African Journal of Biotechnology Vol. 11(79), pp. 14498-14508, 2 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.651
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Detection of quorum sensing molecules from *Vibrio harveyi* and use of synthetic furanone to control *V. harveyi* during shrimp larviculture

Kannappan Sudalayandi[1,2]*, Anil Kumar[2], Uzo Ihemere[2], Vanessa Falcoa[2], Narayanan Narayanan[2], Elisa guerroa[2] and Richard Sayre[2]

[1]College of Biological Sciences, Stanley J Aronoffs Laboratory, Plant Cellular and Molecular Biology Division, Ohio State University, Ohio State, Columbus, USA.
[2]Nutrition, Genetics and Biotechnology Division, Central Institute of Brackishwater Aquaculture, (Indian Council of Agricultural Research) 75 Santhome high road, Raja Annamalai puram, Chennai - 600 028, Tamilnadu, India.

Accepted 30 July, 2012

This paper explores the extraction and detection processes of quorum sensing molecules such as N-aceyl homoserine lactone compounds (AHL) from marine *Vibrio harveyi*. The spent culture of *V. harveyi* was solvent partitioned for AHL, rotary evaporated and re-suspended in 50% acetonitrile then detected with reporter strain using Luminometer. Furanone was tested for antagonism against *V. harveyi* and an inhibitory zone of 9.0 ± 0.25 mm was observed. When, *V. harveyi* grew in Lysogeny broth (LB) with furanone, the growth was decreased from 0.2 to 0.03 optical density (OD) in 16 h. In control, *V. harveyi* growth was increased from 1.33 to 2.3 OD. When furanone was challenged against *V. harveyi* among shrimp post larvae, the cumulative percentage mortality was increased in the control from 0.85 to and 80% for the period of 5th to 30th days. But in the treatment tank the mortality varied from 0.53 to 1.26, 5.51, 8.17, 10.72 and 13.37% till the 30th day. The differences in the cumulative percentage mortalities in the treatment tank were 0.32, 2.34, 28.63, 51.50, 59.20 and 66.71% respectively as compared to the control. The results suggest that furanone can be used as non-antibiotic agent to control shrimp disease caused by *V. harveyi*.

Key words: *Vibrio harveyi*, detection of N-aceyl homoserine lactone compounds (AHL), furanone, antagonism, shrimp larviculture.

INTRODUCTION

Under the coastal aquaculture, shrimp farming is one of the most important profit earning systems. The change in the traditional shrimp grow-out practices to semi-intensive system brought more earnings in higher stocking densities. Due to such intensification, the aquatic bacterial pathogens have developed into very grave distress in shrimp grow-out systems. Halophilic vibrios such as *Vibrio harveyi* are omnipresent in the marine environment and are considered as the cause of a number of diseases in wild and grow-out aquaculture system (Haldar et al., 2011). Vibriosis is one of the foremost diseases in shrimp aquaculture (Karunasagar et al., 1994). *V. harveyi* isolates (10^6 cfu/ml) obtained from diverse geographical locations were pathogenic to rainbow trout and *Salmo salar* fish with mortalities of up to 100% (Zhang and Austin, 2000). Four *Vibrio* species including *Vibrio alginolyticus*, *Vibrio vulnificus*, *V. harveyi* and *Vibrio mimicus* were identified from the hemolymph of lobsters (Raissy et al., 2011). *V. harveyi* is the causative agent of luminous disease on post larvae of shrimps (80 to 100% mortality) larviculture. As control measures, the farmers

*Corresponding author. E-mail: sudalikanna@gmail.com. Tel: + 91- 44 - 24616948, 91- 09677039103. Fax: 91- 44 – 24610311.

used antibiotics in a disorganized way therefore vibrios are becoming more resistant against antibiotics used in aquaculture. Mass mortality was reported among the *Penaeus monodon* post larvae owing to multiple antibiotic resistant of *V. harveyi* (Karunasagar et al., 1994). Therefore, the quest for alternative methods to control infection caused by antibiotic-resistant Vibrios are imperative challenge for the sustainable development of aquaculture. One of the alternative strategies to control infections caused by antibiotic-resistant bacteria is the disruption of cell - to- cell signaling compounds between bacteria called quorum sensing (Defoirdt et al., 2007). Quorum sensing (QS) is a signaling mechanism through which bacteria modulate a number of cellular functions such as sporulation, bio-film formation, horizontal deoxyribonucleic acid (DNA) transfer, extracellular polysaccharides (EPS), enzymes, surfactants, antibiotics, virulence responses and luminescence, etc. Lumine-scence is one of the indications of quorum sensing among the marine vibrios for their presence in higher level (10^{10} to 1011 cfu/ml) and causes mortality among shrimps or their larvae or fish. *Vibrio* reveals quorum sensing by producing auto inducers and acquires a threshold concentration. This is a direct indication of population density, able to set off the genes, bringing into the effect of concentrated phenotypes (Charu and Srivastava, 2006). Luminescence process was attributed to the transcriptional regulation of the enzyme called luciferase, which in turn match up to a threshold density of cells. This whole circuit is based on the estimation of bacterial density by means of releasing auto-inducers. They establish a communication between the cells that gets reflected (Whitehead et al., 2001) in the expression of a particular gene for example, luciferase gene (lux) and fatty acid derivatives called N-Acyl homoserine lactones (AHL). Hence, in place of antibiotics, alternative bio-inhibitors are required to control antibiotic resistant marine vibrios. Arivuselvan et al. (2011) used bark and leaves of Indian mangroves as antagonistic agent against various marine pathogenic bacteria. Shobharani and Renu (2010) used synthetic furanone to control quorum sensing causing vibrios in milk. Since *V. harveyi* was found to cause heavy mortality in shrimp larviculture and grow-out systems, it is necessary to develop control measures. Consequently, quorum sensing causing AHLs were extracted and purified from *V. harveyi* and the inhibitory pattern and virulence production of *V. harveyi* was studied against synthetic furanone.

