EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 12
509-515
Year: 2018

EVALUATION OF EXO POLYSACCHARIDE PRODUCTION BY MARINE BACTERIUM *VIBRIO HARVEYI*

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Article Received on 03/11/2018 **Article Revised on 24/11/2018** **Article Accepted on 14/12/2018**

ABSTRACT
Vibrios are one of the most important pathogens for reared aquatic organisms such as penaeid shrimps, several fish species and molluscs, and also for corals. Some of the luminescent Vibrios, which include *Vibrio cholerae, V. fischeri, V. harveyi, V. logei, V. splendidus, V. mediterranei, V. orientalis, Photobacterium leiognathi* and *P. phosphoreum* have been implicated principally with disease outbreaks in shrimp larviculture facilities, and to a lesser degree in grow-out ponds. *V. fischeri* colonizes the squid light organ and provides bioluminescence for the squid to use as counter shooting in order to evade predators. In return the bacteria gain a protected nutrient environment. The discovery of N-acylhomoserine lactones as quorum sensing signals was first made in *V. fischeri* is the paradigm of gram-negative QS systems even though it is not fond in all vibrios.Fish pathogen *Vibrio harveyi* was isolated from naturally diseased fishes collected from local fish market. The presence of disease symptoms on represented by skin disease and scale detachment. Some fishes shows enlargement of internal organs. Production of exopolysaccharide (EPS) by *Vibrio harveyi* were determined with different cultivation parameters such as different NaCl, pH and utilization of carbon sources. Among the parameters screened pH 6 and 7, 5% NaCl and the carbon source sucrose highly influence the growth and EPS production by *Vibrio harveyi.Vibrio harveyi* also screened for their response to commercially availed antibiotics such as Neomycin, Ampicillin, Penicillin-G and Vancomycin. Among the antibiotic Neomycin strongly inhibit (10mm) of fish pathogen *vibrio*.Molecular characterization of the *Vibrio harveyi* isolates was also done by the sequence analysis and submitted at accession number was obtained.

KEYWORDS: *Vibrio harveyi*, Fish pathogen, Neomycin, EPS (Exopolysaccharides).

INTRODUCTION
Vibrio
Vibrios are one of the most important pathogens for reared aquatic organisms such as penaeid shrimps, several fish species and molluscs, and also for corals. Some of the luminescent Vibrios, which include *Vibrio cholerae, V. fischeri, V. harveyi, V. logei, V. splendidus, V. mediterranei, V. orientalis, Photobacterium leiognathi* and *P. phosphoreum* have been implicated principally with disease outbreaks in shrimp larviculture facilities, and to a lesser degree in grow-out ponds. Only a few *Vibrio* species have been proven to be pathogens for shrimps; the closely related species *V. harveyi* and *V. campbellii* have caused disease in shrimp larvae, while *V. penaeicida* and *V. parahaemolyticus* affected juveniles and adults **(Ben-Haim *et al.*, 2003)**.

Bacteria belonging to the *Vibrionaceae* family are widespread in the marine environment. Today 128 species of vibrios are known. Several of them are inflames for their pathogenicity or symbiotic relationships. Despite their ability to interact with eukaryotes, the vibrios are greatly under explored for their ability to produce bioactive secondary metabolites and studies have been limited to only a few species. Most of the compounds isolated from vibrios so far are non-ribosomal peptides or hybrids with example of N containing compounds produced independent of non-ribosomal peptide synthetass. Through covering a limited chemicals space, vibrios produce compounds with attractive biological activities, including antibacterial, anticancer and anti-virulence activities **(Maria Mansson *et al.*, 2011).**

Vibrios are particularly abundant on the surface of marine macro organisms such as coral, fish, sea grass, sponges and zooplankton, where they form commensal, symbiotic, or pathogenic association**.** A particularly famed *Vibrio* is *V. fischeri* known for its light organ symbiosis with the Hawalian squid *Euprymna scolopes* **(Thompson *et al*., 2004)**.

