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Research Article

Antimicrobial activity and Characterization of Marine bacteria in Coastal sea water

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Abstract: Coastal seawater samples were collected from Rameswaram, Ramanathapuram District. Twenty five bacteria were isolated and identified according to morphology, biochemical tests and selective growth media. The active marine bacteria isolates were assigned to the genera *Alteromonas*, *Pseudomonas*, *Bacillus* and *Pseudoalteromonas* sp. The TLC autobiographic overlay assay implied that the antimicrobial metabolites produced by four strains with wide antimicrobial spectrum were different. The isolates were tested for antimicrobial activity against the test organisms of *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and *Streptococcus mutans*. Only 2 isolates of MB_{11} and MB_{17} showed clean growth inhibition zone against test organisms. These marine bacteria were identified and expected to be potential resources of natural antibiotic products.
Keywords: Marine bacteria, Antibiotic, TLC, Antimicrobial metabolites, Test organisms

INTRODUCTION

Marine ecosystems represent 95% of the biosphere and coastal regions are particularly promising, because of the rightly adapted species found in these harsh environments. Each of these classes of marine bio-products has a potential multi-billion-dollar market value. This dark-green-Thousands of unique chemical compounds have been identified from a relatively small number of the oceans biological and chemical diversity [1]. The oceans represent a virtually untapped resource for discovery of even more novel compounds with useful activity. In the 19th and early 20th centuries cod liver oil was used as food supplement. So far, more than 10,000 bioactive molecules have been discovered form marine sources with hundreds of new compound still being discovered every year [2]. Thousands of marine organisms are known to contains antibiotic substance and less than 1% have been examined for their pharmaceutical activity. Bacteria are known to produce bioactive substances in the marine environment even if they are specifically antibiotic producers. Bacteria exhibiting antibacterial activities have been isolated from various water samples. In recent years marine microorganisms have become important in the study of novel microbial products exhibiting antibacterial, antiviral, antitumour as well as anticoagulant and cardioactive properties. These active compounds may serve as model systems in the discovery of new drugs [3]. For the past 50 years antibiotics have revolutionized medicine by providing cures for formerly lie threatening diseases. However strains of bacteria have recently emerged that are virtually unresponsive to antibiotics. Although many preexisting antibiotics have been modified to yield new derivatives, bacteria have the potential to mutate known resistance mechanisms to combat these. Many marine free-living inhabiting marine bacterial have been shown to produce secondary metabolites that display antibacterial properties [4]. The first antibiotic from a marine bacterium was identified and characterized in 1996. Recently, the marine bacterium *Alteromonas rava* sp. was found to produce new antibiotic Thiomarinol [5]. In 1947, Rosenfeld and Zo Bell (1947) at Scripps reported on antibiotic activity from marine microorganisms found as a result of study to determine why sea water was bacteriostatic (or) bactericidal to some non-marine bacteria in culture. The Department of ocean Development has brought out a vision perspective plan for 2015 and it appears that about 80% of drugs needed for human health care could be derived from natural sources. A great percentage of marine microorganisms have not been described, although marine microorganisms have been shown to have an increasing interest, as a sources of new bioactive molecules [6]. Several studies have been performed to assess the inhibitory activity expressed by strains isolated from living surfaces in the marine waters. For example of *Pseudoalteromonas* (*Pseudomonas* + *Alteromonas*) group. The Gram-negative, motile bacterium *Pseudoalteromonas tunicata* is found worldwide in marine waters [7, 8].

In this study, the distribution, Morphological characteristics and antimicrobial activities of marine bacterial species in coastal seawater of Rameswaram, was tested.

MATERIALS AND METHODS

Collection of samples

To study the morphological characteristics and anti-microbial properties of the marine bacterial species, coastal water was collected in Rameswaram, Ramanathapuram District. Sample water was collected in clean, sanitized and sterilized glass water bottles.

Total viable count

Total viable count (TVC) of aquatic sediment sample was calculated by following spread plate technique. Each sample was serially diluted with dilutions ranging from 10^{-1}-10^{-11} and 1 ml of each dilution was spreader on the nutrient agar plates. Plates were incubated at 37°C for 48 hours in an inverted position and the colonies were counted. The count was expressed as the number of forming units in 1 ml of the original sample. The colony count below 30 was indicated as TSTC [Too few to count] and above 300 as TNTC [Too number to count].