MATERIALS AND METHODS

Bacterial strains, media, chemicals and culture conditions

Wild *V. harveyi* strains were isolated from marine shrimp and identified using various bio-chemical tests (Abraham and Palaniappan, 2004) and compared with standard type strain *V. harveyi* ATCC 25919. *Chromobacterium violaceum* (CV026) and *V. harveyi* BB170 were procured from the Division of Plant Cellular and Molecular Biology, The Ohio State University, Ohio State, Columbus. N-acyl homoserine lactones were procured from Sigma (USA). For regular maintenance of *V. harveyi* and *C. violaceum*, Luria Bertani (LB) agar medium was used. LB agar, Skim milk agar, azocasein and Ethyl acetate were procured from the Oxoid Company (USA). AHL compounds were purified using high-performance liquid chromatography (HPLC) (Shimadzu- Japan), 4-Hydroxy- 2-5-Dimethyl - 3 (2H) - Furanone obtained from Sigma Aldrich Chemical, Co., Inc (W317403). 1 mg of furanone was dissolved into 1.0 ml of 80% ethanol and (Ren and Wood, 2004) used for the antagonism against *V. harveyi*. Wild *V. harveyi* was used for all the experiments after comparing its properties with standard *V. harveyi* ATCC strain.

Detection of quorum sensing AI-2 molecules using Luminoscan - TL plus

The assay is based on the ability of *V. harveyi* BB170 to specifically bioluminate in response to AI-2. At lower cell-densities of BB170 (10^6 to 10^7CFU/ml), the bioluminescence can be detected in response to the added AI-2. Auto induction (AI) medium (Turovskiy et al., 2006) was used for the growth of *V. harveyi* (NaCl 17.5 g/L, $MgS0_4$ 12.3 g/L, Casamino acids 2.0 g/L; these solutions were sterilized adjusting the pH to 7.5 using 3N NaOH. When cooled, filter sterilized 1 M KH_2PO_4, 20 ml/L of 50% glycerol, and 10 ml /L of 0.1 M L- arginine were added. *V. harveyi* cells (OD 2.0) were inoculated on AI medium and incubated at 30°C/12 h. AI broth was used for the growth of *V. harveyi* in 16 h duration and then diluted to 5000 times in fresh AI medium to obtain 10^5 CFU/ml. 1 ml of the cell-free supernatant to be tested for the presence of Auto-induction-assay (AI) was added with 9 ml of 5000 time's diluted cells. This was thoroughly mixed and incubated at 28°C in shaker incubator (140 rpm). Measurement of bioluminescence was observed in every 30 min using Luminoscan TL plus (Thermo Lab systems) and observed after 5.5 h of incubation normalized with *V. harveyi* BB170 (Bassler et al., 1994) as positive control and then expressed as relative bio-luminescence per CFU/ml.

Detection of AI-1 molecules (AHL) production from *V. harveyi* using reporter strain

100 µl of viable auto inducer bacteria (100 µl), *Chromobacterium violaceum* (CV026) was streaked as reporter strain along the center of LB agar plate. The test bacteria, *V. harveyi* (10^9 cfu/ml) intended to check for AHL production was cross streaked (50 µl) to the left and right side of the CVO26 streak (Figure 2). Plates were incubated at 28°C/24 h. Positive assay was judged as the induction of violacein as purple pigment produced by the reporter strain (Morohoshi et al., 2005).

Extraction and detection of AI-1 molecules (AHL) from *Vibrio harveyi* using Luminometer

Wild *V. harveyi*, (5 ml, OD 2.0) was inoculated into 500 ml of LB broth and incubated in shaker incubator at 28°C/48 h. The cells (OD 2.0) were decanted through centrifugation (4213 *g*, 10°C). The supernatant was passed through 0.2 µm filter and treated with equal volume of ethyl acetate (1:1). The extract was then vacuum evaporated at 30°C to a minimal volume and re-suspended in 50% (v/v) acetonitrile: water. The crude extract (1.67 ml) was

