Research Article

Isolation and Identification of Bioluminescent Bacteria from Marine Water at Nagapattinam Sea Shore Area

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ABSTRACT

Luminescence is the emission of light by an object. Living organisms including certain bacteria are capable of luminescence. Bacteria are the most abundant luminescent organisms in nature. Bacterial luminescence is due to the action of the enzyme called luciferase. Other interesting features of marine microorganisms are having their ability to survive at low temperatures and high salinity. In the present study, marine water samples were collected from different sites of Nagapattinam sea shores, and analyzed the physico-chemical characteristics. The effect of salinity, pH concentration on the growth and luminescence of these 10 strains was also studied. From the samples, bioluminescent bacterial strains were isolated using Sea Water Complex Agar and TCBS medium. Ten bioluminescent bacterial strains were isolated and identified based on cultural, morphological and biochemical charactertics which are belonged to the genera of *Vibrio* sp and *Pseudomonas* sp.

Keywords: Marine ecology, sea water, luminescent bacteria, luciferese, oceanography,

INTRODUCTION

Marine life is a vast resource, providing food, medicine, and raw materials. Marine organisms contribute significantly to the oxygen cycle, and are involved in the regulation of the Earth's climate. Bacteria were isolated and cultivated from all possible regions of the earth, on the basis of their habitat, diversity, ecological functions, degree of pathogenicity and biotechnological applications. 70% of the earth's surface is covered by oceans with rich microbial diversity[1]. Bioluminescence is a form of light produced by a chemical reaction in living organisms Sluminescent bacteria are common in the ocean, especially in temperate to warmer waters[2].

Marine microorganisms have unique properties since they have to adapt to extreme marine environment conditions such as high or low temperature, alkaline or acidic water, high pressure and limited substrate in the deep sea water. These distinctive characteristics have attracted many researches to explore in depth since there is the potential of marine microorganisms used in biotechnological applications[3]. Bacterial luminescence is due to the action of the enzyme called luciferase. Excess energy is liberated in this process. The energy is dissipated as a luminescent blue-green light the bacterial luminescence reaction, which is catalyzed by luciferase, involves the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide ($FMNH_2$) with the liberation of excess free energy in the form of a blue-green light at 490nm

$FMNH_2 + RCHO + O_2$ ----> $FMN + RCOOH + H_2O$ + light (490nm).

The bioluminescent intensity reflexes the overall health of the microorganisms and the bioluminescence reaction with reflex reactions sensitive wide variety of toxic substances. Other interesting features of marine microorganisms are their ability to survive at very low temperature and high salinity. The groups exhibiting the above characteristics are referred to as psychrophiles and halophiles respectively. Marine bacteria are also characterized by their pressure tolerance, especially those at depths. These forms belong to the group of barophiles[4]. The generation time of marine bacteria is quite long, ranging from less than one hour to many months. The shortest generation time, 9.8 minutes, has been reported for *Pseudomonas natriegens* at 37°C. In the present study, physico- chemical analysis of the marine water samples, enumeration of bacterial load and the bioluminescent bacteria were isolated from marine water from different areas of Nagapattinam sea shores. Ten bioluminescent bacteria were isolated using sea water complex agar medium and TCBS medium was identified by gram staining and biochemical tests.

MATERIALS AND METHODS

Collection of marine water sample

The marine water sample was collected from different sites of Nagapattinam district, Tamil Nadu, South India. Sampling was done by taking all possible aseptic measures and was stored at 4°C. The samples were processed for isolation of marine luminescent bacteria.

Physico-chemical parameters of the marine water samples

Analysis of the physico-chemical parameters of the marine water samples collected from different sites. The parameters such as pH, temperature, electrical conductivity, dissolved oxygen, carbonate, bicarbonate, chloride, calcium, total dissolved solids, salinity, zinc, copper, iron, nickel, cobalt, total mercury, total arsenic,

total cyanide, total lead, selenium, total silver, nitrate, nitrite, ammonia, inorganic sulphide, and sulphate, were analyzed using the standard methods[5].

Enumeration of bacterial load[6]

The samples were serially diluted as per the method of the suspension from dilutions 10^{-5} , 10^{-6} , and 10^{-7} were inoculated on Thiosulfate Citrate Bile Salt Sucrose agar medium, LM medium and Sea Water Complex agar plates for the isolation of marine luminescent bacteria. The plates were incubated for 24 hrs respectively.

