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Identification and antimicrobial potential of bioluminescent bacteria isolated from the mangrove ecosystem of the Roach Park, Tuticorin, South east coast of India

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Abstract

This study aimed to isolate the bioluminescent bacterial strains from seawater and sediments in near the Roach Park, coastal area of Tuticorin. Further to identify the luminescent bacteria strains and to screen for production of bioactive metabolites and their bioactivity against human bacterial pathogens. Bioluminescent bacteria are wide spread in natural environment. Water and sediment samples were collected from nearby mangrove ecosystem of the Roach Park off Tuticorin coast, Bay of Bengal. The luminous bacteria density was found to be 3 X 10^4 CFU/ml - 5 X 10^4 CFU/g in seawater and sediment respectively. Totally 10 luminescent bacterial strains were identified up to genus levels were selected and screened for the production of antibacterial substance, in the preliminary screening the strains SWLB3, STLB5 and STLB6 strains had shown broad spectrum activity against the human pathogens. Among these strain STBL5 exhibited predominant activity against human bacterial pathogens. The bioactive bacterial metabolites which showed higher activity against human pathogens indicate that luminescent bacteria could be used as a good source for the isolation of bioactive compounds.

Keywords: Bioluminescent, Mangrove ecosystem, Human pathogens, Bacterial pathogens

Introduction

Micro-organisms are essential parts of any ecosystem including the marine ecosystem are represented by groups such as viruses, bacteria, fungi, diatoms, algae, protozoa *etc.* The two fundamental reasons for the indigenous microorganisms are used for biologically indicator of coastal environment. They are responsible for the regeneration of nutrients and the transfer of primary production from phytoplankton to micro zooplankton and to larger organisms [26]. There are few biologically relevant and simple general indicators

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of coastal water quality available to assess the effect of human activities on the functioning of coastal ecosystems, particularly indicators of chemical contamination [14]. The population densities in coastal regions are estimated to be nearly three times higher than the global average density [28].

The vast majority of bioluminescent organisms reside in the ocean because more than 700 genera known to contain luminous species [27]. These occupy a diverse range of habitats, from polar to tropical and from surface waters to the sea floor [15]. Bioluminescence is a form of light produced by a chemical reaction in living organisms. It is exhibited by a diverse group of organisms although their number is very less compared to the total number of known species. It has been estimated that luminous organisms may have come from about 30 different evolutionarily distinct origins [17,6]. However some animals, including crustaceans, squid, jellyfish and fish, release their light-emitting chemicals into the water, producing clouds or particles of light that serve to distract or blind a predator [4].

In the marine environment luminescent bacteria are isolated from seawater, sediment and detritus are distributed widely in shallow coastal environments and deep pelagic water [24, 25].Certain bioluminescent species are established as species-specific symbionts with marine fish and squids and are harbored in highly specialized light organs [20,12].The most luminous bacteria are classified into three major genera such as *Photobacterium* spp. *Vibrio* spp. and *Photorhabdus* spp. Among them the species *Photobacterium* spp. and *Vibrio* spp. are exist in marine environment and the *Photorhabdus* spp. are terrestrial species [10].

The *Vibrio* species are a diverse group of bacteria found in abundance in the marine environment and associated with aquatic plants and animals to which they may provide a chemical defense for the host and some of them are showing bioluminescence [13]. Research to isolate bioactive compounds from the 74 species of this group has already shown promise with the isolation and identification of many antibiotic compounds [8]. This study aimed to isolate the bioluminescent bacterial strains from seawater and sediments in near the Roach Park, coastal area of Tuticorin. Further to identify the luminescent bacteria strains and to screen for production of bioactive metabolites and their bioactivity against human bacterial pathogens.

Materials and Methods

Sample collection

The samples were collected from near Roach Park (Lat. 08˚46’ 58 N and Long. 78˚ 09’36 E) of the Tuticorin coast, Bay of Bengal (Fig. 1). The surface water samples were collected in 30 ml sterile screw capped bottles. Enough space was left in the bottles to allow thorough mixing. Precautionary measures were taken to avoid contamination through handling. Sediment samples were collected at 1m depth from surface using the sterile polyvinyl corer (10cm diameter) and these samples were transported to sterile vials and tightly sealed. The collected samples brought to the lab in an ice-box.