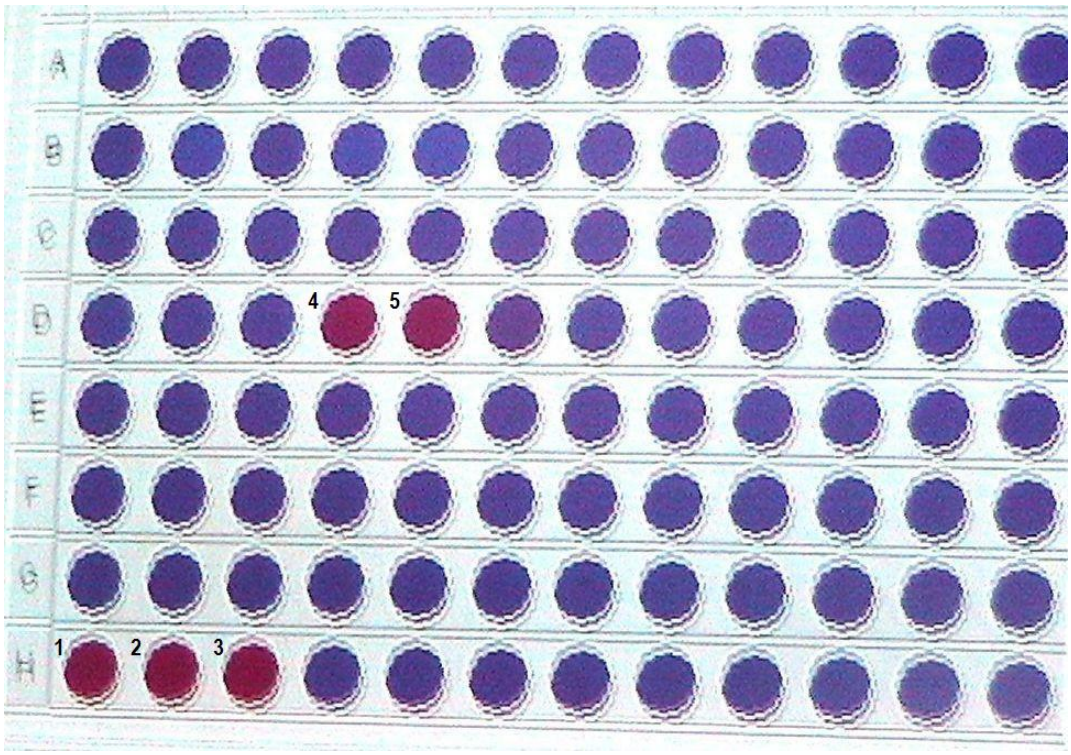

Figure 1. The luminometer readings for AHL compounds extracted from *V. harveyi* (D row 4 and 5 two pink spots under Lux mode). Controls : H row 1: N-butanoyl –L- homoserine lactone 500 ng/ml = 10^{-3}dilution; H row 2, N- Octanoyl – L- homoserine lactone 5ng/ml =10^{-5} dilution; H row 3, N- dodecanoyl – L - homoserine lactone 50 ng/ml = 10^{-4}dilution.

fractionated on a reverse-phase C18 (RP- C18) analytical HPLC column (5 Km, 250 - 4 LiChrospher 100) mounted with C18 guard column. The fractionated extract was eluted for 5 min with water, then 40 min with 41% (v/v) acetonitrile, followed by third elution with 100 % (v/v) acetonitrile. AHL lactonase enzyme activity of the sample was assayed by adding 100 µl of sample in the micro titre plate with reporter strain, *Burhkolderia cepacia* CepR (protein function as AHL receptor, NCBI Accession number: CAD 19536 - Cognate signal C8-HSL) with green flourescence protein (GFP). Positive control of AHL 100 µl (500 ng/ml) was also taken in the micro-titre plate separately, then read (Figure 1) by Luminometer (Teplitski et al., 2004). The presence of red color spots in the centre of the plate showed AHL from the samples. The positive control samples showed three red color spots in the first row of the plates where standard N-acyl homoserine lactone spotted in the same concentration.

Protocol description for Luminometer

Protocol Name: MM-Red Shift P (488/510), Protocol number: N/A, Name of the plate type: Generic 8x12 sizes, Delay of repeats: 1, Delay between repeats: 600 s, Measurement height: Default. Shaking duration: 5.0 s, Shaking speed: fast, Shaking diameter : 0.10 mm, Shaking type: orbital, Repeated operation: yes (thrice), Name of the label : Red-shift GFP (1 s), Label technology : Prompt Flurometry, CW-lamp filter name: F485, CW-lamp filter slot: A5, Emission filter name: F510, Emission filter slot: A3, Measurement time: 0.1 s, Emission aperture: Normal, CW- lamp energy: 9000, Second measurement CW-lamp energy: 0, Emission side: Above, CW-Lamp control: Stabilized energy, Excitation aperture: N/A.

Disruption of virulent protease from *Vibrio harveyi* against furanone treatment

V. harveyi; 50 µl (OD 2.0) was treated with furanone (1 mg/ml, dissolved in 80% ethanol) spotted on the surface of Skim milk agar (25 ml of nutrient agar with 5% skimmed milk powder). The plates were allowed to remain at 37°C/2 h and then incubated at 28°C/12 h. The formation of clear zone around the bacterial spot was the indication for protease production. For quantification of protease from *V. harveyi*, the fermentation culture medium was prepared (w/v) by adding 0.75% glucose, 0.75% peptone, 0.5% magnesium sulphate, 0.5% potassium dihydrogen phosphate, and 0.01% Ferrus sulphate. 1 ml of *V. harveyi* (OD 2.0) was inoculated to the fermentation broth (150 ml) with furanone of 1 mg/ml and incubated at 28°C for 72 h in shaker incubator at 140 rpm. Cells were decanted from the fermentation broth and supernatant was used as crude protease. The activity of crude protease was analyzed with azocasein as substrate (Folasade and Olajuyigbe, 2005). The assessment of protease enzyme activity was done using 500 µl of 0.5% azocasein in Tris HCl buffer with 100 µl enzyme solution and incubated for 60 min at 37°C. The reaction was stopped by the addition of 500 µl of 15% TCA. This solution was incubated at 30°C/15 min and then centrifuged at 4°C/15 min at 3000 rpm. 1 ml of supernatant was added to 1.0 ml of NaOH and the absorbance was read at 440 nm (One unit of protease activity is defined to be

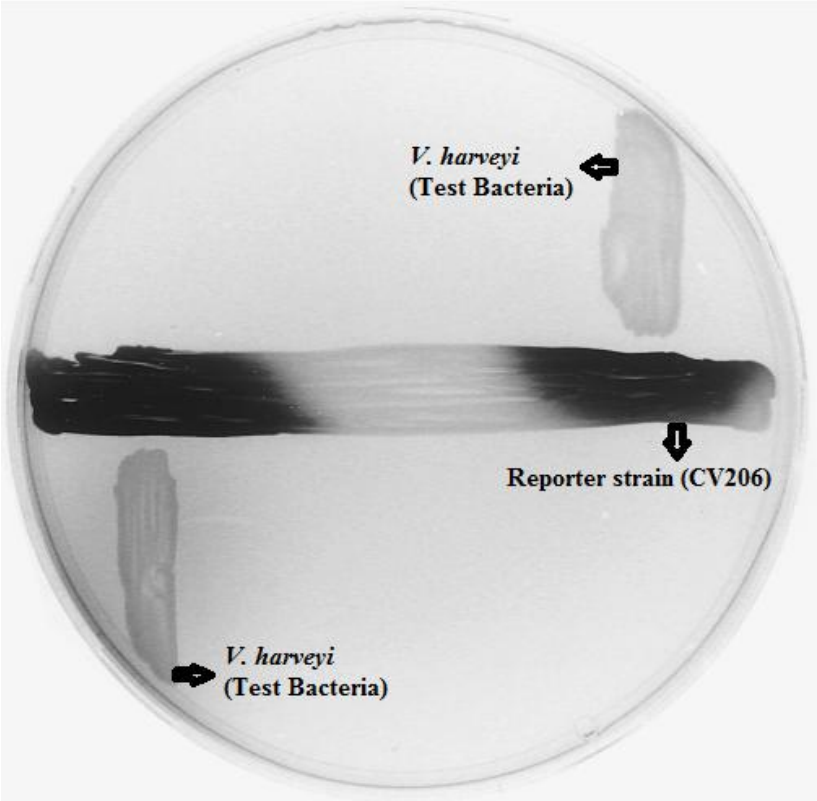

Figure 2. Induction of AHL on CV 206 by *V. harveyi* both sides.

