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Antagonism of marine *Actinomycetes* bacteria against virulence and bio-luminescence disease causing *Vibrio harveyi*

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ABSTRACT

Luminescence is caused by *Vibrio harveyi,* which is responsible for causing mass mortality (80 - 100 %) among *Penaeus monodon* hatcheries. An alternative strategy is the use of marine *Actinomycetes* to control *V. harveyi*. Eighty three marine *Actinomycetes* bacteria were isolated from marine and brackishwater of Muttukadu, Chennai and treated against growth and virulence factors produced by *V. harveyi.* Among 83 *Actinomycetes* isolates, 2 (CBWA3 and CBWA10) showed higher zone of inhibition against *V. harveyi*. When the *Actinomycetes* isolates were co-cultured with *V. harveyi*, the growth of *V. harveyi* was reduced to 4.66 log cfu mL^{-1} by CBWA3 and 3.75 log cfu mL^{-1} by CBWA10. The virulence factors like luminescence, crude bacteriocin, protease production and phospholipase activity were determined during co-culture and it was observed that phospholipase activity was very high in control and virulence was weak in the treatment. This study confirms that marine *Actinomycetes* bacterial isolates can be effectively utilized to control luminescence disease in the aquaculture system (**Keywords**: Marine *Actinomycetes* bacteria, antagonism, *Vibrio harveyi*, growth, virulence factors).

INTRODUCTION

Marine microorganisms are promising sources of biologically active substances (Bavya *et al.,* 2011). It was reported that more than half of the bioactive secondary metabolites including antimicrobial, antitumour, immunosuppressive agents and enzymes (Solanki *et al.,* 2008) was discovered from *Actinomycetes* bacteria (Berdy, 2005). *V. harveyi* is a Gram-negative, luminescent bacterium causing mass mortality (80 - 100 %) in post larvae of *Penaeus monodon* hatcheries (Raissy *et al.,* 2011). However, the use of antibiotic agents has led to the emergence of antibiotic-resistant bacteria which may lead to transfer of resistance among other bacteria and accumulation of antibiotics in shrimp products. Therefore, developing an alternative strategy to control infections is necessary. Marine *Actinomycetes* are prolific producers of antibiotics and other therapeutic compounds (Selvakumar, 2011). In the present study, the antagonistic potential of *Actinomycetes* isolated from marine and brackishwater was assessed against luminous *V. harveyi*.

MATERIALS AND METHODS

Collection of water samples

Marine and brackishwater samples were collected from Muttukadu (Latitude 12.806 °N; Longitude 80.248 °E), experimental station of Central Institute of Brackishwater Aquaculture (CIBA) at Chennai. Sample collection was done from below 30 cm depth and transported to the laboratory in a sterile glass container and stored in room temperature.

Isolation of V. harveyi

V. harveyi strains (50) were isolated from *P. monodon* larviculture tanks. The isolates were pre-enriched in alkaline peptone water for 12 h and serially diluted with normal saline (0.85 %) and then surface spread on thiosulphate citrate bile-salt sucrose agar medium (TCBS) (Abraham and Palaniappan, 2004) and seawater complex (SWC) agar medium (Harris *et al.,* 1996). Luminescent colonies were observed after 20 h of incubation at 28 °C in SWC agar. The isolates were confirmed by various biochemical tests such as arginine

dihydrolase (-), lysine (+), ornithine decarboxylase (+), gelatinase (+), Vogesproskauer (-) and D-glucosamine (-) (Abraham and Palaniappan, 2004). The isolates were further confirmed by PCR (Maiti *et al.,* 2009). The pathogenicity of the *V. harveyi* was determined by spotting in 3 % blood agar. The isolates were stored in sterile glycerol solution (15 % v/v).

