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Studies on characterization and antibacterial potential of epibiotic bacteria from marine animals

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Abstract

Life in our planet is dependent upon the ocean, which are the sources of wealth, opportunity and abundance. About 70 % of the earth surfaces of this planet are covered by salt water. They provide us food, energy and water and sustain the livelihoods of hundreds of millions of organisms. Open Ocean can be subdivided vertically and horizontally. The marine environment covers a wide thermal, pressure and nutrient ranges and it has extensive photic and non-photic zones. The marine environment for exceeds that of the terrestrial environment, research in to the use of marine natural agent is still in its infancy. In the early sixties, the increasing needs for drugs able to control new illnesses or dead set against strains of microorganism stimulated to look for unconventional new sources of bioactive natural products. Recently, studies have also suggested that some bioactive compounds isolated from marine organisms have been showed to exhibit anti- cancer and other pharmacological activities. Most recently marine fauna associated microbial population excert unique biological activity. Lobster and Jelly fish were collected from Muthupettai is one of the Villages in Tiruppullani Taluk in Ramanathapuram District in Tamilnadu State and used as sources for the isolation of associated bacterial population. This study investigates the bacterial diversity in the lobster (*Pannilurus homarus*) and Jelly fish (*Rhizostoma*. sp).

The conditions for optimal growth and salt- loving were established for the epibiotic bacteria. Over all twenty six isolates were obtained for two samples from each animal. Among these isolates totally eight strains produced pigmented colonies. Biochemically, the isolates

resembled the genus *Vibrio, Pseudomonas, Aeromonas, Bacillus* and Altromonas. All of the salt concentration optimum growth of 1%, 20%, 40% and 60% NaCl for growth. Based on the salt concentration optimum growth of isolates observed in 20% of salinity. Epibiotic bacterial isolates on different cultivation medium, growth on different pigmentation were observed. The antibacterial activity of marine animal (lobster, jelly fish) epibiotic marine bacterial metabolites against bacterial pathogens was determined by agar diffusion method, all the 26 isolates exhibited varied ranges of antibacterial activity. The zone of inhibition ranged between 1-13mm. The minimum inhibitory and minimum bactericidal assay was performed with metabolites of selected bacterial isolates which exhibit maximum antibacterial activity and screened against test organisms at different concentration, Such as 10µg, 100µg and 1000µg. Time kill assay was performed against most susceptible bacterial pathogens by using potential epibiotic bacterial metabolites with 1x, 4x and 16x concentration for 12 hours.

Introduction

The Ocean encompasses diversified macro and microorganism. Marine microbial technology is the emerging field in which marine microorganisms are act as source to extract novel metabolite and to synthesis the compounds in large scale through fermentation technology or by means of mass cultivation. The importance of marine bacteria and as sources of valuable bioactive metabolites is very well established for more than 20 years (Akagawa-Matsushita *et al.,* 1992*).* The marine environment, animate and inanimate surfaces are rapidly covered with an organic layer as a result of molecular adsorption of dissolved organic matter, and colonization by bacteria, protozoa, algae and fungi (Wahl and Clare e*t al.,*1992). Provided this association occurs between living organisms, this phenomenon is referred to as epibiosis. Bacterial films are act as quarum sensors for larval settlement of many benthic marine invertebrates (Maki and Wieczorek *et al.,* 1998) bacterial epibiosis may promote subsequent colonization by macroorganisms. Novel secondary metabolites from marine epibiotic bacteria are attracting attention because of the growing demand for new compounds of natural origin, having potential applications in different fields. Such bacteria living on the surfaces of marine organisms have been documented to include a percentage of antibiotic producing bacteria higher than that observed in free living bacteria isolated from marine ecosystem (Lemos *et al.*, 1986 and Zheng *et al.,* 2005). In the last 25 years, marine organism's algae, invertebrates and microbes have provided key structures and compounds that proved their potential in several fields, particularly as new therapeutic agents for a variety of diseases. During the past