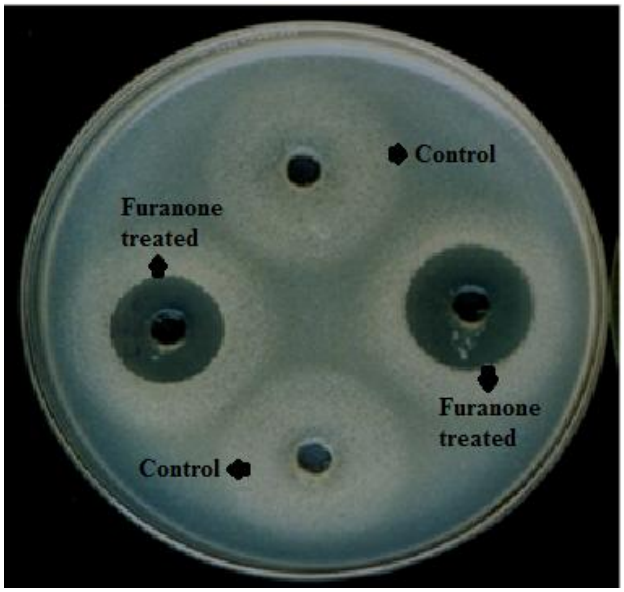

Figure 3. Furanone (300 µl of 1 mg/ml), inhibiting *V. harveyi*; the control well did not show inhibition.

the amount of enzyme required to produce an absorbance change of 1.0 in a 1 cm cuvettee under the condition of the assay)

Inhibition of *V. harveyi* by furanone through "Well diffusion assay"

Viable cells of *V. harveyi* (50 µl of 1.8 OD) were inoculated into the Petri plate. LB agar was poured and mixed on it. This medium was solidified and wells of 6.0 mm dia were made using sterile steel borer. Three hundred µl of furanone solution (1mg/ml, dissolved in 80% ethanol) was added into each well. The plates were incubated at 28°C/16 h. Formation of clear zones (Figure 3) around the well indicates inhibition of *V. harveyi* (Kannappan and Manja, 2004). Control did not show any inhibition.

Growth reduction of *Vibrio harveyi* by furanone in LB broth

100 µl of *V. harveyi* (OD 1.8) was inoculated into 25 ml of LB broth in 100 ml flask. Furanone (1 mg/ml) was added (dissolved in 80% ethanol) into the flask. Suitable control and replicates were kept with 80% ethanol. The flasks were incubated at 28°C in a shaker incubator (100 rpm). The growth pattern of *V. harveyi* against furanone was measured using Spectrophotometer (Cary 3E UV Visible Spectrophotometer- USA) for 3 h interval for 48 h (Hentzer et al., 2002).

Determination of haemolytic activity of *Vibrio harveyi* against furanone

The furanone (1 mg/ml) treated *V. harveyi* cells were tested for haemolysis using Columbia agar incorporation with 5% defibrinated

sheep blood. 20 µl of *V. harveyi* cells were spotted on the surface of the medium. The replicate plates were incubated at 28°C/24 h. Formations of lytic zone around the spots were observed as haemolytic activity of *V. harveyi* (Nakayama et al., 2006).

Scanning electron microscopy (SEM) picture of *Vibrio harveyi* after furanone treatment

The test bacteria (1.0 ml of 1.80 0D) *V. harveyi* was serially diluted to 10^{-4}. A sterile Eppendorf tube containing 0.5 ml of 10^{-4} dilution (5×10^3 CFU/ml) was incubated with 0.5 ml of furanone of 1:1 ratio at 37°C for 24 h in a shaker. The cells after incubation were centrifuged at 6000 rpm for 15 min and washed twice with 0.01 M potassium phosphate buffer at 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 furanone.

The slides containing the cell were dried in desiccators. 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 furanone against *Vibrio harveyi* in the experimental conditions of larviculture of *Penaeus monodon*

25 L aquarium glass tanks were washed with 1% $KMN0_4$ solution. The tanks were filled with 10 L of 20 ppt seawater (Tank 1). Disease free post larvae (PL 18 days) of *P. monodon*, procured from commercial shrimp hatchery were acclimatized in 20‰ seawater for 4 days before starting the experiment at 29°C with aeration. The post larvae (PL) had an average weight of 0.1 to 0.2 g and 1000 numbers were stocked in all the three experimental tanks. 10 g of furanone was dissolved in 80% ethanol and added as such into (1 mg/ml concentration with active ingredient composition was 99%) into the treatment tank with 5.0 ml of *V.harveyi* (OD 1.9 = 2.2×10^7 cfu/ml).

The tanks were provided aeration continuously not more than 4mg/L. PL feed was given for the 20 days period and the experiment was conducted for 20 days without exchanging water. The dead PL's were collected and ascertained as cumulative mortality from all the tanks for 30 days. A control tank was kept in this way without adding furanone (Tank 2). Furanone alone was also inoculated in another tank with PL without *V. harveyi* (Tank 3) for estimating stress if any among PL due to furanone. Water quality parameters like salinity, temperature, and pH were measured using salinometer, thermometer and pH meter respectively.

Total heterotrophic bacteria and total *V. harveyi* count were determined in both treatment and control tanks with five days of time interval (Defoirdt et al., 2006).

