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Biological control of vibriosis by antagonistic actinobacteria - an *in vitro* study

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Vibriosis is a common disease in aqua farms with high mortality rate. In the current study we focused on the bio control of vibriosis by actinobacteria. The actinobacteria and *Vibrio* sp. were isolated from the water of aqua farms in Vellore, TN, India. The isolation was performed by serial dilution and plating technique on TCBS and SCA medium for *Vibrio* sp. and actinobacteria respectively. The isolates were identified on the basis of their morphology and biochemical properties. The *Vibrio* sp. was checked for the drug susceptibility by disc diffusion method, isolate was found sensitive toward ciprofloxacin (33.3±1.13), chloramphenicol (26.6±0.57) and streptomycin (27.6±0.57), whereas, resistant to bacitracin, rifampicin, penicillin and ampicillin. The antagonistic activity of actinobacteria isolates was checked by agar well diffusion method on TCBS medium. The ethyl acetate extract of S1 isolate of actinobacteria showed maximum inhibition (61.0±1.73) against *Vibrio* sp., whereas, petroleum ether and ethanol extract of S3 and S4 isolate didn't show any inhibition. Ethyl acetate extract of S1 showed high relative percentage inhibition (335.56 %) whereas, petroleum ether and ethanol extract of S3 and S4 isolate didn't show any relative percentage inhibition. The results indicate that the isolated actinobacteria can be use as a bio control agent for vibriosis.

Keywords: Vibriosis; biological control; *Vibrio* sp.; actinobacteria

Introduction

Aquaculture is the cultivation of fresh water and sea water animals and plants for food or other purpose. The aquaculture animal includes fish, prawn, squids, crabs, mollusks etc. Vibriosis is a major pathogenic disease in the aquaculture animals with high mortality rate (Muroga and Egusa, 1988). Vibriosis is caused by the various species of genus *Vibrio*, includes *Vibrio alginolyticus, V. anguillarum, V. harveyi, V. splendidus, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, V. campbelli, V. fischeri, V.*

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damsella, V. pelagicus, V. orientalis, V. ordalii, V. mediterrani, V. logei, V. listonella, V. icthyoenteri, *V. parahaemolyticus* and *Vibrio haemoliticus* (Egidius et al., 1986).

Vibrio is a Gram negative, comma shaped bacteria (Don et al., 2005) that usually present in water in very low count, and sudden changes in the physiochemical conditions of the water may leads to the rapid proliferation of the bacterium. High *Vibrio* count causes vibriosis in aqua farm animals (Sizemore et al., 1985). Before 1980 *V. anguillarum* was the major cause of vibriosis in fish but most recently other species of genus *Vibrio* reported to cause vibriosis (Egidius, 1987; Akayh and Timur, 2002). In fish, the disease passed through an acute and often chronic phase. In acute phage the symptoms includes loss the appetite, lethargy, skin haemorrhages, the haemorrhages are very deep and necrotic. Vibriosis features enlargement of the spleen and kidney, focal hemorrhages on the surface of the heart, and the gills are usually pale. In chronic stage exophthalmos, ulcers, and intestinal inflammation are common.

The bacteria can be controlled efficiently by the use of antibiotics therapy, chloramphenicol and erythromycin are the drugs of choice, since these drugs are usually given orally, fishes are not treated with these antibiotics. Alternative therapies for vibriosis include, use of natural therapeutic agent like saponin (Bo-Kun and Jiann-Chu, 2008), bacteriophage therapy (Vinod et al., 2006). *V. anguillarum* MVAV6203 is a mutant strain of *Vibrio sp*., and used as live attenuated vaccine for the prevention of vibriosis (Yang et al., 2007). The disease can be controlled biologically by the use of the antagonistic actinobacteria.

In this study we focused on the isolation of actinobacteria from the water samples collected from different aquaculture farms in Vellore, TN, India, that having antagonistic activity against *Vibrio* sp. which was isolated from the same aquaculture farms water sample.

Materials and methods

Sample collection

Total five water samples were collected from different aquaculture farms in Vellore, TN, India, during October 2008. Samples were collected in sterilizes glass bottles and transported to the Molecular and Microbiology Research Laboratory, VIT University, Vellore, TN, India. The water samples were stored at 4˚C up to further processing.

Isolation of *Vibrio* sp.