Isolation of marine Actinomycetes

Water samples were pre-enriched in marine *Actinomycetes* growth medium (MAG) for 48 h. The enriched samples were diluted in physiological saline and spread plated on MAG agar medium (starch - 2.0 g, yeast extract - 0.8 g , peptone - 0.4 g , agar - 4.0 g, aged seawater - 100 mL, pH - 7.0) (Dhanasekaran *et al.,* 2009). The plates were and incubated for 2 days at 30 °C. Isolated colonies were inoculated into MAG broth (glycerol - 5.0 g, beef extract - 1.0 g, peptone - 1.0 g, yeast extract - 2.0 g, $MgSO_4$, $7H_2O$ - 0.1 g, K_2HPO_4 - 0.1 g, $CaCO_3$ - 0.2 g, aged seawater - 100 mL, pH - 7.0) (Kumar *et al.,* 2006). A total of 83 *Actinomycetes* strains were isolated. The cell pellets and spent cultures of *Actinomycetes* were obtained by centrifugation (10,000 rpm for 20 min at 25 °C) and checked for antagonism against *V. harveyi* (1.8 OD at 600 nm, 2.17×10^7 cfu mL^{-1}).

Biochemical characterization of Actinomycetes

Isolates that were Gram-positive, branching and filamentous nature, methyl red positive, indole positive, starch hydrolysis positive, vogus-proskauer test positive, catalase positive, alkaline slant and acid butt on triple sugar iron agar, and showed negative result in fermentation of citrate were confirmed as *Actinomycetes* (Dhananjeyan *et al.,* 2010). Seawater *Actinomycetes* isolates were named as Ciba seawater *Actinomycetes* (CSWA1 to CSWA 41) and brackishwater isolates were named as Ciba brackishwater *Actinomycetes* isolates (CBWA 1 to CBWA 42).

Antimicrobial assay

Marine *Actinomycetes* bacterial isolates were cultured in 5 mL of MAG broth at 30 °C for 2 days. Aliquot (10 µL, OD 2.3, 3.0×10^9 cfu mL^{-1}) of the cell pellet was spotted onto agar plates containing 10 mL of MAG solid medium. After 18 h incubation at 30 °C, the plates were overlaid with 5 mL of the MAG soft agar (0.8 %) inoculated with the cell suspension (20 µL, OD 1.8 at 600 nm, 4.1×10^7 cfu mL^{-1}) of the *V. harveyi*. The plates were further incubated at 27 °C for 48 h. The zone of inhibition around the spot was observed (Selvakumar, 2011). Likewise four *V. harveyi* isolates were tested against *Actinomycetes* spp. Further, the spent culture of *Actinomycetes* from MAG medium was tested for antagonism against *V. harveyi* by the "Well Diffusion Assay". The cells of *Actinomycetes* were also grown in LB broth (Casein enzymichydrolysate 10 g, Yeast extract 5 g and NaCl 10 g in 975 mL deionized water, pH 7.5) (Kannappan *et al.,* 2013) supplemented with aged seawater (100 %) and the inhibition values were compared (Jensen *et al.,* 2005) with MAG medium.

Effect of Actinomycetes against growth and virulence of V. harveyi during co-culture

V. harveyi of 1.8 OD (100 µL) was inoculated into 100 mL of MAG broth and *Actinomycetes* (OD 2.3) was inoculated at 100 µL. The inoculums were shaker incubated at 27 °C for 4 days at 100 rpm as co-culture. Inoculum was taken and plated in respective medium after serial dilution. *V. harveyi* selective agar was used for *V. harveyi* and MAG agar for *Actinomycetes* bacteria. The plates were incubated at 30 °C for 48 h. For control, *V. harveyi* and test *Actinomycetes* isolates were grown in MAG broth separately. Each test was performed in triplicates and values are expressed as average of 3 determinations. The estimations of growth and virulence factors like luminescence, crude bacteriocin, protease production, phospholipase produced by *V. harveyi* was followed (Soto-Rodriguez *et al.,* 2012; Madhusudana Rao *et al.,* 2013; Kannappan *et al.,* 2013). The luminescence produced by *V. harveyi* was measured by luminometer and expressed as counts per second (CPS i.e., photons/second).