Estimation of the total heterotrophic bacteria against furanone treatment (THB) during larviculture

2 ml of water sample was taken in sterile tubes from treatment and control tanks and 1.0 ml was serially diluted with 9.0 ml normal saline. 1 ml of each dilution was taken in separate Petri plate. Zobel marine agar was added and mixed thoroughly. The plates were allowed to solidified and incubated at 37°C for 24 h and counts were determined.

Estimation of *V. harveyi* against furanone treatment during larviculture

2 ml of water sample was taken in sterile tubes from the larviculture tanks. 1 ml was inoculated into 25 ml of alkaline peptone water medium and shaker incubated at 250 rpm 28°C /12 h for enrichment. One ml was taken from the enrichment and serially diluted in 9.0 ml normal saline. From the serial dilution, 1ml was taken and inoculated into the petri plates and *V. harveyi* selective agar medium was added (Lachlan et al., 1996). The plates were allowed to solidified and incubated at 28°C for 24 h. Growth of the blue azure colonies were confirmed as *V. harveyi.*

Statistical analysis

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) × 100.

RESULTS AND DISCUSSION

Auto-induction 2 assay

After treating with furanone, *V. harveyi* did not produce any zone when tested for pathogenicity. Blue green light produced by *V. harveyi* could be better visualized in the auto induction (AI) medium as luminescence during the absence of light (in the dark room). The luminescent measurement by means of Luminoscan-T plus showed the most pronounced difference between the positive and negative control. During 5.5 h incubation, the cells grew well and showed max OD of 1.0. AI -2 bio-assay is based on the ability of reporter strain BB170 to specifically bio-luminate in response to AI-2 at higher cell densities (10^9 cfu/ml). During higher cell-densities (10^{10} cfu/ml) the bioluminescence can be detected in response to the chemical components added into the AI medium (Bassler et al., 1994). It was found that the signal of the reporter strain under AI-2 assay was minimal (relative bio-luminescence) between 5 and 5·5 h after inoculation (interference by endogenous AI-2 of the reporter was < 1% of the bioluminescence signal). Similar to detection, AI medium can also be applied to ascertain the growth of *V. harveyi* (10^{10}cfu/ml) even in any food samples and can also be used in number of tests for AI-2 production among vibrios and non- vibrios (Turovskiy et al., 2006).

Auto-induction 1 assay and antagonism against *Vibrio harveyi*

During the AI-1 assay, the AHL fractions were found positive in two wells and were comparable with the control AHL (first row of the plate). The AHL compounds

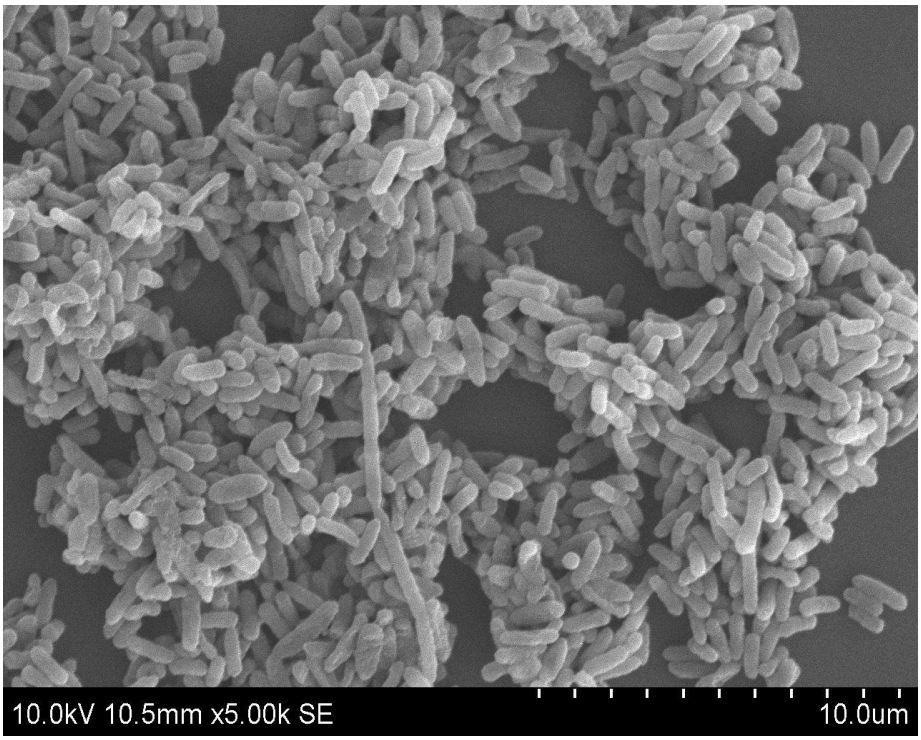

Figure 4. The SEM picture of *V. harveyi* as control.

extracted from *V. harveyi* were stored at -80°C for two months. The structure and sequential confirmation of AHL compounds is under progress in the lab. In the control, three different derivatives of AHL compounds were used as mentioned in Figure 1. The target bacteria *V. harveyi* streaked next to the CV206 streak produced diffusible AHL which induces the strain (Figure 2) CV206 to produce violet pigment (Morohoshi et al., 2005).

Growth pattern of furanone treated *Vibrio harveyi*

The furanone showed an inhibitory zone of 9.0 ± 0.25 mm (excluding the well) against *V. harveyi*, whereas no inhibition was observed using alcohol (80%) as control against *V. harveyi* (Figure 3). The minimum inhibitory concentration (MIC) of furanone was found to be 50 µg of 1mg/ml against 50 µl of 10^9 cfu/ml of *V. harveyi* through "Well diffusion assay". The growth of *V. harveyi* in the control varied from 1.33 ± 0.01 OD in 4 h and further increased to 1.62 ± 0.01/8 h, 1.9 ± 0.02 /16 h and 2.1 ± 0.1 /24 h respectively, when *V. harevyi* was grown in LB medium with furanone as media component. But in the furanone treatment, the OD for growth changed from 1.33 ± 0.01 OD / 4 h, 1.0 ± 0.01 OD in 8 h, 0.9 ± 0.02 OD /16 h and 0.9 ± 0.02 OD / 24 h respectively (Figure 6). Figure 4, shows the structure of *V. harveyi* without furanone treatment. Figure 5, shows the distorted shape of *V. harveyi* cells after treating with furanone. Furanone was also produced by Australian macro marine red algae, *Delisea pulchra* which antagonize against luminescence and the toxin produced by *V. harveyi* (when furanone is treated with *V. harveyi,* it may disrupt the bacterial signal by mimicking the AHL produced by bacterial signals and competing for the same receptor site (Manefield et al., 2002).