The *Vibrio* sp. was isolated from the water samples by serial dilution and spread plate technique. The water samples were serially diluted to get a concentration range from 10^{-1} to 10^{-6}. A volume of 0.1 ml of the diluted sample was transferred to the Thiosulfate citrate bile salts sucrose (TCBS) agar plates from each dilution. The Petri plates were rotated clockwise and anticlockwise to spread the sample uniformly. Plates were incubated at 37ºC for 48 hours.

The bacterial isolates were further sub cultured on TCBS plates in order to obtain pure culture. Pure isolates were maintained in TCBS slants at 4°C for further studies.

Identification of Vibrio sp.

The colonies were observed and recorded with respect to color, shape, nature and pigmentation of colony. The bacterial isolates were Gram stained to observe morphology. Motility test was performed to observe the motility. The bacterial isolates were biochemically characterized by performing catalase, oxidase, nitrate reduction, indole, methyl red, voges proscauer's, Simmons citrate, Urease, H_2S production, Nitrate reduction, Starch hydrolysis and carbohydrate fermentation test (Cappuccino and Sherman, 1996).

Antibiotic susceptibility test

The *Vibrio* sp. isolate was screened for their susceptibility towards the standard antibiotics drugs such as bacitracin, chloramphenicol, streptomycin, rifampicin, penicillin, ciprofloxacin and ampicillin. The antibiotic susceptibility test was performed by disc diffusion method. The *Vibrio* sp. isolates were inoculated in alkaline peptone water and incubated for 8 hours at 37ºC. The turbidity of the broth was adjusted at 0.5 McFarland turbidity standards and lawns cultured on TCBS plates by using sterilize cotton swabs. The antibiotic discs were placed on agar surface by using sterilized forcep. Plates were incubated at 37°C for 48 hours. The plates were examined for the presence of zone of inhibition. Experiment was performed in triplicates.

Isolation of actinobacteria

The actinobacteria were isolated from water samples by serial dilution and spread plate method on Starch casein agar (SCA). Water samples were serially diluted in sterilized distilled water to get a concentration range from

10^{-1} to 10^{-6}. A volume of 0.1 ml of each dilution was transferred aseptically to SCA plates. The Petri plates were rotated clockwise and anticlockwise to spread the sample uniformly. The plates were incubated at room temperature for 7 days. The plates were observed for the presence of actinomycetes (Karthik et al., 2010).

The actinobacteria isolates were further subcultured on SCA plates in order to obtain pure culture. Pure isolates were maintained in SCA slants at 4°C for further studies.

Identification of actinobacteria

The colony of actinobacteria isolates were observe under a high power magnifying lens and colony morphology was noted with respect to colour, mycelium, size, shape, pigment. Gram staining was performed to check the morphology of the cells. Spore chain morphology was identified by cover slip culture technique. Actinomycetes isolates were biochemically characterized by carbohydrate fermentation test (Nonomura, 1974).

Fermentation process

Actinomycetes isolates were inoculated in 100 ml of Starch casein broth in Erlenmeyer flasks. Flasks were lodged on the flask shaker at a speed of 110 rpm at room temperature for 7 days. After fermentation, the medium was harvested and centrifuged to remove growth and debris. Filtrate was collected in a sterilized screw cap bottle and stored in freeze at 4°C for further use.

Isolation of antibacterial metabolites

The bioactive compounds were recovered from the harvested medium by solvent extraction method. The filtrate was mixed with methanol, ethyl acetate, petroleum ether and ethanol (1:1 v/v) and shaken vigorously for 1 hour in a solvent extraction funnel. The solvent phase that contains the extracted compound was separated from the aqueous phase (Umasankar et al., 2010).

All the solvent extracts were concentrated by using rotary evaporator and dried using lyophilizer. The powered extract was mixed in 10 % DMSO to get a concentration of 1000 µg/ml.

Positive and negative control

Ciproflaxin (10 µg/disc), chlorampenical (10 µg/disc) was used as positive control, 10% DMSO was used as negative control.

In vitro Vibriocidal activity

The Vibriocidal activity of the actinomycetes isolates was determined by agar well diffusion method (Gaurav et al., 2010). All the isolates of *Vibrio* were inoculated in alkaline peptone water and incubated for 8 hours at 37ºC. The turbidity of the broth was adjusted at 0.5 McFarland turbidity standards. The Vibrio cultures were inoculated on TCBS plates using sterilized cotton swabs. In each of these plates, wells were cut out using a sterilized gel borer. Using a micropipette, 100 µl of each Actinobacteria supernatant was poured in to each well. Plates were incubated at 37°C for 48 hours. After the incubation, all plates were examined for the presence of zone of inhibition around the Wells. Each experiment was performed in triplicates.