thirty to forty years, numerous novel compounds have been isolated from marine organisms and many of these have been reported to have biological activities, some of which are of interest from the point of view of potential drug development (Fenical, 1993). Special interest is pointed to the peptides and modified peptides with origin from marine microorganisms. An intriguing group of bioactive peptides are cyclic peptides, which exhibit a wide range of biological activities including cytotoxic, antibiotic and antifungal activity (Ireland *et al.,* 1993). The marine environment also considered as a largely unexplored source for isolation of new microbes (bacteria, fungi, actinomycetes, microalgae-cyanobacteria and diatoms) that are potent producers of bioactive secondary metabolites. Lobster are small decapods crustaceans, closely related to the slipper lobsters and spiny lobsters (Ferran palero *et al.*, 2008). Lobsters have ten walking legs, the front three pairs bear claws, first of which are larger than others. Lobsters live in a muddy environment at the bottom of the oceans. Lobsters are invertebrates, with a hard protective exoskeleton like most arthropods, lobsters must molt in order to grow, which leaves them vulnerable. Spiny lobsters commonly called as Rock lobster (*Palinuridae*), which are one of the world's most valuable seafood with high market appeal in Asia, Europe and America. (Phillips, 2005). The discovery of potential source of cnidarians and the fact that they were indicated as early as the 1970s as a potential source of natural bioactive compounds of pharmacological concern useful to develop new drugs or biomedical materials (Mariscal *et al.*, 1994). Scyphozoan jellyfish belonging to the order rhizostomeae are harvested for food. About 12 of the approximately 85 species are harvested and sold on international market. The Muttai chori jellyfishes are invertebrates belonging to phylum cnidarians. Muttai chori jellyfish is a whit is medusa with flattened bell, violet or pink margins provided with several tentacles and four oral arms with fringing in the lower portion, four half moon shaped gonads can be easily seen Muttai chori jellyfish is a cosmopolitan species living in temperate and temperate cold waters, with temperatures varying from -0.5°C to 30°C, and carry out vertical migrations under influence of light. Muttai chori jellyfish Size approx 50cm, the jelly fishes are commonly found in the inshore waters of Pondicherry and Tamil Nadu coasts. At present processed jelly fish are exported from Pondicherry and South Arcot in Tamil Nadu only. The exports were made mainly to Japan, Thailand and Hong kong (Kelecom *et al.,* and Kideys *et al.*, 2001). Since marine organism possesses diversified biological activity, in this present investigation marine animal associated marine bacterial population were evaluated for their bioactive potential.

Materials and Methods

Collection site

Lobster and jellyfish were collected from Muthupettai site. This site occurs in the region of south eastern coast of Tamilnadu India.

Description of study Area

A study was carried out in the Muthupettai to evaluate the epibiotic bacterial population of marine animals. Muthupettai is 12.1 km far from its District Main City Ramanathapuram. It is 447 km far from its State Main City Chennai. Nearest Towns are Ramanathapuram (14.4 k.m.), Bogalur (20.6 k.m.), Mandapam (30.7 k.m.), Mudukulathur (37.6k.m.), Alangulam, Ekkakudi, Kalimankundu, Kanjirangudi A/B, Koraikuttam, Kudakottai. The villages along with this village in the same Tiruppullani Taluk. It spreads to an area of about 6,800ha. A total of 39 species of macro benthic fauna were recorded. Among them, 17 species belonged to polychaetes, 10 species to crustaceans, 10 species to molluscs, single species to sipunculida, fish larvae and hermit crab. The macro fauna population density varied from 17 to 409 individuals/sq m, respectively.

Collection of marine animal

Lobster (*Pannilurus homarus*) and jellyfish (*Rhizostoma.* sp) were collected from Muthupettai region of Tamilnadu. Collected samples were transferred in zip-lock bags on ice. The lobster collected from rock side. Jelly fish from coastal region, which help of the fisherman.