RESULTS AND DISCUSSION

Antimicrobial activity of marine *Actinomycetes* isolates against the *V. harveyi* was given in Table 1 and Figure 1. A total of 83 *Actinomycetes* isolates were isolated and named as CSWA1 to CSWA41for seawater isolates and

Table 1. Zone of inhibition on *V. harveyi* by marine *Actinomycetes* cells grown in SLB, MAG and SC-MAG broths

Antagonistic *Actinomycetes* isolates	Zone of inhibition (mm) against *V. harveyi*		
	*SLB	*MAG	*SC-MAG
Brackishwater isolates			
CBWA3	43.33 ± 1.66	38.00 ± 1.20	37.00 ± 1.25
CBWA4	ND	11.33 ± 0.32	11.33 ± 0.42
CBWA5	12.66 ± 0.45	13.00 ± 0.45	14.33 ± 0.52
CBWA6	15.00 ± 0.60	12.50 ± 0.45	12.66 ± 0.52
CBWA7	24.00 ± 0.90	20.50 ± 0.79	19.83 ± 0.82
CBWA9	10.00 ± 0.26	14.16 ± 0.50	14.33 ± 0.52
CBWA10	33.66 ± 1.24	30.33 ± 1.12	30.33 ± 1.15
CBWA14	18.00 ± 0.64	14.00 ± 0.50	14.00 ± 0.51
CBWA17	20.50 ± 0.72	15.00 ± 0.60	15.00 ± 0.62
Seawater isolates			
CSWA1	21.66 ± 0.85	14.00 ± 0.50	14.00 ± 0.42
CSWA2	ND	7.00 ± 0.15	7.00 ± 0.15
CSWA7	ND	8.50 ± 0.32	8.33 ± 0.32
CSWA8	ND	12.33 ± 0.42	12.33 ± 0.41
CSWA9	ND	11.00 ± 0.36	12.50 ± 0.41
CSWA10	26.00 ± 0.89	13.66 ± 0.52	14.33 ± 0.51
CSWA12	ND	12.66 ± 0.45	13.50 ± 0.52
CSWA14	2.00 ± 0.05	15.00 ± 0.55	16.00 ± 0.62
CSWA15	1.33 ± 0.03	6.00 ± 0.25	6.16 ± 0.20
CSWA16	ND	4.83 ± 0.14	5.50 ± 0.19
CSWA17	ND	10.33 ± 0.45	10.00 ± 0.25
CSWA18	ND	8.33 ± 0.32	8.50 ± 0.31
CSWA19	5.00 ± 0.15	5.00 ± 0.15	5.00 ± 0.13
CSWA20	ND	ND	3.23 ± 0.12

*SLB - Sea water LB broth; *MAG - Marine *Actinomycetes* growth medium; *SC-MAG - Spent culture of MAG; ND - Not detected

Activity of isolates : High level (Zone of inhibition > 11 mm); Moderate level (Zone of inhibition 5 - 11 mm); Low level (Zone of inhibition < 5 mm)

CBWA1 to CBWA42 for brackishwater isolates. Ten strains, out of 41 seawater *Actinomycetes* isolates showed moderate (5 - 11 mm) antagonistic activity, whereas out of 42 isolates from brackishwater, 4 isolates (CBWA3, CBWA4, CBWA5 and CBWA7) showed better inhibitory activity (> 11 mm) against *V. harveyi* and 11 showed moderate (5 - 11 mm) activities (Table 1).

A total of 83 *Actinomycetes* isolates were named as CSWA1 to CSWA41 for seawater isolates and CBWA1 to CBWA42 for brackishwater isolates. Among seawater *Actinomycetes* isolates, 7 isolates showed high level of activity, 6 isolates exhibited moderate level and one isolate showed low level of