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Determination of relative percentage inhibition

The relative percentage inhibition with respect to positive control was calculated by using the following formula (Ajay et al., 2002; Gaurav *et al*., 2010).

Relative percentage inhibition of the test extract = $\frac{100 \times (X - Y)}{(Z - Y}$

where, **x:** area of inhibition of the test extract
y: area of inhibition of the solvent
z: area of inhibition of the standard drug

The area of the inhibition was calculated by using area = πr^2;

where, r = radius of zone of inhibition.

Results and discussion

Vibrio is Gram negative, motile bacteria causes vibriosis in fish and other aqua farm animals. Vibriosis stayed a worst threat among many of the fishery forms/aqua forms, causing the death of the fishes resulting in great economic lose. In this study we studied the Biological control of vibriosis by the antagonistic activity of actinobacteria.

The *Vibrio* sp. was isolated on TCBS agar medium, as TCBS is the selective medium for the isolation of *Vibrio* sp. The isolates were characterized on the bases of microscopic, cultural and biochemical characters (Table 1), the isolate was identified as *Vibrio* sp (Cappuccino and Sherman, 1996).

The isolates of *Vibrio* sp. was checked for their susceptibility toward standard antibiotic drugs. The *Vibrio* sp. was find sensitive to ciproflaxin, chloramphenicol and streptomycin, whereas, resistant toward bacitracin,

rifampicin, penicillin and ampicillin. Ciproflexin showed the maximum zone of inhibition (33.33±1.3). The results of the study are reported in Table 2 and Fig. 1.

Actinobacteria were isolated on the SCA medium from the same water sample. The actinobacteria isolates were characterized on the bases of their morphology, cultural and biochemical characters (Table 3). The four isolates with different colony morphology were characterized as *Streptomyces* sp.

The actinobacteria isolates were screened for the vibriocidal property and all four isolates showed great vibriocidal activity against the *Vibrio* sp. the results are expressed as mean ± SD of three values of the zone of inhibition (Table 4 and Fig. 2).

The antagonistic activity of actinobacteria was compared with standard antibiotic drugs and relative percentage inhibition was calculated, the results are listed in table 5 and Figure 3. The results of RPI are expressed in percentage.

Further research can be carried out in the studies on biological control of vibriosis by carrying out the bio control procedure *in vivo* and also by performing the molecular characterization of the isolates.

Table 1. Characterization of *Vibrio* sp.

	Characters	**Result**
Cultural characters	Colony morphology	Yellow coloured, slimy and slightly elevated colonies.
Microscopic characters	Gram staining	Gram negative rods
	Motility	Motile
Biochemical characters	Indole	Negative
	Methyl Red	Positive
	Voges proskauer	Negative
	Citrate utilization	Positive
	Catalase	Positive
	Oxidase	Negative
	Urease	Negative
	Nitrate reduction	Positive
	Starch hydrolysis	Positive
	H_2S production	Negative
	Glucose	Positive
	Sucrose	Positive
	Maltose	Positive
	Fructose	Positive

Table 2. Antibiotic susceptibility test.

Antibiotics	Antibiotic susceptibility test	
	Zone of inhibition (mm)	Susceptibility of drug
Ciproflexin	33.3±1.13	Sensitive
Chlorempenical	26.6±0.57	Sensitive
Streptomycin	27.6±0.57	Sensitive
Penicillin	00.0±0.00	Resistant
Rifampcin	00.0±0.00	Resistant
Ampillicin	00.0±0.00	Resistant
Bacitracin	00.0±0.00	Resistant

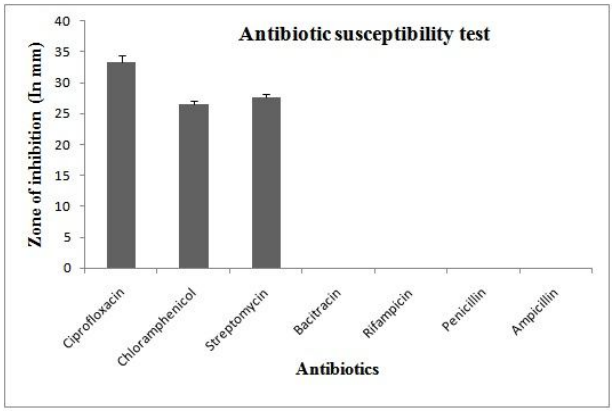

Fig. 1. Antibiotic susceptibility test.