Marine *Vibrio* and its bioactive potential

The genus vibrio is a major taxa of culturable heterotrophic bacteria commonly found in marine and estuarine environments. *Vibrio sp*., are characterized by being small and are comma-shaped rods. The genus *Vibrio* includes several pathogenic species that can cause disease in human as well as marine animals. The first *Vibrio* species was discovered by the Italian physician Filippo Pacini i.e., *V. cholera* as the causative agent of cholera. Cholera had killed thousands of people in England between the 1830s and 1850s. The genus is classified into the family *vibrionaceae*, which, as of 2012, is the only family in the order vibrionales. It includes the genus *Vibrio* and other 10 genera: Aliivibrio (6 species), Allomonas (1 species), Beneckea (11 species), Catenococcus (1 species), Enterovibrio (4 species), Grimontia (1 species), Listonella (3 species), Lucibacterium (1 species), Photobacterium (24 species), and Salinivibrio (4 species) **(Bragaru A *et al*., 2011)**.

Vibrio are widely distributed in aquatic environments from brackish to deep sea water, commonly found associated with marine organism and as the important pathogen to aquaculture farm animals and human consuming contaminated seafood grown in polluted water. They are thought to evolve from marine environment as they require sodium as an important growth factor. *Vibrio* are frequently deleted in summer than winter **(Brain Austin.2011).**

Exopolysaccharides and quorum-sensing regulates biofilm formation and influences attachment to biotic surfaces in a number of *Vibrio* species. The presence of multiple signaling pathway for regulating EPS and biofilm formation indicates that different pathways operate in diverse environments or selection of different strains occurs under certain condition. They also exhibit an elaborate and highly developed starvation adaptation mechanism **(Heithoff and Mahan, 2004)**.

Fish on marine environment

Experimental studies using live, intact creatures have played, and continue to play, an essential role in developing new knowledge and better understanding of life processes, life form, and the environment in which these forms and processes occur. The enormous evolutionary radiation of fishes comprises at least 25,000 species. Fishes exist in myriad forms and have developed in any unique physiological, behavioral, and ecological specializations. Fishes occupy a variety of niches in virtually every kind of aquatic habitat. Understanding their biology simply cannot be accomplished in the absence of experimentation with live, intact animals.

Among the reasons for studying fishes are the following: fishes are useful indicators of environmental quality and ecological integrity; fishes provide an important source of food for many of the world's humans; catching and observing fishes are very popular and economically important recreational and commercial activities for million people; the unique adaptation and physiological specializations of fishes make them especially suitable for use as physiological and the unique adaptations and functions in the world's ecosystems, an understanding that cannot be accomplished without accurate and detailed knowledge of the biology of fishes **(Bethesda, 2014).**

Bacterial disease of fish

Epidemics of bacterial diseases are common in dense population of cultured food or aquarium fish. Predisposition to such outbreaks frequently is associated with poor water quality, organic lading of the aquatic environment, handling and transport of fish, marked temperature changes, hyporia or other stressful conditions. Most bacterial pathogens of fish are aerobic, gram negative, rod. A number of bacteria produce a similar syndrome, generically referred to as hemorrhage septicemia and characterized by external reddening and hemorrhage in the peritoneum, body wall and viscera. Morbidity and mortality are highly variable, depending on predisposing conditions such as low dissolved oxygen, other water quality problems, handling stress or trauma. Ulcerative lesions are common as disease progresses, and mortality can be significant if stress is not controlled. Antibiotic therapy is recommended if fish are drying. Common bacterial isolates from affected fish include *Aeromonas* spp and *Pseudomonas* spp which are common in freshwater animals and *Vibrio* spp which are more commonly isolated from marine fish **(Ruth Francis, 2011).**

Importance of EPS

The exopolysaccharides (EPS) secreted from bacteria might plays a potential role in improvement of agricultural productivity, which is yet unexplored. EPS have ubiquitous nature of alginates, which is widely known for its industrial applications. It is used in plant tissue culture to produce artificial seeds, immobilizing enzymes by entrapment, as food and wound dressing material. EPS secreted from bacteria plays a key role in encystment of artificial seeds, which protects against desiccation and predation by the protozoon's, phage attack, and also affect the penetration of antimicrobial agents and toxic metals. However, its application in agriculture with respect to its role in plant growth and activity is less explored. The exo-polysaccharides (EPS) secreted from bacteria has shown enormous effect on various soil properties and plant productivity. EPS possess unique water holding and cementing properties. Therefore, it play a vital role in the formation and

stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation.