Sample preparation

The Lobster and jelly fish were taken from the ice box. The tissue samples were suspended in sterilized distilled water and kept in shaker for 30min to detached epibiotic bacterial population.

Isolation of marine animal associated marine bacterial population

The samples were taken out from the shaker and subjected for serial dilutions up to 10^{-8}. A volume of 0.1 ml of respected diluted sample was plated on Zobella marine agar (Mool, Himedia, Mumbai), Halophilic agar medium and the plates were incubated at 28°C for three days. Single bacterial colonies with different morphological characteristics, such as colony

elevation, color, shape, margin and surface texture were analyzed and transferred on to fresh zobella marine agar plates.

Morphological Identification

Bacterial cultures grown on zobella marine agar were examined based on their gram reaction by conventional staining techniques (Harvell *et al.*, 1999). A series of selective mediums which are marine oxidase fermentation agar (MOF), Casein soya meal agar (CASO), Sea water yeast extract peptone agar, Trypticase soya agar (TSA), Nutrient agar + Sea water medium, YEPG medium, Minneral medium, Sea water medium, Pseudomonas isolation agar, Marine agar + Congo red, MacConkey agar, TCBS agar, Eosin Methylene Bule agar were used to characterize these isolated (Giovannoni *et al.*, 2000).

Biochemical test for marine animal associated organism

Isolated strains were characterized by conventional microbiological methods (Rohwer *et al.,* 2003). Involving following characteristics assays, Catalase test, Oxidase test, Nitrate reduction test, Indole production test, Degradation of starch, Urea, Casein, Gelatin, Gas and Acid production from D-sucrose, D-maltose, D-lactose, D-galactose, D-arabinose, D-maltose, D-fructose, D-mannitol and dextrose, utilization of citrate, Triple sugar iron agar. In this assay, the bacteria were grown on the standard preparation protocol with minor modification. All media used were added with filter sterilized sea water using nitrocellulose membrane to fulfill the halophilic requirement of marine bacteria. The pH was adjusted according to the type of media used.

Bio assay of antibacterial activity from marine bacteria

All isolated marine bacteria were screened for antimicrobial activity, using commom bacterial test pathogens including *Escherichia coli, Staphylococcus aureus, Klebsiella.* sp*, Proteus.* sp and *Pseudomonas.* sp as test microorganisms. Antimicrobial activity was assayed into well cut of using method. The dried crude extracts were dissolved in DMSO to a concentration of 100 mg/ml. The samples were used to saturate the anti microbial well cut with a period.

Broth tube dilution method to determine minimum inhibitory concentration (MIC)

The start of research on marine bacteria used a broth tube dilution method as recommended by the NCCLS to determine minimum inhibitory concentrations (MIC). Marine bacteria

powder in MHB (25 mg/ml) was serially diluted to achieve the following concentration; 10µg, 50µg, 100µg, 200µg and 1000µg. One millilitre bacterial suspension (1×10^5 CFU/ml) was incubated with 1ml marine bacteria in sterile 5ml bijoux at 37° C for 16h. The MIC was taken as the lowest concentration of antimicrobial agent that inhibited bacterial growth. Growth inhibition was determined visually (Eisin shakir, 2007).

Minimum bactericidal concentration (MBC)

For each MIC broth tube without visible growth, 10 µg from each tube was plated onto nutrient agar plates. Following overnight incubation, the plates were examined for colony growth. Lack of growth indicates that the tested drug is bactericidal, while growth indicates that the drug is bacterio static at a particular dilution. The MBC is the lowest concentration that kills the bacteria.