Isolation of bioluminescent bacteria

The isolation of luminous bacteria was performed by the dilution plate method[Abraham, 2003] using seawater complex agar (SWA) comprising peptone 0.5 g, yeast extract 0.3 g, glycerol 0.3 ml, seawater 75 ml, distilled water 25 ml and agar agar1.5 g and maintained pH 8.0±0.1. The Petri dishes were then incubated at room temperature for 48 hrs and the luminescent colonies were observed in dark room after 16-20 hrs of incubation. Luminous colonies were aseptically picked, streaked repeatedly on SWC agar until pure. The pure cultures were transferred to SWC agar slants and preserved at 4^0C. Respective 4 and 6 luminous bacterial strains were selected from seawater and sediment for screening antibacterial activity against human pathogens and were given designated codes i.e., SWLB (luminous bacteria isolated from sea water) and STLB (luminous bacteria isolated from sediment).

Preliminary antibacterial screening

Antibacterial assay was used as a preliminary screening to isolate antagonistic bacteria against

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human pathogen such as *Staphylococcus aureus* (MTCC 3160), *Micrococcus luteus* (MTCC 4821), *Streptococcus pyogenes* (MTCC 1923), *Bacillus subtilis* (MTCC 1133), *Enterobactor faecalis* (MTCC 2729), *Escherichia coli* (MTCC 50), *Klebsiella pneumoniae* (MTCC 3384), *Shigella sonnei* (MTCC 2957), *Salmonella typhimurium* (MTCC 1357) and *Vibrio cholerae* (MTCC 3906) obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.

The screening of supernatant was carried out employing agar well technique followed by [5]. The isolated and selected bacterial strains were inoculated into 50 ml of Zobell Marine Broth (ZMB) and incubated on a shaker at 120 rpm for 48 hrs. At the end of incubation period the broth was centrifuged at 5000 rpm for 15 min to separate cell mass from fermentation medium. The collected cell free supernatants were concentrated. The residue was diluted with one ml of distilled water and 100 µl of the supernatant was loaded on to the respective 5 mm wells of the medium seeded with 24 hrs test pathogens. The plates were incubated at room temperature for 18 to 24 hrs, and then observed. The zone of inhibition was measured from the end of the well to the end of the clear zone in mille meter. Based on the activity in supernatant, active strain was further selected for extraction of bacterial metabolites using different solvents.

Extraction of bacterial metabolites

The extraction of bacterial metabolites was carried out following the method of [33]. The selected antagonistic bacterial strains were inoculated into 100 ml ZMB broth, and incubated in a shaker at 120 rpm for 48 hrs. After incubation period the broth culture was centrifuged at 5000 rpm for 15 min. The supernatant was extracted twice with equal volume of hexane, ethyl acetate, chloroform and butanol. The solvent phases were then separated using separating funnel and concentrated by evaporation. The concentrate (crude extract) dissolved in one ml of respective solvents was then impregnated at 100 µg/disc concentration on to sterile WhatmanNo. 1.6 mm diameter disc and the antibacterial activity were assayed following the disc diffusion assay [9, 19]. The solvents alone in the disc were used as control. The inhibition zone was measured from the border of the disc to edge of the clear zone in mm.

Results

Table 1: Total luminous bacterial count in seawater and sediment

Sample	No of bioluminescent bacteria
Seawater	3 X 10^4 CFU/ml
Sediment	5 X 10^4 CFU/g

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Table 2: Antibacterial activity of luminous bacteria isolated from seawater

S. No	Human pathogens	SWLB1	SWLB2	SWLB3	SWLB4
		Zone of Inhibition level (mm) at 100µl/disc			
1	*Staphylococcus aureus*	1	-	2	-
2	*Micrococcus luteus*	-	-	2	-
3	*Streptococcus pyogenes*	-	-	4	-
4	*Bacillus subtilis*	-	-	3	-
5	*Enterobactor faecalis*	-	-	1	-
6	*Escherichia coli*	2	-	-	-
7	*Klebsiella pneumoniae*	1	-	2	-
8	*Shigella sonnei*	-	-	4	-
9	*Salmonella typhimurium*	-	-	-	-
10	*Vibrio cholerae*	1	-	-	-