Effect of furanone against virulent protease produced by *V. harveyi*

A zone of 12 ± 0.2 mm (excluding the spot) showed as the protease production by *V. harveyi* in the control, whereas in furanone treatment, *V. harveyi* did not produce any zone. Furanone treated *V. harveyi* in the broth produced protease enzyme from 0.1 to 0.06 OD in 24 h. But, in the control, the protease production varied from 3.1 ± 0.1 to 2.0 ± 0.2 OD from the first day to the fourth.

Effect of furanone against *Vibrio harveyi* during shrimp larviculture.

In the challenge experiment, the cumulative percentage mortality was calculated which increased in the control

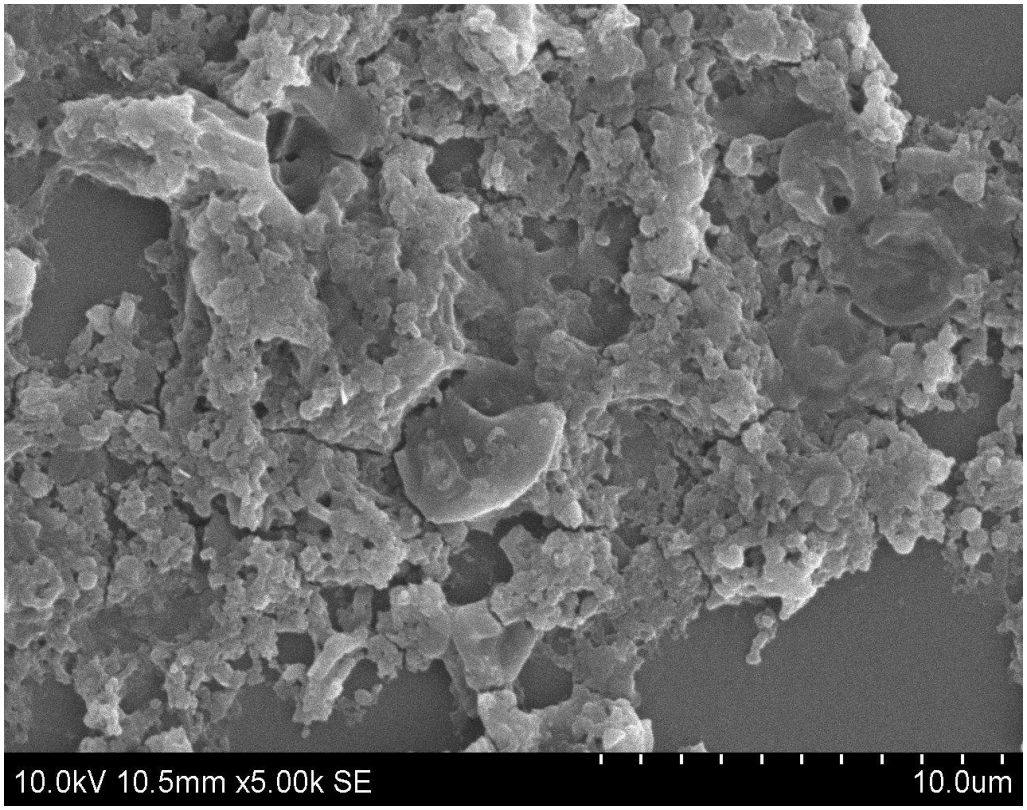

Figure 5. Disruption of *V. harveyi* by furanone.

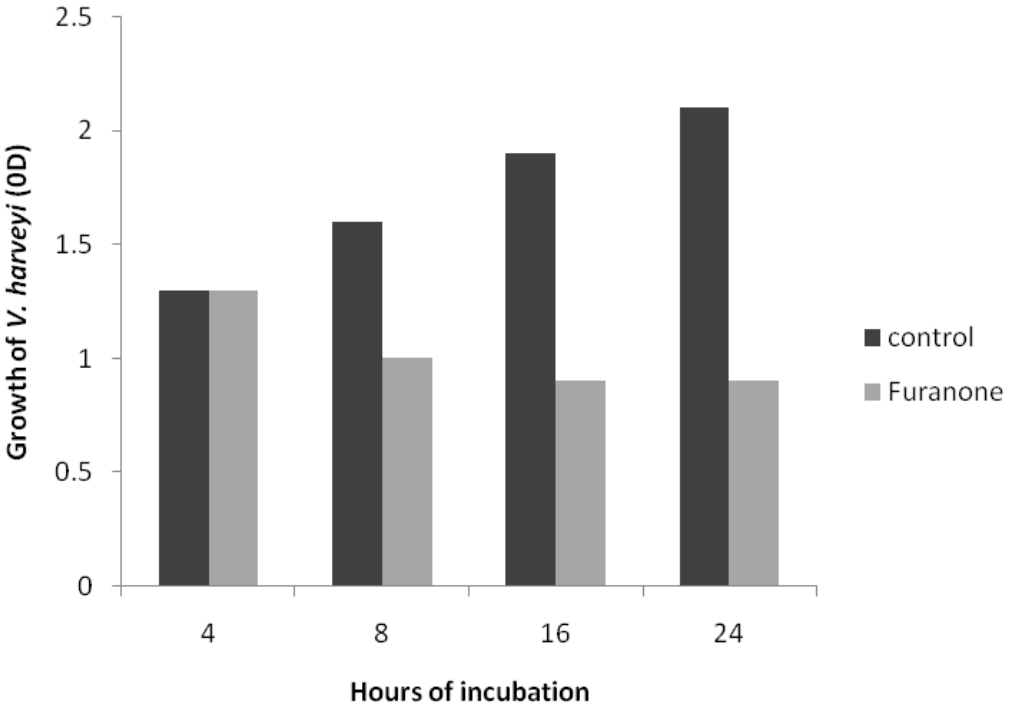

Figure 6. Effect of furanone treatment against *V. harveyi.*

Table 1. Changes of cumulative percentage mortality on post larvae of *P.monodon* by *V. harveyi*