Antibacterial time kill assay

The potential candidate species of SLP3 (Lobster), SJP1and SJP6 (Jelly fish) extracts carryover during the process was selected for its effect on the more susceptible bacterial pathogens *Pseudomonas.* sp and *Klebsiella.* sp. The inoculums of *Pseudomonas.* sp and *Klebsiella.* sp at the concentration (10^{-8} CFU/ml) starting from a 0.5 Mc Farland was prepared from fresh colonies. The extract of SLP3, SJP1 and SJP6 was prepared at a concentration of $\mu g.ml^{-1}$. And different strength of the extract- 1x (6.25), 4x (25) and 16x (100 mg) was prepared with nutrient broth and inoculated with equal volume of bacterial inoculums and incubated at 35ºC for 24 h. Plating was made at every 3 h interval and the colony forming unit was calculated (NCCLS, 1990).

Result and Discussion

Lobster and Jelly fish tissue samples found to be 18×10^{-8} to 55×10^{-2} CFU and 11×10^{-8} to 68×10^{-2} CFU. Most of the bacterial isolates were pigmented. Such as red, yellow, brown, pure white, yellow mixed white and orange colour colonies were observed (Table 1). Eze *et al.,* (2011) reported that the incidences of various isolates from mackerel fish (*Scomber scombrus*) found to be 1.135×10^{-6} CFU g^{-1} to 5.0×10^{-5} CFU g^{-1}. The kind and number of microorganism found with marine animal dependent on the source habitat, ecological cycling, temporal succession, the defense mechanism of marine animal against most of the fouling organism nutritional behaviors. Twenty six epibiotic bacterial isolates were enumerated from two marine animals were characterized. They biochemical as well as based

on their fatty acid similarity. Among twenty six isolates of marine epibiotic bacteria five of the isolates were Gram positive, and remaining epibiotic twenty one isolates were Gram negative in nature. Twenty six epibiotic bacterial isolates were enumerated from two marine animals were characterized. They biochemical as well as based on their fatty acid similarity. Among twenty six isolates of marine epibiotic bacteria five of the isolates were Gram positive, and remaining epibiotic twenty one isolates were Gram negative in nature. Epibiotic bacterial isolates were grown in different salinity concentrations, such as 1%, 20%, 40% and 60%. It was observed that all the salinity levels support the growth of marine isolates. However better growth was observed it 20% NaCl concentration. It was observed that bioflim forming bacterial isolates (*Halodule pinifolia)* grown well on the NaCl concentration from 6% to 40% (Medomeria *et al.,* 2010).

Table: 1 Morphological characteristic of epibiotic bacterial isolates of marine animals

Marine animal	No. of bacterial isolates	Shape and marginal and No of isolates	Surface and No of isolates	Pigmentation and No of isolates
Lobster	Ten	Round- Six Irregular- Four	Mucoid- One	Orange- Three
Jelly fish	Sixteen	Round- Five Irregular – Eleven	Mucoid- Three	Yellow - Three Red - One

Plate: 1a) Heterotrophic epibiotic bacterial colonies on marine agar
b) Pigmented bacterial isolates

(a) **(b)**

Table: 2 Growth of marine animal (Jelly fish) epibiotic bacterial population in different salinity concentration

Characteristics	SJP1	SJP2	SJP3	SJP4	SJP5	SJP6	SJP7	SJP8	SJP9	SJP10	SJP11	SJP12	SJP13	SJP14	SJP15	SJP16
NaCl requirement 1%	+	++	+++	++++	++++	++++	++++	+++	++	++	+++++	+++++	+++	+++	++++	++++
20%	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+
40%	++	++	++	++	+++	++++	+++	+++	+++	++++	++	++++	+++	++	+++	++
60%	+++	+++	+++	+++	++++	++	+++	++	+	++	+	++	++	++++	+++	++++

-; Nil, +; Growth, ++; High Growth, +++; Abundant

Table: 3 Growth of marine animal (Lobster) epibiotic bacterial population in different salinity concentration

Characteristics	SLP1	SLP2	SLP3	SLP4	SLP5	SLP6	SLP7	SLP8	SLP9	SLP10
NaCl Requirment 1%	++	++	+++	++	++++	+++	+++++	+++	++	+++
20%	+	+	+	+	+	+	+	+	+	+
40%	+++	+++	++	+++	++	++	++	++	+++	++
60%	++++	++++	++s	+++	++++	++++	++	+++	++++	+++