*Low activity (1 mm); moderate activity (2-5mm); - no activity

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Table 3:Antibacterial activity of luminous bacteria isolated from sediment

S. No	Human pathogens	STLB1	STLB2	STLB3	STLB4	STLB5	STLB6
		Zone of Inhibition level (mm) at 100µl/disc					
1	*Staphylococcus aureus*	2	-	-	-	3	1
2	*Micrococcus luteus*	-	-	-	-	2	1
3	*Streptococcus pyogenes*	-	1	-	-	-	-
4	*Bacillus subtilis*	-	-	-	-	5	-
5	*Enterobactor faecalis*	1	-	-	-	-	-
6	*Escherichia coli*	-	-	-	-	4	2
7	*Klebsiellapneumoniae*	1	1	-	-	2	1
8	*Shigellasonnei*	1	-	-	-	3	1
9	*Salmonella typhimurium*	-	-	-	-	2	-
10	*Vibrio cholerae*	-	1	-	-	5	1

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Table 4: Antibacterial activity exhibited by crude extracts of *Vibriomediterranei*STLB5

S. No	Human pathogens	STLB5			
		Zone of Inhibition level (mm) at 50µg/disc			
		H	C	EA	B
1	*Staphylococcus aureus*	2	1	4	3
2	*Micrococcus luteus*	-	-	3	3
3	*Streptococcus pyogenes*	-	-	-	-
4	*Bacillus subtilis*	1	1	5	2
5	*Enterobacterfaecalis*	-	-	-	-
6	*Escherichia coli*	-	-	4	2
7	*Klebsiellapneumoniae*	2	1	3	3
8	*Shigellasonnei*	-	-	4	-
9	*Salmonella typhimurium*	-	-	2	1
10	*Vibrio cholerae*	2	3	5	4

*H – Hexane; C- Chloroform; EA – Ethyl acetate; B - Butanol

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Table 5: Morphological and biochemical characters of isolated luminescent bacterial strains

Strain code	Gram stain	Motility	Oxidase	Catalase	TSI	Growth on TCBS agar	Tentative identification
SWLB1	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
SWLB2	Rod /gram negative	Motile	+	+	Acid butt & Alkaline slant	Green color	*Vibrio*sp
SWLB3	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
SWLB4	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Green color	*Vibrio*sp
STLB1	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
STLB2	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
STLB3	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
STLB4	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
STLB5	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp
STLB6	Rod /gram negative	Motile	+	+	Acid butt & Acid slant	Yellow color	*Vibrio*sp

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Table 6: Morphological and biochemical characters of *Vibriomediterranei*(STLB5)

Biochemical characters	***Vibrio mediterranei*** **(STLB5)**
Gelatin hydrolysis	-
Amino acids Decarboxylase test	
Arginine decarboxylase	-
Lysine decarboxylase	+
Ornithine decarboxylase	-
Growth on gelatin agar	
Gelatin agar with 3% NaCl	+
Gelatin agar without NaCl	-
Pigment production	
Seawater complex agar	-