MATERIALS AND METHODS

Sample collection

Fishes of marine origin were randomly collected from local fish market after immediate fishing at Tuticorin. All the samples were transferred individually into new polythene bags and immediately transported to the laboratory in a portable pack with ice cold condition within 5 hours of collection (L. Banupriya *et al.*, 2014).

Sample processing for bacteriological examination

Bacteriological analysis of collected marine fish were performed within four hour of sampling. The samples such as fish were washed thoroughly with sterile distilled water prior to bacteriological examination. Fish samples were cut into small pieces using sterile scissors. Approximately 25g of each sample was homogenized and serially plated on TCBS plates, incubated for 24 hours al 37^0 C (L. Banupriya *et al*., 2014).

Phenotypic characterization of the test isolates

The strains were characterized based on their growth on selective media (TCBS agar), colony characteristics, gram staining, motility and series of biochemical tests, such as Hugh-Leif son's test, ONPG (o-nitro phenyl β-D-galactoside), *V. parahaemolyticus* sucrose agar, alkaline peptone water, tryptone sucrose tetrazolium agar base (TSTA), saline nutrient agar.

Screening of *Vibrio harveyi* for exopolymer production

Vibrio bacterial culture for EPS production, grown and maintained as batch culture in 200ml of MSM medium supplemented with NaCl to a final concentration of 1-5% (w/v), 0-2% glucose in 500ml of Erlenmeyer flask on a rotary shaker at 28 ± 2^o C for 2 days. The pH of the medium was adjusted to 7-0 with 1 mol 1^{-1} NaOH. The medium was dispensed in 500ml Erlenmeyer flasks and inoculated with 2% (v/v) of an overnight grown culture in the same medium at room temperature on a rotary shaker at 160 rev min^{-1}. The subsamples of 5 ml aliquots were drawn at regular interval and the turbidity measurement of bacterial growth and EPS production were determined.

Extraction of *V. harveyi* exopolysaccharide

The culture volume of *V. harveyi* isolate 200 ml was centrifuged at 15,000 g for 20 min at 4^oC. the cell pellets were freeze dried and weighed. The supernatant were pressure-filtered through cellulose nitrate filters with the following pore size: 0.8, 0.45 and 0.25 µm. EPS were precipitated from the final filtrate after the addition of three volumes of cold ethanol and the solution was chilled at 4^oC overnight. The resulting precipitate was recovered by vacuum filtration through a scintered glass apparatus. An additional 100 ml of ice cold ethanol was added to the filtrate and the solution was placed at -20^oC overnight. The precipitate was recovered as above. The precipitate were washed with 70-100% ethanol- water mixture.

Molecular characterization of efficient strains

Molecular characterization of most efficient bacterial isolates was done by sequencing of their 16S rRNA gene.

PCR amplification of 16S rRNA gene

The 16S rRNA gene of the target endophytic actinobacterial isolate was amplified by using universal eubacterial primers as reported by Heddi. (1998). The base sequences of the primers used are presented. These were custom synthesized (Sigma, Pvt. Ltd.).

Agarose gel electrophoresis of PCR products

The amplified PCR product was resolved by electrophoresis using 1 per cent agarose gel in 1X Tris acetate EDTA buffer (2 M Tris base, 57.10 ml acetic acid and 0.5 M EDTA, pH 8.0, 50 X). Agarose gel was mixed with ethidium bromide (0.5 µg/ ml) before pouring. 1 kb DNA ladder (Bangalore Genei) was used as a marker. The gel was run at 120V for 45 minutes using Bangalore Genei Power Pac system. The ethidium bromide stained gel was viewed and image captured using gel documentation system.