Larvi-culture in days	Furanone treatment against *V. harveyi* (T_1)	Control (T_2)	Furanone treatment without *V. harveyi* (T3)	Growth of *V. harveyi* against furanone treatment (CFU/ml)	Growth of *V. arveyi* in control (CFU/ml)	THB against furanone treatment (CFU/ml)	Temp	pH	PSU
1st	0	0	0	2.2×10^7	2.2×10^7	2.1×10^6	29 ± 0.3	7.2 ± 0.1	20 ± 0.2
5th	0.53 ± 0.01	0.85 ± 0.03	0.63 ± 0.02	2.1×10^6	2.1×10^7	3.1×10^7	28 ± 0.2	7.4 ± 0.2	19 ± 0.1
10th	1.26 ± 0.05	3.60 ± 0.09	1.08 ± 0.03	1.5×10^6	2.1×10^7	2.1×10^6	27 ± 0.3	7.5 ± 0.3	20 ± 0.2
15th	5.51 ± 0.20	34.14 ± 1.11	1.73 ± 0.05	1.1×10^4	1.2×10^6	2.2×10^5	28 ± 0.3	7.1 ± 0.2	17 ± 0.1
20th	8.17 ± 0.26	59.67 ± 1.91	2.29 ± 0.09	1×10^3	1.2×10^4	2.1×10^5	28 ± 0.2	7.2 ± 0.1	18 ± 0.2
25th	10.72 ± 0.33	69.92 ± 2.51	2.74 ± 0.11	1×10^3	1.1×10^4	1.1×10^4	26 ± 0.3	7.5 ± 0.1	19 ± 0.3
30th	13.37 ± 0.53	80.08 ± 3.11	3.17 ± 0.13	1×10^3	1.31×10^4	1.3×10^4	28 ± 0.3	7.4 ± 0.2	18 ± 0.2

PSU, Practical Salinity Unit; values are average of three determinations with SD; THB, total heterotrphic bacteria.

experiment from 0.85 to 3.60, 34.14, 59.67, 69.92 and 80.08% for the period of the 5th day to 30th day. But in the treatment tanks, the CPM varied from 0.53 to 1.26, 5.51, 8.17, 10.72 and 13.37% till 30^{th} days of experiment. But the difference in the cumulative percentage mortalities in the treatment tank was 0.32, 2.34, 28.63, 51.50, 59.20 and 66.71% respectively as compared to control. Furanone treatment alone without *V. harveyi* showed no abnormal symptoms or mortality among post larvae (Table 1).

The water quality parameters were normal. Much variation was not observed among the water quality due to furanone treatment. The initial load of *V. harveyi,* was 2.2×10^7 CFU/ml during the first day of both treatment and control tanks. The growth was then reduced to 1×10^3 CFU/ml in the treatment tank during the 20th day and further remained constant for 10 more days. In the treatment tank, the growth pattern of *V. harveyi* was reduced from 2.2×10^7 CFU/ml to 2.1×10^7 in the fifth day, 1.5×10^6 CFU/ml in the the 10th day, 1.1×10^4 in the 15th day and 1×10^3 CFU/ml in the 20th day. The difference in the growth reduction levels by furanone treatment against *V. harveyi* was from 10^4 to 10^6 CFU/ml from 15 to 20 days of the experiment. The level of furanone used was able to antagonize10^4 to 10^6 CFU/ml of *V. harveyi* in the treatment tank. In the control tank, the growth of *V. harveyi,* varied to 2.1×10^7 CFU/ml/10^{th} day, 1.2×10^6 CFU/ml/ 15^{th} day, 1.2×10^4 CFU/ml/ 20th day, and 1.1 × 104 CFU/ml /25th day. The THB counts increased to 3.1 × 107 CFU/ml during the 5th day, and then decreased to 1.31×10^4 CFU/ml in the 30th day. It is well documented that furanone also protects the brine shrimp *Artemia franciscana* from the infection caused by the pathogenic *V. harveyi*, *V. campbellii*, and *V. parahaemolyticus* (Defoirdt et al., 2006). Furanone also inhibit biofilm formation through interfering quorum sensing produced by *Staphylococcus epidermis* (Stensrud et al., 2009). A significant reduction in the toxicity of concentrated supernatant from *V. harveyi* in the presence of the furanone signal antagonist has been measured by *in-vivo* toxicity assays in shrimps and mice (Manefield et al., 2000). Furanone has been used as effective disease control agent against luminescent causing vibrios in aquaculture system (Defoirdt et al., 2007) and have beneficial effect on the marine environment (Dobretsov et al., 2009). Addition of furanone in fermented milk as inhibitor of bacterial spoilage caused by *Pseudomonas* spp are also well documented (Shobharani and Renu, 2010). Tendencia and Pena, (2003) investigated the components of the green water system and their effects on the population of luminescent bacteria. Their experiment suggests that both components of tilapia water and *Chlorella* spp alone are effective against luminescent bacteria over a short period of time.

Conclusion

Furanone is a chemical preservative considered as the next generation antimicrobial agent (Bijarnsholt and Givskow, 2008). It can be directly applied to the shrimp larviculture or grow-out systems for controlling the luminescence produced by *V. harveyi.* Furanone, may also have

potential to control the multi cellular behavior of bacteria. Further, novel antimicrobial strategies could be intended based on this study using furanone.

ACKNOWLEDGEMENTS

The first author thank the Director CIBA and ICAR for their permission to carry out this work at The Ohio State University, Plant Cellular and Molecular Biology Division, Columbus, USA under the DBT Overseas Associateship offered by the Department of Biotechnology, India, during 2006.