Figure 1: Map showing the study area

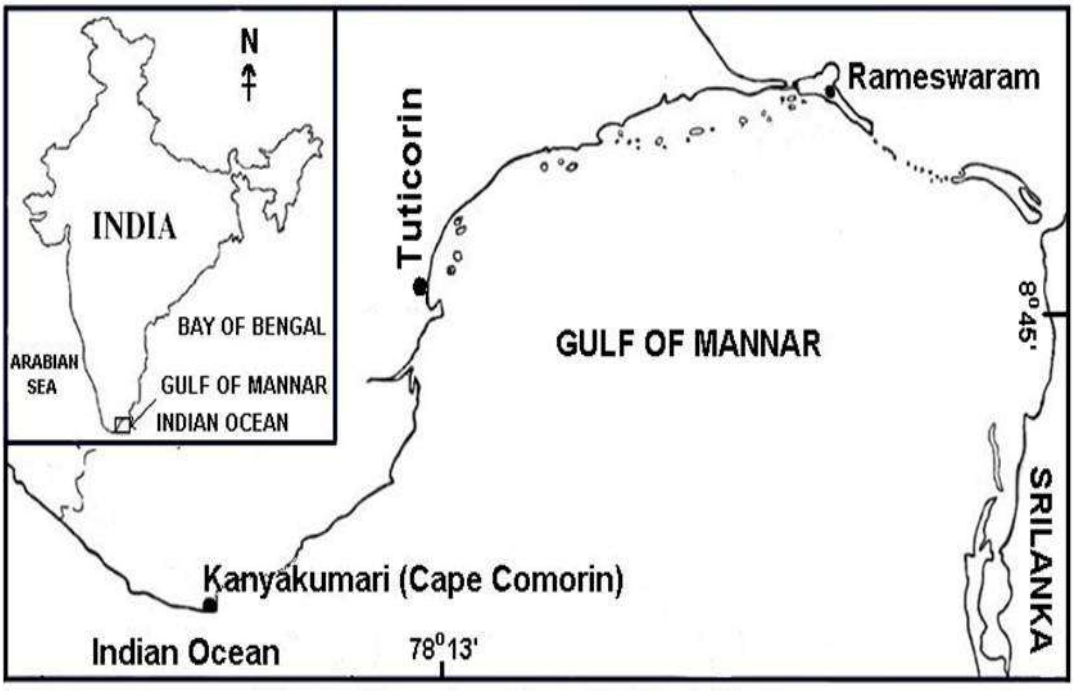

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Figure 1: Isolated pure culture of luminescent bacteria on normal light and dark on the seawater complex agar medium

Bacterial Colonies

Luminescent Bacteria

Pure culture of luminescent bacteria in normal light

Pure culture of luminescent bacteria in dark

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Figure 2: Antibacterial activity exhibited by crude extracts of *Vibriomediterranei*(STLB5)

Staphylococcus aureus

Micrococcus luteus

Streptococcus pyogenes

Bacilussubtilis

Enterobactor faecalis

Escherichia coli

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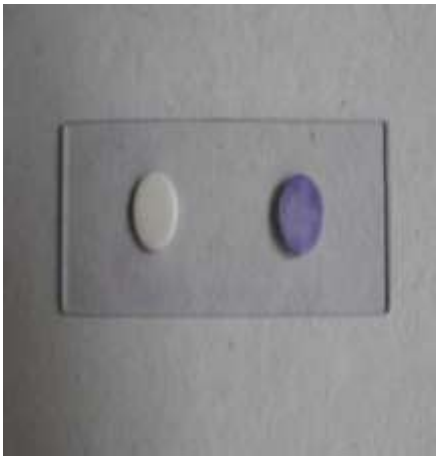
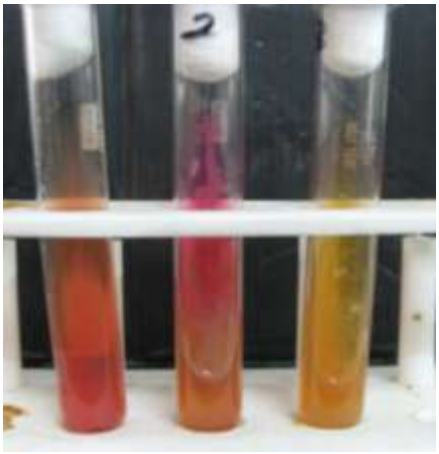
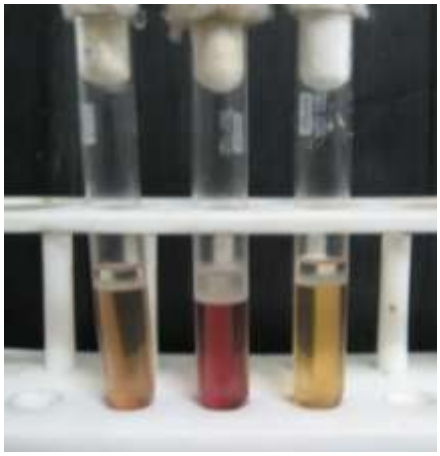
Figure 3: Morphological and Biochemical Characters of Isolated Bioluminescent Bacteria



Oxidase test

Catalase test





Triple sugar iron test

Figure 4: Growth of luminescent bacteria on TCBS agar

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Luminous bacterial density

The total luminous bacterial density enumerated from different sources is given in table 1. The luminous bacterial density was found to be 3 X 10^4 CFU/ml and 5 X 10^4 CFU/g in seawater and sediment samples respectively. Only 10 luminous bacterial strains were selected and screened for antibacterial activity against human bacterial pathogens. The isolated luminous bacterial strains were given designated codes from SWLB (luminous bacteria isolated from sea water) and STLB (luminous bacteria isolated from sediment).