Nucleotide sequence analysis

The 16S rDNA sequences of different bacterial isolates were BLAST (Basic local alignment search tool) searched against the sequences of 16S rRNA of bacterial isolates available in the Genbank Nucleotide Database (http://www.ncbi.nih.gov/blast) for sequence comparison. The sequences were aligned by Clustal W program using website http://www.ebi.ac.uk/clustalw/ and pair wise per cent nucleotide sequence similarities between the potential PGPS isolates and other selected bacterial sequences were determined.

Phylogentic Tree Construction on Potential PGPS strains

Phylogenetic analysis was performed with neighbor-joining method using program in Molecular Evolutionary Genetics Analysis (MEGA) version 4. Confidence limits on grouping were done by the bootstrapping technique (1,000 repeats). Sequencing of bacterial isolate done at Progen Biotech Salem.

RESULTS AND DISCUSSION

Cultivation profile of *Vibrio harveyi*

The fish pathogen *Vibrio harveyi* was grown on Zobell marine broth medium supplemented with glucose to evaluate their growth profile as well as production EPS with reference to their glucose utilization.

A total of 72 isolates of *Vibrio* in 6 species including *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. harveyi, and V. mimicus* were examined. The results

revealed that all isolates were expressing multiple antibiotic resistance. (Raissy *et al*. 2012).

Antibiotic susceptibility

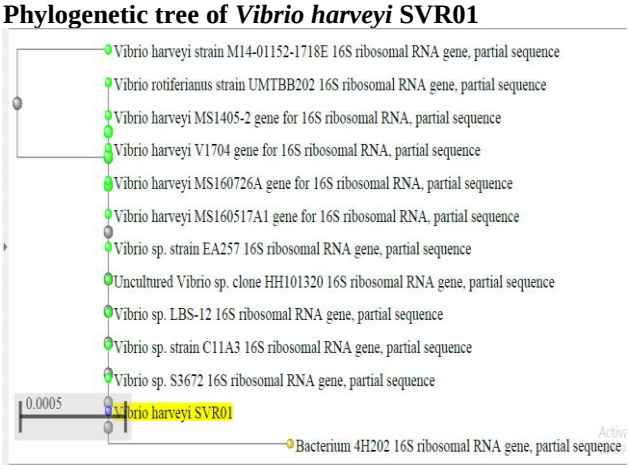
Vibrio harveyi also screened for their response to commercially availed antibiotics such as Neomycin, Ampicillin, Pencillin-G and Vancomycin. Among the antibiotic Neomycin strongly inhibit (10mm) of fish pathogen *Vibrio.* (Plate 2).

An investigation was undertaken to determine the extent of antibiotic resistance exhibited by *Vibrio harveyi*, isolated from diseased *Penaeus monodon*, collected from culture ponds located in East Godavari District of Andhra Pradesh. A total of 159 isolates of *Vibrio harveyi*, of which 110 are from four Modified-Extensive ponds (ME1, ME2, ME3, ME4) and 49 are from four Semi-Intensive ponds (SIA, SIB, SIC, SID), were screened for their susceptibility to 22 antibiotics. All the isolates from ME and SI ponds were resistant to penicillin G and 100% susceptibility was observed in the case of all the isolates of ME ponds towards Ciprofloxacin and Norfloxacin.

