 0.90 = 1.40)	27 ± 1.0	8.1 ± 0.1	20 ± 0.2	1.9×10^5	1.0×10^3
25th	20 (1+ 3.50 = 4.50)	200 (10 + 27 = 37%)	9 (0.45 + 1.40= 1.85)	26 ± 0.4	8.0 ± 0.1	20 ± 0.2	1.6×10^4	1.0×10^3
30th	20 (1+ 4.50 = 5.50)	100 (5 + 37 = 42 %)	8 (0.40 + 1.85 = 2.25)	27 ± 0.7	8.1 ± 0.1	20 ± 0.1	1.4×10^4	$1 .0 \times 10^3$

Values are means ± standard deviations of three independent determinations (n = 3), mortality showed here as total dead numbers of PL counted every day, the values represented as once in five days.

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