Antibacterial activity exhibited supernatants of luminous bacteria

The antibacterial activity of supernatant of bacteria was considered as a good tool to screen active strains. In preliminary screening, agar well method against ten human pathogens was used and the results are represented in table.2 and 3. The activity exhibited by supernatants is ranged from low to moderate level activity according to their zone of inhibition against pathogen. Out of 10 strains, 3 strains showed broad spectrum antibacterial activity. Interestingly, the human pathogen *Salmonella typhimurium* was found to be most resistant and has not been inhibited by any of the supernatant. Strains SWLB1 and SWLB3 which was isolated from seawater showed broad spectrum activity against human bacterial pathogens. The strains SWLB3 showed low and moderate level activity against seven human pathogens. The zone of inhibition was ranged between 1 mm and 4 mm. The maximum zone of 4 mm was observed against *Streptococcus pyogenes* and *Shigellasonnei*. The minimum activity of 1 mm was exhibited against the *Enterobacterfaecalis*. The SWLB1 strains showed low activity against four pathogens. Other two strains inhibited none of the pathogens (Table 2).

Invariably growth all the human pathogen was inhibited by the selected isolates. The four strains (STLB1, STLB2, STLB5 and STLB6) isolated from sediments were found to have broad spectrum activity against human bacterial pathogens. The three strains STLB1, STLB2 and STLB6 were observed to exhibit low level activity against three to six pathogens. The STLB5 showed broad spectrum activity against eight human pathogens. The zone of inhibition was ranged from 2 mm and 5 mm. The maximum level of zone was observed against *Bacillus subtilis* and *Vibrio cholerae;* minimum zone was exhibited against *Micrococcus luteus, Klebsiellapneumoniae* and *Salmonella typhimurium* was only inhibited by this strain. But it did not inhibit both *Streptococcus pyogenes and Enterobactor faecalis* which were inhibited by STLB2 and STLB1 respectively. The other strains did not show activity against none of the pathogens. From these strains, the STLB5 was further selected for extraction of secondary metabolites (Table 3).

Antibacterial activity of bacterial metabolites

The active luminous bacteria strain (STLB5) was subjected to solvent extraction and screened against ten human pathogenic bacteria and results are depicted in Table 4. In this study, ethyl acetate extracts were shown to have broad spectrum activity against eight pathogens. The zone of inhibition was ranged from nil to 5 mm. The higher activity was observed against *Bacillus subtilis* and *Vibrio cholerae* followed by *Staphylococcus aureus, Escherichia coli, Shigellasonnei, Micrococcus luteus, Klebsiella pneumonia* and lowest against the *Salmonella typhimurium*. Even though butanol, chloroform and hexane extract showed activity against seven and four pathogens respectively, this was considered to be low level activity. Butanol extract exhibited 4 mm zone of inhibition against *Vibrio cholerae*. In general, the maximum inhibition zone was recorded against *Vibrio cholerae* by all the four extracts. But none of the extracts showed activity against both *Streptococcus pyogenes and Enterobactor faecalis*.

Identification of luminous bacterial strains

All the isolated luminous bacterial strains were identified as *Vibrio* spp; the results are represented in Table 5. The antagonistic strain (STLB5) was identified as *Vibrio mediterranei* (Table6).As the strain *Vibrio mediterranei* grew in Gelatin agar with 3% Nacl and not grows in Nacl free gelatin agar plate. It showed negative result in gelatin hydrolysis, L-arginine

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decarboxylase, L-ornithine decarboxylase and positive in L-lysine decarboxylase test by color change from yellow to dark purple color. They did not produce yellow orange pigment in seawater complex agar plate (Table 6).