Molecular characterization of *Vibrio harveyi*

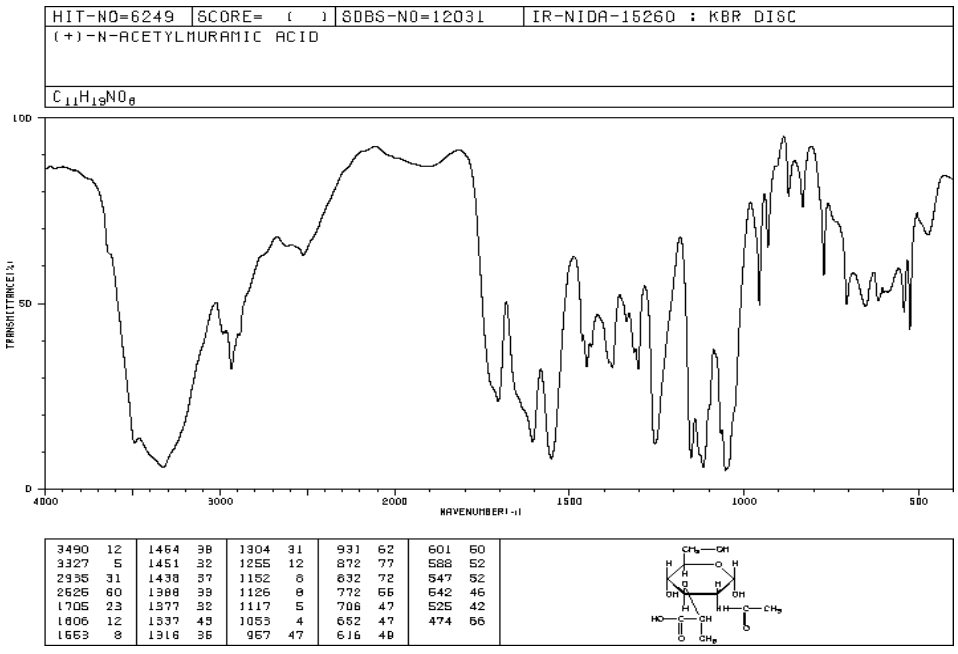
Taxonomical identification of potential bacterial strain identified by phenotype includes morphological, physiological and biochemical properties of the microorganism. In order to, the biochemical tests in bacterial identification include the relationship with oxygen, fermentation reactions and nitrogen metabolism. Other tests may be performed as appropriate, depending on the bacterial strains studied. However, the genotypic characters was analysed by 16S rRNA molecule comprises of variable and conserved regions, and universal primers for the amplification of full 16S rRNA gene are usually chosen from conserved region while the variable region is used for comparative taxonomy. The 16S rRNA gene sequence is deposited in databases such as Ribosomal Database Project II (http://rdp.cme.msu.edu/) and GenBank (http://www.ncbi.nlm.nih.gov/). Sequences of related species for comparative phylogenetic analysis can also be retrieved from these databases.

Phylogenetic tree of *Vibrio harveyi* SVR01



EPS characterization by FTIR

The FTIR spectrum of the purified EPS of *V. harveyi* strain VB23 revealed characteristic functional groups, such as broad stretching hydroxyl group at 3412 cm^{-1} and a weak C-H stretching peak of methyl group at 2925 cm^{-1}. Further, an asymmetrical stretching peak was noticed at 1636 cm^{-1}, which corresponds to carboxyl groups and a peak at 1546 cm^{-1} could be assigned to amide II. A broad stretching of C-O-C, C-O at 1000–1200 cm^{-1} corresponds to the presence of carbohydrates. Specifically, the peaks at 1000– 1125 cm^{-1} range ascertain the presence of uronic acid, o-acetyl ester linkage bonds.



CONCLUSION

Vibrio harveyi is a serious pathogen for a wide range of marine animals. Associated with intestinal microbiota of marine animals and highly pathogenic to aquatic fauna. Exopolysacharides play an important role in their cell where they persist and involved in the numerous biological activity including interaction and pathogenesis and they are highly species specific in nature. Their abundance in each bacterial cells vary with the culture condition and physiological state of the organism. Most of the polysaccharides used in industry are extracted from plants algae and animals. Now a days polysaccharides from marine microbial population have significant interest because of their diver's bioactive potential and also in industrial and biomedical applications. *Vibrio harveyi* is the cause of vibriosis and is the most common pathogenic bacteria in the cultured shrimp in aquaculture. Since complete characterization of the this bacterial isolate were done in this present investigation will give an insight for the further identification of the pathogenesis caused by the gene of interest and also genes involved in exopolysacharide production will give a tool to metabolic engineering for the overproduction of the exopolysccharide for biomedical application.

ACKNOWLADGEMENT

The authors are would like to thank and acknowledge of Centre for Marine Science and Technology, Manonmaniyam Sundharanar University, Sri Paramakalyani College for giving facility to carry out this work

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