-; Nil, +; Growth, ++; High Growth, +++; Abundant

Table: 4 Antibacterial activity of lobster associated marine bacterial isolates against test organisms

Test Organisms	Zone of inhibition(mm)									
	SLP1	SLP2	SLP3	SLP4	SLP5	SLP6	SLP7	SLP8	SLP9	SLP10
E. coli	6±1.3	4±1.1	5±1.9	4±1.9	6±4.5	5±1.9	4±0.5	9±5.1	5±2.4	5±1.1
Klebsiella.sp	4±0.9	3±1.3	6±2.3	2±0.5	4±0.8	5±1.3	4±1.5	3±0.9	5±1.9	2±1.5
S.*aureus. sp*	4±0.8	3±0.7	5±1.5	3±1.4	4±0.3	6±2.0	4±1.2	3±0.4	6±1.2	3±0.8
Proteus.sp	3±0.5	2±0.3	5±1.2	5±3.1	4±2.3	3±1.1	4±1.7	2±0.7	2±0.3	3±0.3
Pseudomonas.sp	7±3.1	7±4.1	12±3.7	3±1.6	6±3.6	3±0.7	2±0.5	4±2.1	7±3.2	2±0.2

Mean ± standard deviation

Table.5 Antibacterial xsactivity of Jellyfish associated bacterial isolates.

Test Organisms	Zone of inhibition (mm)															
	SJP1	SJP2	SJP3	SJP4	SJP5	SJP6	SJP7	SJP8	SJP9	SJP10	SJP11	SJP12	SJP13	SJP14	SJP15	SJP16
E. coli	12±3.1	13±3.9	7±2.9	4±0.6	5±1.8	10±3.2	9±4.1	7±1.0	1±0.2	1±0.5	9±3.9	8±2.8	5±1.9	5±1.5	6±2.3	6±2.9
Klebsiella .sp	3±0.8	4±1.9	2±0.4	3±0.4	2±0.9	4±0.5	3±1.4	6±2.5	4±1.9	5±2.4	3±1.9	6±2.3	8±4.2	6±2.6	6±1.9	3±0.7
S.aureus	4±2.1	3±0.6	5±1.8	3±0.2	4±1.4	4±1.2	2±1.8	2±0.9	2±0.5	3±1.6	3±1.3	2±0.9	4±2.1	5±1.7	2±1.0	5±1.9
Proteus. sp	2±0.9	2±0.3	4±1.3	3±0.5	4±1.7	5±1.9	5±2.1	6±2.6	4±1.3	5±1.8	4±2.1	4±1.8	6±2.5	7±3.2	3±1.3	5±2.1
Pseudomonas.sp	1±0.3	2±0.2	2±0.3	1±0.5	1±0.3	1±0.3	3±1.4	3±0.7	4±1.6	4±1.7	4±1.9	5±2.1	6±2.9	6±2.1	1±0.5	1±0.8

Mean ± standard deviation

Plate: 2 Antibacterial activity of marine animal associated bacterial isolates against *E.coli*

Control plate

SJP1 to SJP16 extract against *E.coli*

SLP1 to SLP10 extract against *E.coli*

Among the twenty six isolated screened against bacterial pathogens for antibacterial activity five of the isolates extracts better anti bigram was selected and subjected for Biochemical