Discussion and conclusion

Luminous bacteria are the most ubiquitous of all bioluminescent organisms. The marine luminescent bacteria are diversified in either as free living bacteria or in symbiotic association with certain marine organisms. The prevalence of luminescent bacteria considered as indicative of congenial physic chemical conditions and increased availability of nutrients. These associated bacterial luminescent genes are considered as biosensors for marine environmental studies, with special emphasis on the micro toxicity assay [Halldorson VSN and Duran NL, 2003]. In this study the average density of luminous bacteria in seawater was 3 X 10^4 CFU/ml. The present observation of luminous bacterial population was higher when compared with [2] Abraham *et al.* (2003) who reported 20 to 1050 cells/ml of luminous bacteria from seawater of Tuticorin bay, 3.0 X 10^3 CFU/ml in port Harcourt in Nigeria and 1.3 X 10^4 to 3.0 X 10^4 in Bonny estuary Nigeria [3], 20 to 90 cells/100 ml in surface seawater of the Seto Inland sea [30].

These results noted that the luminous bacteria found on seawater and sediment samples from near-shore environment of Tuticorin Bay probably reflected low level of toxicants. The present observation concurrent with the [22, 23] reported that luminous bacteria lose their ability to emit light in the presence of a toxicant even at very low concentration. As the presence of luminous bacteria in high numbers in coastal waters indicated healthy and pollution free condition.

In the next aspect of my study was to screen antibacterial activity of supernatant from 10 luminous bacterial strains. Out of 10 strains, the SWLB3, STLB5 and STLB6 showed broad spectrum activity against the human bacterial pathogens, other strains were nil to low level activity. Among these strain STLB5 inhibited growth of eight human bacterial pathogens than other broad spectrum strains. A similar study [31] reported antibacterial activity of *Vibrio ruber* supernatant against 100% gram positive organisms and 75% gram negative organisms.

The present observation was further supported by [21] who have revealed the antibacterial activity of *Bacillus licheniformis* supernatant against human pathogens. The observation was concurrent with the report stating that the antibacterial activity of marine bacterial supernatant from Arabian Sea of Pakistan [5]. It is to be noted that the production of biologically active compounds of marine bacteria depends on the pressure, temperature, salinity, and depletion of micronutrients, with survival and proliferation [11].

The antibacterial activity of luminescent bacterial supernatant was obtained from standard medium because the antibacterial compound productions depend on the specialized media. It is to be noted that the loss of specific micronutrients in culture media, changes in the mode of culture for broth, mixing velocity, changes to specific temperature requirements, pH, light or pressure, that lead to the quantitative and qualitative isolation of a modified marine natural product[7,18].

The subsequent study was to extract bacterial metabolites from active strain STLB5 with solvents such as hexane, ethyl acetate, chloroform and n-butanol. The ethyl acetate extracts showed broad spectrum activity against the eight human bacterial pathogens. The maximum zone was observed against *Bacillus subtilis* and *Vibrio cholerae*. In a similar study [29] reported that the ethyl acetae extract of *Pseudomonas aeruginosa* showed wide spectrum activity against human pathogens and the maximum activity against *Staphylococcus epidermidis.*

The *Vibrio* sp are reported to be most common luminescent bacteria. It supports this work that all luminous bacterial strains were identified as *Vibrio* spp. and the prominent antibacterial active strain (STLB5) was further identified as *Vibrio mediterranei* based on the morphological and biochemical characteristics. This obtained result is backed by the observation of [1, 2] who reported different species of *Vibrio* such as *Vibrio harveyi, V. orientalis, V.splendidus,*

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V. fischeri and *V. mediterranei* in seawater and sediment samples from Tuticorin bay. The results of the present study are in close agreement with the observation of [32] who isolated and identified some luminous bacteria such as *Vibrio harveyi, Vibrio orientalis*
V. splendidus, Photobacterium phosphorium and *photobacterium leiognathi*.The present study has revealed the distribution of luminous bacteria in seawater and sediments, suggested the healthy environment in near Roach park coastal area, Tuticorin. Also, the luminous bacterium could be used as a good source for the production of antibacterial substances. Further works has to be done to purify and characterize active antibacterial compounds.

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