The number of colonies per ml was calculated by the following formula:

The number of colonies per ml by		Number of colonies × dilution factor
	=	----------
		Weight of the sample

Isolation and identification of marine luminescent bacteria

Evaluation of different Media used for growth of bioluminescence bacteria

Thiosulfate Citrate Bile Salt Sucrose agar medium, LM medium, SWC medium, was prepared and it was poured on to the sterile Petri plates. After solidification, the marine water samples were spread on the medium. Then, the plates were incubated at 37°C for 24 hours for the isolation of bacteria. Bacterial colonies were purified by repeated streaking the purified colonies were preserved at 4°c for further experiments[8].

Morphological characteristics[9]

1 .Gram staining

2. Test for Motility (Hanging Drop Method)

3. Biochemical tests

The isolated organisms were subjected to biochemical test for identification. The biochemical tests include, Methyl red, Indole, Voges proskauer test, Citrate utilization test, Glucose, Sucrose, Catalase, Oxidase and Nitrate.

Identification of the bacteria

Growth of luminescent bacteria in TCBS medium

TCBS medium and Sea Water Complex Agar medium is a selective medium that allows the selective growth of bacteria belonging to the genera *Vibrio* sp and *Pseudomonas* sp. TCBS medium and SWCA medium was prepared and poured in petri plates and 10 different strains were streaked and observed the result after 24 hours. Appearance of yellow color colonies in this medium indicates the bacterial strain as *Vibrio* sp, and green colour colonies in this medium indicate the bacterial strain as *Pseudomonas sp*.

The ten isolates obtained and identified by gram staining, motility test, biochemical tests. According to "Bergey's manual of determinative bacteriology, 9th ed.," Luminescent, there are three genera and five species of luminous bacteria, *Vibrio cholerae biotype, Pseudomonas, Vibrio fischeri, Lucibacterium harveyi, Photobacterium phosphoreum* and *Photobacterium mandapamensis*[7].

Effect of external factors on the luminescent bacteria

Effect of salinity (Varying conecntrations of NaCL)

SWCA medium TCBS medium was prepared by adding different amounts of NaCl to obtain the final concentrations of salinity such as 0%, 3%, 6%, 9% and 12%. The medium was poured in Petriplates and ten bacteria were streaked per plate with clear divisions between them. Likewise, all the 10 organisms were tested. The plates were incubated for 24 h and the intensity of luminescence was assessed by visual scoring[10].

Effect of pH

SWCA medium was prepared with four different pH values such as 5, 7, 9 and 11. The pH of the medium was adjusted with appropriate acid or base and pH was adjusted, the medium was added with respective amount of agar and then sterilized. The medium was poured in Petriplates and 10 bacteria were streaked per plate with clear divisions between them. Likewise, all the 10 organisms were tested. The plates were incubated for 24 h and visual scoring as described previously assessed the intensity of luminescence. The same experiment was done once in broth medium in test tubes without agar[10].

RESULTS AND DISCUSSION

In the present study, marine water samples were collected from different sites of Nagapattinam sea shore areas.

Physico-chemical parameters of the marine water samples

Analysis of the physico-chemical parameters of the collected marine water samples using the standard methods and the results were showed in the Table -1.

Enumeration of bacterial load marine water samples

The samples were subjected to serial dilution and the bacterial count was made in the plate counting techniques. The bacterial loads obtained were enumerated from four different sites. Some colonies were counted particularly in the dilution rate of 10^{-5}, 10^{-6}, 10^{-7} (Table-2). (Buchriesesr and Kaspar, 1993) reported that the direct viable count, a microscopoic method for enumeration of viable bacteria. The modified direct viable count will be useful in growth and survival studies of bacterial cells in marine water samples.

Biochemical Tests for the Identification of the Bacterium

All the strains were tested and showed negative result for Gram staining. Hence, all the isolates were belonging to the group of gram negative bacteria .The isolated colonies were identified by biochemical tests (Table-4).

The isolated bacterial colonies such as LB1, LB2, LB3, LB4, LB5, LB6, LB7, LB8, LB9, and LB10 and their cultural, morphological and biochemical characteristics results were compared with Bergey's manual of systematic bacteriology. Based on the isolated colonies were identified such as *Pseudomonas* sp and *Vibrio* sp.

Isolation and Identification of isolated bacterial colonies

The 10 different luminescent colonies were noted after the incubation. The isolated colonies were named as LB1, LB2, LB3, LB4, LB5, LB6, LB7, LB8, LB9, and LB10. The isolated bacterial colonies were identified by cultural, morphological and biochemical characteristics of bacterial cell. The results were presented in (Table 3).