characterization. It was observed that the strain SJP1 as *Vibrio gazogenes, SJP6 as Bacillus subtilis, SLP3 as Aeromonas hydrophila, SLP7 as Pseudoalteromonas.* Ethyl acetate fraction of marine epibiotic bacteria was used for antibacterial activity against *Escherichia coli, Staphylococcus aureus, Klebsiella. sp, Proteus. sp and Pseudomonas.sp* pathogens was determined by agar diffusion method, all twenty six isolates exhibits varies ranges was observed zone of inhibition ranged between 1- 13 mm. marine animal associated epibiotic bacterial extracts passed out in thin layer chromatography spots were resolved. The Rf value is calculated, the high Rf value of epibiotic bacterial strains SJP1 0.46 and SLP3 0.41 was observed. The minimum inhibitory and minimum bactericidal assay was performed with metabolites of selected bacterial isolates which exhibit maximum antibacterial activity while screened against test organism at different concentration. The growth inhibition of test organism was analyzed by measuring optical density through UV- visible spectrophotometer at 620 nm. The optical density was noted as 0.625 to 0.701 respectively.

Minimum bactericidal concentration of epibiotic bacterial metabolites extracts against test organisms such as *Pseudomonas. sp* and *Klebsiella. sp*. Growth inhibition of test organism was found at 100 µg concentration of metabolites. Time kill assay performed against most susceptible bacterial pathogens by using epibiotic bacterial metabolites, are reduction the of test organism count was observed.

Figure: 1 Minimum inhibitory concentration of marine animal lobster associated bacterial extract against test organisms

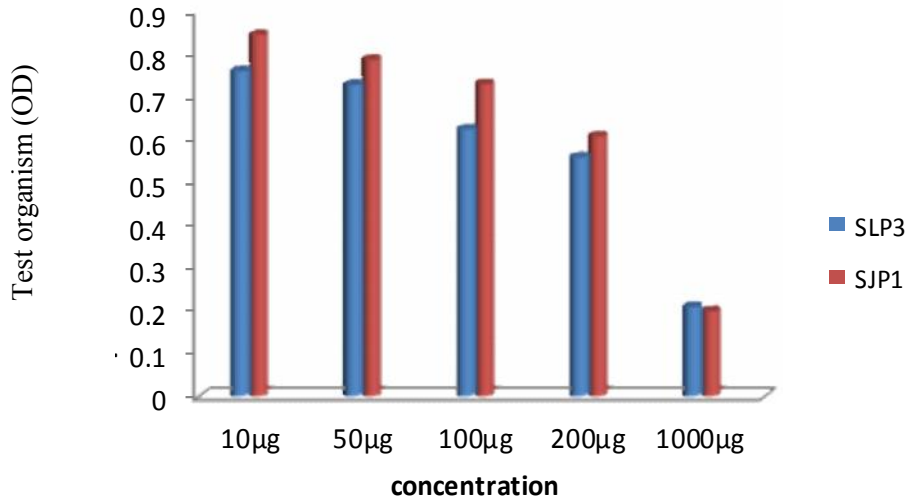
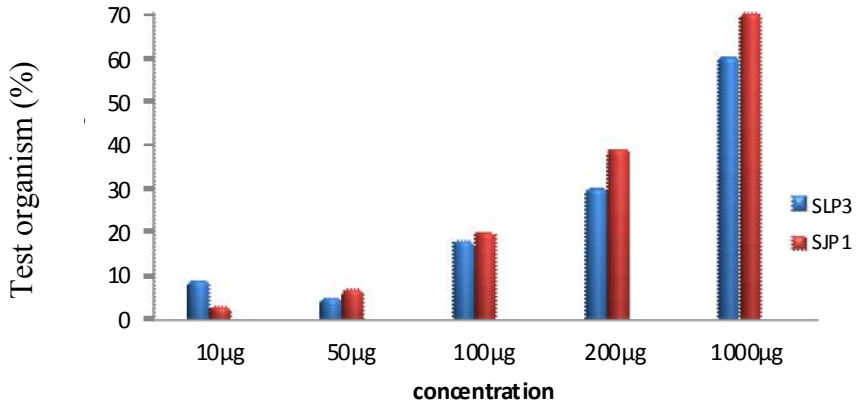


Figure: 2 Minimum bactericidal concentration of Marine animal lobster associated marine bacteria extract against test organisms



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