Table 3. Characterization of *Streptomyces* sp.

	Characterization	S1	S2	S3	S4
Colony morphology on SCA medium	a) Aerial mass color	White	Brown	Grey	Sandal
	b) Reverse side pigment	Brown	Black	Black	Brown
Morphology	Gram staining	GP	GP	GP	GP
	Spore chain morphology	Spiral	RA	RA	RF
Carbon utilization test	Xylose	+	++	+	+
	Rhamnose	+	–	++	–
	Raffinose	+	–	+	+
	Arabinose	–	+	+++	+
	Sucrose	++	–	++	+
	Fructose	+	+	++	++
	Mannitol	+++	–	+	+
	Inositol	+	–	+	++

GP:Gram positive

Table 4. Vibriocidal activity of *Streptomyces* sp. Isolates.

	Zone of inhibition (mm)				
	ME	**EE**	**PE**	**ET**	
S1	54.6±0.57	61.0±1.73	12.3±0.57	14.0±0.0	
S2	23.6±1.15	39.6±0.57	0.0±0.0	8.3±0.57	
S3	26.0±1.0	23.6±0.57	0.0±0.0	0.0±0.0	
S4	27.6±0.57	31.3±1.15	0.0±0.0	0.0±0.0	
PC	–	–	–	–	33.3±1.13
NC	–	–	–	–	0.0±0.0

ME: methanol extract, EE: ethyl acetate extract, PE: petroleum ether extract, ET: ethanol extract, PC: positive control, NC: negative control
S1, S2, S3 and S4 are the actinobacteria isolates

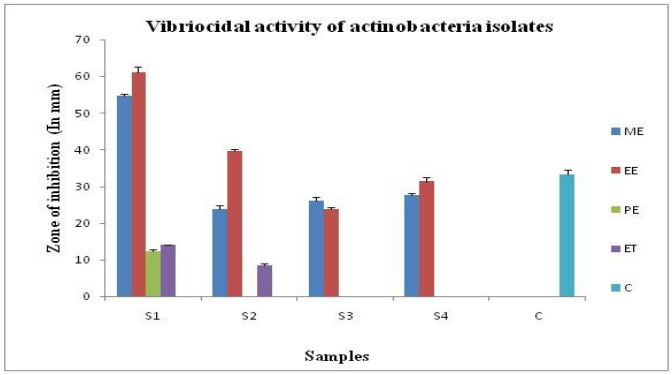

Fig. 2. Vibriocidal activity of *Streptomyces* sp. Isolates.
ME: methanol extract, EE: ethyl acetate extract, PE: petroleum ether extract, ET: ethanol extract, C: positive control
S1, S2, S3 and S4 are the actinobacteria isolates

Table 5. Relative percentage inhibition in comparison of ciprofloxacin.

	Relative percentage inhibition (in %)			
	ME	**EE**	**PE**	**ET**
S1	268.84	335.56	13.64	17.67
S2	50.22	141.41	0	6.21
S3	60.96	50.22	0	0
S4	68.69	88.34	0	0

ME: methanol extract, EE: ethyl acetate extract,
PE: petroleum ether extract, ET: ethanol extract
S1, S2, S3 and S4 are the actinobacteria isolates

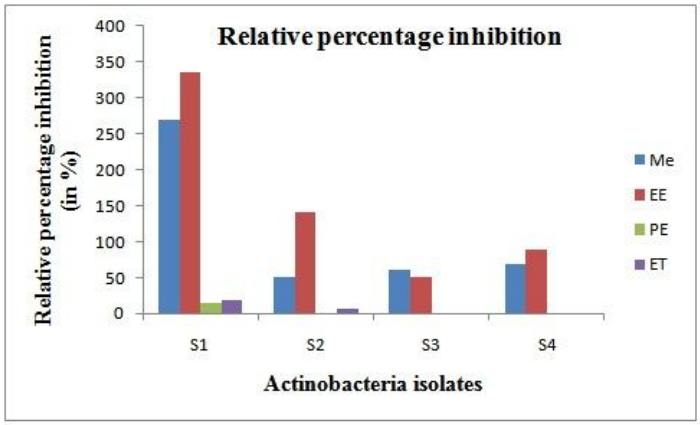

Fig. 3: Relative percentage inhibition in comparison of ciprofloxacin.
ME: methanol extract, EE: ethyl acetate extract
PE: petroleum ether extract, ET: ethanol extract
S1, S2, S3 and S4 are the actinobacteria isolates

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