Table 1: Physico chemical analysis of water samples

S.No	Name of the Parameters	Sampling site at nagapattinam district			
		Poompukar	Nagapattinam	Velankanni	Kodiakkarai
1	pH	6.75	6.9	7.14	7.1
2	Temperature	31.1	31	31.4	31.5
3	Electrical conductivity	17.9	19.3	18.5	17.9
4	Dissolved oxygen	146.58	112.8	124.5	68.8
5	Total dissolved soilds	15.08	16.6	16.9	15.25
6	Salinity (ppm)	17.8	21.9	22.2	18.1
7	Total zinc (mg/l)	2.72	4.52	4.87	3.79
8	Total copper (mg/l)	1.63	2.45	2.61	1.79
9	Total iron (mg/l)	10.69	16.8	19.21	12.49
10	Total manganese (mg/l)	8.5	12.36	13.49	10.42
11	Total Boron (mg/l)	0.27	0.32	0.33	0.49
12	Total Molybdenum (mg/l)	0.20	0.23	0.22	0.36
13	Total Chromium (mg/l)	0.03	0.05	0.07	0.06
14	Total nickel (mg/l)	0.12	0.09	0.10	0.16
15	Total Cadmium (mg/l)	0.04	0.02	0.03	0.03
16	Total Cobalt (mg/l)	0.05	0.04	0.06	0.03
17	Total Mercury (mg/l)	0.001	0.001	0.001	0.002
18	Total Arsenic (mg/l)	BDL	BDL	BDL	BDL
19	Total cyanide (mg/l)	BDL	BDL	BDL	BDL
20	Total lead (mg/l)	0.001	0.002	0.003	0.004
21	Selenium (mg/l)	0.12	0.16	0.18	0.13
22	Total Silver (mg/l)	0.02	0.03	0.03	0.06
23	Nitrate (mg/l)	0.012	0.31	0.02	0.020
24	Nitrite (mg/l)	0.079	0.019	0.009	0.009
25	Ammonia (mg/l)	0.35	0.107	0.102	0.114
26	Inorganicphosphorus (mg/l)	1.149	0.062	0.087	0.070
27	Sulphide (mg/l)	0.035	0.053	0.83	0.078
28	Sulphate (mg/l)	1.100	1.00	1.113	1.215
29	Calcium (mg/l)	120	200	120	200
30	Magnesium (mg/l)	288	168	408	288

BDL-Below Detectable Level

Table 2: Shows different sites of collected samples in varying dilution level 10^{-5} to 10^{-7} and bacterial count

Sample	Dilution factor	No. of colonies (CFU/ml)
Poompukar	10^{-5}	200×10^{5} CFU/ml
Nagapattinam	10^{-6}	180×10^{6} CFU/ml
Velankanni	10^{-5}	200×10^{5} CFU/ml
kodiakkarai	10^{-7}	160×10^{7} CFU/ml

Table 3: Morphological characteristics (Colony observation)

Isolation of bacteria	Colony Observation						
	Shape	Margin	Elevation	Size	texture	Appearance	pigment
S1	Circular	Entire	Flat	Small	Smooth	Glistening	Pigmented
S2	Irregular	Lobate	Raised	moderate	Rough	Dull	Non pigmented
S3	Circular	Entire	Flat	Small	Smooth	Glistening	Pigmented
S4	Circular	Filamentous	Flat	Small	Smooth	Glistening	Pigmented
S5	Filamentous	Filamentous	Flat	Small	Smooth	Glistening	Pigmented
S6	Circular	Entire	Flat	Small	Smooth	Glistening	Pigmented
S7	Circular	Filamentous	Flat	Small	Smooth	Glistening	Pigmented
S8	Filamentous	Entire	Flat	Small	Smooth	Glistening	Pigmented
S9	Filamentous	Entire	Flat	Small	Smooth	Glistening	Pigmented
S10	Circular	Entire	Flat	Small	Smooth	Glistening	Pigmented

Table 4: Biochemical characterization

Strain	*Gram nature*	*Cell shape*	*Motility*	*MR*	*Indole*	*VP*	*Citrate test*	*Glucose*	*Nitrate*	*Sucrose*	*Catalase*	*Oxidase*	*Identification*
1	-	Curvedrod shape	Non motile	+	+	-	-	-	+	-	+	+	*Vibrio sp*
2	-	Curedrod shape	Non motile	+	+	-	-	-	-	-	+	+	*Vibrio sp*
3	-	Straight short rods	motile	-	-	-	+	-	-	-	+	+	*Vibrio sp*
4	-	Straight short rods	motile	-	+	-	+	-	-	-	+	+	*Vibrio sp*
5	-	rod	motile	+	-	+	+	-	+	+	+	+	*Vibrio sp*
6	-	Straight short rods	Motile	+	+	-	+	+	-	-	-	+	*Pseudomonas sp*
7	-	Straightshort rods	Motile	+	+	-	+	+	+	+	+	-	*Pseudomonas sp*
8	-	Straight short rods	Motile	-	+	-	+	-	+	+	+	-	*Pseudomonas sp*
9	-	Cocci shape	motile	+	+	+	+	-	-	+	+	+	*Pseudomonas sp*
10	-	rod	motile	+	+	+	+	-	-	+	+	+	*Pseudomonas sp*