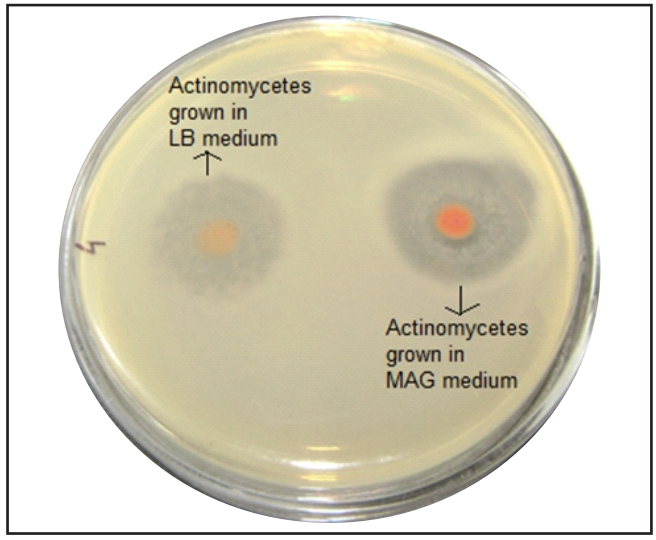

Figure 1. Inhibition of *V. harveyi* by *Actinomycetes* CBWA10 grown in seawater LB and MAG medium

activity. Four seawater isolates (CSWA1, CSWA10, CSWA12 and CSWA14) were considered as better. It was observed that out of all *Actinomycetes* isolates, 9 brackishwater isolates showed more inhibition, whereas 4 isolates (CBWA3, CBWA7, CBWA10 and CBWA17) showed best inhibitory activity against *V. harveyi* (Table 1). Most of the *Actinomycetes* strains belonged to the genus *Streptomyces*. However, the species level confirmation was not performed.

Actinomycetes strains were isolated from sea sediment and seawater from Konkan coast of Maharashtra (Gulve and Deshmukh, 2012) and seawater from East coast of Andhra Pradesh and their antimicrobial activity was determined against many pathogenic bacteria (Reddy *et al.,* 2011). The isolates of *Actinomycetes* grew in seawater - LB broth and MAG broths were compared for inhibition against *V. harveyi* (Table 1). Further it was found that the spent culture of *Actinomycetes* isolates gave better inhibition as maximum 24 mm against human pathogen (Rakshanya *et al.,* 2011) than that of cell pellets (Table 1), because the spent culture contains many extra cellular products with antibiotics (Bali *et al.,* 2011).

The highest zone of inhibition was observed by two *Actinomycetes* strains isolated from brackish water [CBWA3 (43.33 ± 1.66) and CBWA10 (33.66 ± 1.24)]. The growth of *V. harveyi* was reduced in the MAG