REFERENCES

Abraham TJ, Palaniappan R (2004). Distribution of luminous bacteria in semi-intensive penaeid shrimp hatcheries of Tamilnadu, India. Aquaculture 232:81-90.

Arivuselvan N, Jegadeesan D, Govindan T, Kathiresan K, Anantharaman P (2011). *In vitro* antibacterial activity of leaf and bark extracts of selected mangroves against fish and shrimp pathogens. Glob. J. Pharmacol. 5:112 -116.

Bassler BL, Wright M, Silverman MR (1994). Multiple signaling systems controlling expression of luminescence in *V. harveyi*: sequence and function of genes encoding a second sensory pathway. Mol. Microbiol. 13:273-286.

Bijarnsholt T, Givskow M (2008).Quorum sensing inhibitory drugs as next generation Antimicrobials: worth the effort ? Curr. Infect. Dis. Rep. 10:22-28.

Charu G, Srivastava S (2006). Quorum sensing the phenomenon of microbial *communication*. Curr. Sci. 90:666-676.

Defoirdt T, Boon N, Sorgeloos P, Verstraete W, Bossier P (2007). Alternatives to antibiotics to control bacterial infections: luminescent vibrios*is* in aquaculture as an example. Trends Biotechnol. 25:472-479.

Defoirdt T, Crab R, Wood K T, Sorgeloos P, Verstraete W and Bossier P (2006). Quorum Sensing-disrupting brominated furanones protect the Gnotobiotic brine Shrimp *Artemia franciscana* from pathogenic *V. harveyi*, *V. campbellii*, and *V. parahaemolyticus* isolates. Appl. Environ. Microbiol. 72:6419-6423.

Dobretsov S, Teplitski M, Paul V (2009). Mini review: quorum sensing in the marine environment and its relationship to bio-fouling. Biofouling 25:413-427.

Folasade MO, Joshua OA (2005). Production dynamics of extracellular protease from *Bacillus* species. Afr. J. Biotechnol. 4:776-779.

Haldar S, Chatterjee S, Sugimoto N, Das S, Chowdhury N, Hinenoya A, Asakura M, Yamasaki S (2011). Identification of *V. campbellii* isolated from diseased farm-shrimps from south India and establishment of its pathogenic potential in an Artemia model. Microbiol. 157:179-188.

Hentzer M, Riedel K, Rasmussen TB, Heydorn A, Andersen JB, Parsek MR, Rice SA, Eberl L, Molin S, Høiby N, Kjelleberg S, Givskov M (2002). Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiol. 148:87-102.

Kannappan S, Manja KS (2004). Antagonistic efficacy of lactic acid bacteria against seafood-borne bacteria. J. Food Sci. Technol. 41:50-59.

Karunasagar I, Pai R, Malathi GR, Karunasagar I (1994). Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *V. harveyi* infection. Aquaculture 128:203-209.

Lachlan H, Leigh O, Sandra S (1996). A Selective and differential medium for *Vibrio harveyi*. Appl. Environ. Microbiol. 62(9):3548-3550.

Manefield M, Harris L, Rice SA, de Nys R, Kjelleberg S (2000). Inhibition of luminescence and virulence in the black Tiger Prawn (*Penaeus monodon*) pathogen *V.harveyi* by intercellular signal antagonists. Appl. Environ. Microbiol. 66:2079-2084.

Manefield M, Rasmussen TB, Henzter M, Andersen JB, Steinberg P, Kjelleberg S, Givskov M (2002). Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover. Microbiology 148:1119-1127.

McDougall LA, Holzapfel WH, Schillinger DF, Feely J, Ruphow H (1994). Scanning electron microscopy of target cells and molecular weight determination of bacteriocin produced by *Lactococcus lactis* D53. Int. J. Food Microbiol. 24:295-300.

Morohoshi T, Ebata A, Nakazawa S, Kato N, Ikeda T (2005). N-acyl homoserine lactone producing or degrading bacteria isolated from the intestinal microflora of Ayu fish (*Plecoglossus altevelis*). Microbes Environ. 20:264-268.

Nakayama T, Nomura N, Matsumura M (2006). Study on the relationship of protease production and luminescence in *V. harveyi*. J. Appl. Microbiol. 101:200-205.

Raissy M, Momtaz H, Moumeni M, Ansari M, Rahimi E (2011). Molecular detection of *Vibrio* spp in lobster hemolymph. Afr. J. Microbiol. Res. 5:1697-1700.

Ren DC, Wood TK (2004). 5Z - 4 – bromo - 5 - (bromomethylene) – 3 – butyl – 2 (5H) - furanone reduces corrosion from *Desulfotomaculum orientis*. Environ. Microbiol. 6:535-540.

Shobharani P, Renu A (2010). Interception of quorum sensing signal molecule by furanone to enhance the shelf life of fermented milk. Food Control 21:61-69.

Stensrud JL, Landin MA, Benneche T, Petersen FC, Scheie AA (2009). Furanones, potential agents from preventing *S. epidermis* biofilm infection. J. Antimicrob. Chemother. 63:309-316.

Tendencia EA, Pena MD (2003). Investigation of some components of the green water system which makes it effective in the initial control of luminous bacteria. Aquaculture 218: 115 - 119.

Teplitski M, Chen H, Rajamani S, Gao M, Merighi M, Sayre RT, Robinson JB, Rolfe BG, Bauer WD (2004). *Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. Plant Physiol. 134:137-146.

Turovskiy Y, Chikindas ML (2006). Autoinducer-2 bioassay is a qualitative, not quantitative method influenced by glucose. J. Microbiol. Methods 66:497-503.

Whitehead NA, Barnard AM, Slater H, Simpson NJ, Salmond GP (2001). Quorum sensing in Gram negative bacteria. FEMS. Microbiol. Rev. 25:365-404.

Zhang XH, Austin B (2000). Pathogenicity of *V. harveyi* to *Salmonids.* J. Fish Dis. 23:93-102.