+ = positive, - = negative

Table 5: Effect of different concentrations of NaCl on the luminescence of luminescent bacteria

Isolate code	Luminescence in different concentrations of Nacl (%)				
	0	3	6	9	12
LB1	++	++	++	-	-
LB2	++	++	++	-	-
LB3	++	++	++	-	-
LB4	++	++	++	-	-
LB5	++	++	++	-	-
LB6	++	++	++	-	-
LB7	++	++	++	-	-
LB8	++	+++	++	-	-
LB9	++	++	++	-	-
LB10	++	++	++	-	-

- No luminescence; + Dull luminescence; ++ Good luminescence; +++ Luxuriant luminescence

Test for motility

All the 10 luminescent bacteria were found to be actively motile (Table 3).

Effect of external factors on the luminescent bacteria

Effect of salinity

It has been found that up to 6% of NaCl concentration the intense of luminescence was good and thereafter it declined (Table-5). Further, in some strains it was completely ceased beyond 9% of salinity.

Effect of pH

Luminescence was not greatly affected by pH in liquid medium. However, the same result was observed in solid medium (Table-6). pH 7 and 9 were found optimum for the favorable sustenance of luminescence by luminescent bacteria. Interestingly, all the isolates have exhibited considerable luminescence in broth with pH 11.

Table 6: Effect of different pH on the luminescence of luminescent bacteria

Isolate code	Luminescence of bacteria in different pH			
	5	7	9	11
LB1	++	++	++	+
LB2	++	++	++	+
LB3	++	++	++	+
LB4	++	++	++	+
LB5	++	++	++	+
LB6	++	++	++	+
LB7	++	++	++	+
LB8	++	+++	++	+
LB9	++	++	++	+
LB10	++	++	++	+

- No luminescence; + Dull luminescence; ++ Good luminescence; +++ Luxuriant luminescence

10 luminescent bacteria have either grown or produced yellow color colonies and green colour colonies in TCBS agar SWCA medium and which is very selective for *Vibrio* sp, *Pseudomonas* sp and So, it has been confirmed that of the 10 strains were belonging to the genera *Vibrio* sp. Luminous bacteria are the most ubiquitous and widely distributed of all bioluminescent organisms and are found in marine, freshwater, and terrestrial environments[13]. The majority of luminescent bacteria inhabit the ocean. Two genera of marine bacteria, *Vibrio* sp and *Photobacterium,* are among the most abundant luminous bacteria. *Vibrio* sp the most studied luminescent bacterium. Members of the *Photorhabdus* are mostly insect pathogens that exist in a complex symbiotic relationship with a family of entomopathogenic nematodes[15]. Marine luminous bacteria comprise gram-negative motile rods, the single, most unique trait of which is the emission of light[12]. Recognized the unique nature of bioluminescence and proposed that all light-emitting bacteria be placed into a single genus, *Photobacterium.* Taxonomic studies have since revealed new luminous bacterial species possessing a large number of phenotypic characters common to members of the *Enterobacteriaceae* and *Vibrionaceae.* They can be found in seawater and in the intestinal tract and on the body surfaces of marine animals. In order to apply bioluminescence of luminous bacteria to industrial use, isolation of luminous bacteria from various sources was carried out on the basis of strong light intensity, and 18 strains were obtained. Eleven of these strains were identified as *Photobacterium phosphoreum* and seven as *Vibrio fischeri*.

CONCLUSION

This study confirmed that care should be taken when using SWCA and TCBS medium to determine the presence of *Vibrio* sp, *Pesudomonas* sp species in processed sea water. Many of the putative *Vibrio* isolates obtained during this study did not belong to this group of bacteria and the selectivity of TCBS needs to be improved to minimize growth of *Pseudomonas, Aeromonas*, *Shewanella* and members of the *Enterobacteriaceae*. They can be found in sea water and in the intestinal tract and on the body surfaces of marine animals. In order to apply bioluminescent of luminous bacteria from various sources was carried out on the basis of strong light intensity. Looking into the depth of microbial diversity, there is always a chance of finding microorganisms producing novel enzymes with better properties and suitable for commercial exploitation. The multitude of physio-chemically diverse habitats has challenged nature to develop equally numerous molecular adaptations in the microbial world. Microbial diversity is a major resource for biotechnological products and processes.

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