Sample preparation

10ml bacterial broth sample was centrifuged at 10000 rpm for 15 minutes. Solids were allowed to settle down by centrifugation, the supernatant was collected and transferred immediately into screw capped glass tubes. Then the supernatant from centrifuged sample was passed through the bacterial filter and the filtrate was numbered. Thus there were 25 samples that contained material that did go through the bacterial filter. Filtered samples were ready to test anti-microbial activity against selected terrestrial bacteria.

Antimicrobial activity from marine bacteria

All the isolated marine bacteria were screened for antimicrobial activity, using terrestrial microbes including *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and *Streptococcus mutans* (Agricultural Culture Collection of Tamilnadu) as the test microorganisms. Antimicrobial activity was assayed in duplicate using a standard paper disc assay [9]. The dried crude extracts were dissolved in EtOAc to a concentration of 100 mg ml^{-1}. The samples (20 µl) were used to saturate the antimicrobial assay paper disks (6 mm) with a period of drying between each application. The disks were placed onto the agar surface containing the test microorganisms, and incubated at 37 °C for 24 h after a diffusion process for 10 h at 8°C. The diameters of any inhibition zones formed around the paper disks were then measured.

TLC autobiography overlay assay and Identification of bacteria

The crude extracts of four marine bacteria (MB_1, MB_{11}, MB_{14}, MB_{15}, MB_{16} and MB_{17} with wide antimicrobial spectrum were used in TLC autobiography overlay assay [10]. Each crude extract was dissolved in EtOAc and made up to a concentration of 100 mg ml^{-1}. The solution (2 µl) was submitted to TLC analysis on a 3.5 x 5 cm silica gel plate (TLC aluminium sheets, 20 x 20 cm, Silica Gel 60F_{254} Merck Co, USA) using to some types of chemicals are (DCM and MeOH) dichloromethane (DCM):EtOAc:Methanol (MeOH) 5:5:1, v/v) as the mobile phase. UV/Vis absorption was used for detection at wavelengths of 254 nm and 365 nm. The developed TLC plates were sterilized by UV lamp for 30 min before enchased in the base nutrient agar in a Petridish (9 mm). It was then covered by melting nutrient agar (46 °C) containing test microorganism *Staphylococcus aureus*. After 10 h diffusion process at 8 °C, the plate was then incubated at 37°C for 24 h and the upper agar was sprayed with 5 mg ml^{-1} of methylthiazoletetrazolium to convert to a formazan dye by the test microorganism. Inhibition zones were observed as clear spots against purple background and their Rf values were calculated.

The isolated bacteria were identified based on the colony morphology, gram staining property and biochemical characteristics. Finally they were confirmed by their growth on selective media.

RESULTS

Antimicrobial activity and identification

Twenty five marine bacteria were isolated from sea water around Rameswaram seawater samples. The antimicrobial activity showed that 25 strains (**Table 1**). After taxonomic study it was concluded that the bacteria with antimicrobial activity belong mainly to the genera *Alteromonas* (9 strains), *Pseudomonas* (11 strains), *Bacillus* (3 strains) and *Pseudoalteromonas* (2 strains). These 25 strains were then preserved in a slant, broth cultures and identified according to antimicrobial activity against the test organisms of *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and *Streptococcus mutans*. Only MB_{11} and MB_1 organisms were used in studying the colony, morphology, microscopical characteristics, gram staining, motility and biochemical tests. MB_{11} and MB_{17} gram negative rod shaped and showed motility (**Table 2).** MB_{11} showed oxidase, catalase, Indole and Voges-proskauer were positive except Methyl red and Citrate utilization were negative. MB_{17} showed oxidase and catalase were positive except Indole, Methyl red, Voges- proskauer and Citrate utilization were negative (**Table 3).** Only MB_{11} and MB_{17} showed clear zone of antimicrobial activity against test organisms **(Table 4).** Based on theirthe two strains MB_{11} and MB_{17} were identified as *Pseudoalteromonas tunicata* and *Pseudoalteromonas luteoviolacea* respectively. The separation and identification of bioactive compounds with wide antimicrobial spectrum from these marine bacteria were undergoing.

Antimicrobial metabolites of different strains

Crude extracts of two strains MB_{11} and MB_{17} with wide antimicrobial spectrum were subjected to autobiographic overlay assay, and the results were presented in **Figure 1**. Each extract of different strains showed one or several inhibition spots under the TLC

development system (DCM:EtOAc:MeOH, 5:5:1 v/v), and the R_f values of these spots were all different. For extracts of strain MB_{11} and MB_{17} the Rf values of inhibition spots were 0.55 and 0.61 respectively.