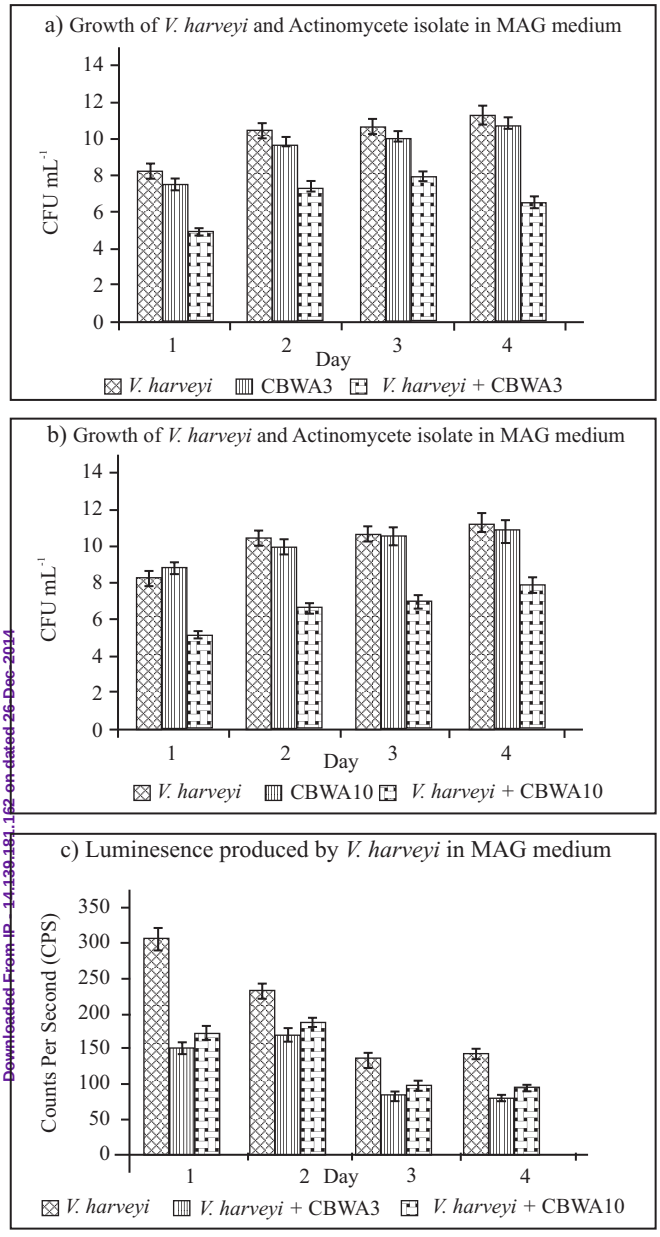

Figure 2. Changes on the growth and virulence factors of *V. harveyi* co-cultured with *Actinomycetes* isolates (a & b - Growth of *V. harveyi* and Actinomycete isolate in MAG medium ; c - Luminesence produced by *V. harveyi* in MAG medium)

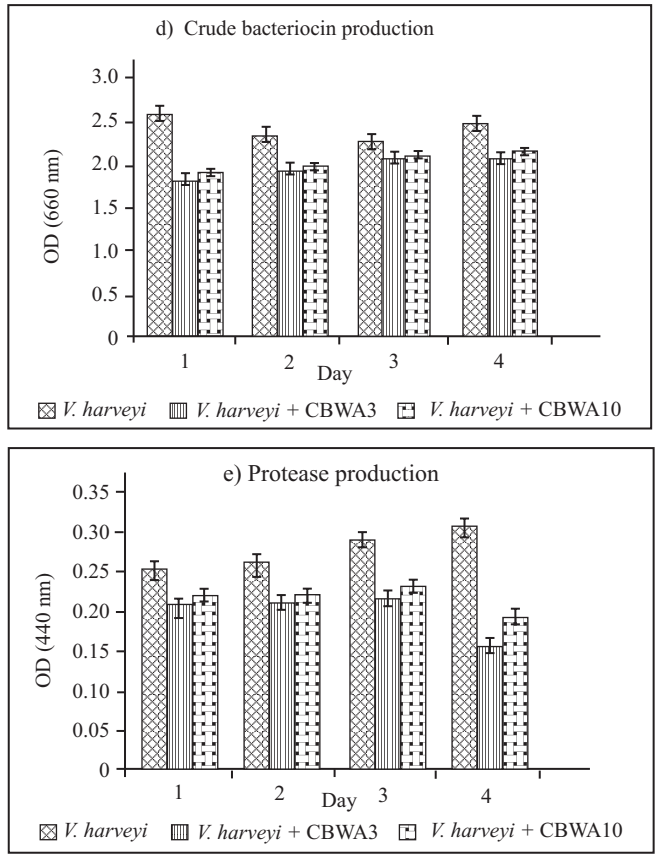

Figure 2 (Contd...). Changes on the growth and virulence factors of *V. harveyi* co-cultured with *Actinomycetes* isolates (d - Crude bacteriocin production; e - Protease production)

Table 2. Phospholipase activity of *V. harveyi* against treating with *Actinomycetes* isolates in MAG medium

Days	Phospholipase activity				
	Control			Treatment	
	V. harveyi	CBWA3	CBWA10	*V. harveyi* + CBWA3	*V. harveyi* + CBWA10
1	++++	++++	++++	++	++
2	++++	++++	++++	++	++
3	++++	++++	++++	+	+
4	++++	++++	++++	+	+

medium by co-culturing *Actinomycetes* strains of CBWA3 and CBWA10. There was 4.66 log growth reduction of *V. harveyi* on the 4th day by CBWA3, but isolate CBWA10 showed reduction to 3.75 log on the 2nd day. The inhibition may be due to the secondary metabolites produced by *Actinomycetes* spp. (You *et al.,* 2005) or their spent cultures.

Quorum sensing (QS) is a regulation of bacterial virulence in some bacterial species based on cell density (Brackman *et al.,* 2009). *V. harveyi* expresses bio-luminescence in response to cell density and the cell-free culture fluid from a number of bacterial species of both marine and terrestrial origin would stimulate *V. harveyi* to produce light (Bassler *et al.,* 1997). QS inhibition may be gaining potential as alternative

strategy for the treatment of bacterial infections. The strain CBWA3 was reduced the luminescence produced by *V. harveyi* and the differences of CPS was154.54, 61.05, 50.88, 64.03 for 4 days when compare to control (305.26, 231.58, 136.84, 143.86 CPS), whereas the strain CBWA10 showed reduced level of luminescence to 132.83, 45.82, 37.46, 48.94 CPS for 4 days as compared to control (305.26, 231.58, 136.84, 143.86 CPS) (Figure 2). The results of this study suggest that *Actinomycetes* strains are capable of controlling luminescence production by *V. harveyi*.

Inter-strain and intra-species inhibition mediated by bacteriocin-like inhibitory substances from a pathogenic *V. harveyi* strain VIB 571 was demonstrated against four isolates of the same species, and also vibrio isolates such as *V. fischeri*, *V. gazogenes* and *V. parahaemolyticus*. *Actinomycetes* strain RB72^T isolated from woodland bluff soil in northern Alabama, USA, produced a broad spectrum of bacteriocin (Farris *et al.,* 2011). When *V. harveyi* was co-cultured with the strain CBWA3, it reduced the crude bacteriocin values as 0.673, 0.348, 0.164, 0.334 (OD 660 nm) for 4 days as compared to the control (2.573, 2.328, 2.264, 2.494). But strain CBWA10 showed reduced OD (0.763, 0.398, 0.184, 0.394) for 4 days as compared to the control (Figure 2).

In many studies the pathogenicity of *V. harveyi* was examined with bacterial inoculums or extracellular products because they are considered as the most important virulence determinants of some *V. harveyi* strains. These extracellular products contain proteases, phospholipases, lipases, siderophores, chitinases and hemolysins (Soto-Rodriguez *et al.,* 2012). In the present study, CBWA3 was reduced the protease values to 0.049, 0.052, 0.075 and 0.151 for 4 days as compared to control (0.252, 0.261, 0.289 and 0.305). CBWA10 exhibited protease reduction (0.038, 0.041, 0.058, 0.114) for 4 days (Figure 2). Austin and Zhang (2006) studied about virulences such as pathogenicity, proteases, phospholipase, haemolysins and other exotoxins between isolates of *V. harveyi* obtained from the tiger prawn. When *V. harveyi* was co-cultured with CBWA3 and CBWA10 for 4 days , showed moderated level (++) of phospholipase activity on the 1st and 2nd day and weak level (+) of enzyme activity on the 3rd and 4th day, but the control (very high level ++++) exhibited very strong level of phospholipase activity (Liuxy *et al*., 2008) for 4 days (Table 2). Since *Actinomycetes* species produce a wide variety of secondary metabolites including antibiotics, parasiticides and herbicides, they are likely to aid in their survival in the presence of other microbes, insects and even animal hosts. It has been proved that *Actinomycetes* strains employ extracellular protease to reduce the biofilm formation on *Staphylococcus aureus* (Park *et al.,* 2012).

CONCLUSION

Considering the values obtained, *Actinomycetes* strains exhibit antagonistic activity against the pathogenic *V. harveyi.* Since, chemical preservatives are not universally accepted as aquaculture therapeutic agents, beneficial bacteria from marine resources may be the better alternative agent as this does not cause any microbial resistance or impart any residual effects to the aquaculture system. Moreover, bacterial preservative are cheaper than any other preservatives. Hence, the indigenous *Actinomycetes* isolates can be used for antagonism against aquaculture pathogenic bacteria.

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