DISCUSSION

Pseudoalteromonas is a genus commonly represented in the marine environment. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from microorganisms, many based on their use in tradition medicine. The studies made by the scientists at the Scripps institution of Oceanography show that marine bacteria are capable of producing unusual bioactive compounds that are not observed in terrestrial sources [6]. Likewise, in the 25 isolates were showed in the antibacterial activity test against *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and *Streptococcus mutans*. Only MB_{11} and MB_{17} were showed clear zone in the antibacterial activity test against *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and *Streptococcus mutans*. Recently the marine bacterium *Alteromonas rava* was found to produce new antibiotic thiomarinol. *P. luteoviolacea* and *P. tunicata* were able to inhibit the growth of most of the *Pseudoalteromonas* species included in this study. These species were also strongly inhibitory against other marine epiphytic bacteria and then on marine strains. The ecological significance of this strong inhibitory activity displayed by these species may colonisation of a broader range of habitats compared to the other *Pseudoalteromonas* species. Assuming these organisms are ejective competitors, the questionarises why they do not become the predominant species on marine surfaces. With respect to *P. tunicata* [11] and *P. luteoviolacea* studies have demonstrated that they produce autoinhibitory compounds. The antibacterial activity of *P. luteoviolacea* is known to be highly strain dependent [12].If the production of self control compounds are closely related to the production of antibacterial compounds or even the same compound, strain variation of *P. luteoviolacea* might explain why an autoinhibitory activity could not be demonstrated in this study. The variation in the activity of the *Pseudoalteromonas* species against different target organisms suggests that different antibacterial compounds are produced by each species. Marine microorganisms as model systems offer the potential to understand and develop treatments for disease based on the normal physiological role of their secondary metabolites and are currently being applied to the development of new drugs [13].

Table 1: **Screening of antimicrobial activity of marine bacteria using agar diffusion assay**

Strain No	**Genus**	**Antimicrobial activity**				
		ST	SA	EC	EA	SM
MB 1	*Alteromonas* sp.	+	++	–	++	+
MB 2	*Alteromonas* sp.	–	–	–	+	–
MB 3	*Alteromonas* sp.	+	–	–	–	–
MB 4	*Pseudomonas* sp.	–	+	–	+	–
MB 5	*Pseudomonas* sp.	+	+	–	+	–
MB 6	*Alteromonas* sp.	–	+	–	+	–
MB 7	*Alteromonas* sp.	+	–	–	+	–
MB 8	*Pseudomonas* sp.	+	+	–	+	–
MB 9	*Pseudomonas* sp.	+	–	–	+	–
MB 10	*Alteromonas* sp.	+	–	–	–	–
MB 11	***Pseudoalteromonas* sp.**	+++	++	++	++	+++
MB 12	*Alteromonas* sp.	+	+	–	+	+
MB 13	*Pseudomonas* sp.	+	++	–	–	–
MB 14	*Pseudomonas* sp.	+	+	+	–	+
MB 15	*Bacillus* sp.	+	+	+	+	–
MB 16	*Bacillus* sp.	+	+	–	+	+
MB 17	***Pseudoalteromonas* sp.**	+++	+++	++	++	++
MB 18	*Pseudomonas* sp.	+	–	–	+	–
MB 19	*Alteromonas* sp.	–	–	–	+	–
MB 20	*Alteromonas* sp.	+	+	+	-	+
MB 21	*Pseudomonas* sp.	–	–	–	+	–
MB 22	*Bacillus* sp.	+	–	–	+	–
MB 23	*Pseudomonas* sp.	–	–	–	+	–
MB 24	*Pseudomonas* sp.	–	++	–	–	–
MB 25	*Pseudomonas* sp.	+	+	–	+	–

The test microorganisms are: ST, *Salmonella typhi*; SA, *Staphylococcus aureus*; EC, *Escherichia coli*; EA, *Enterobacter aerogenes*; SM, *Streptococcus mutans*.
–: no inhibition; +: inhibition zone was 0~10 mm; ++: inhibition zone was 10~15 mm;+++: inhibition zone was≥ 15 mm.

Table -2: **Isolated organisms bases on colony Morphology and other Characteristics**

S.NO	Organisms	Colony morphology	cell shape	Motility	Gram Staining
1	MB_{11}	White coloured colony	Rod	+	-
2	MB_{17}	Green coloured colony	Rod	+	-

+ Indicates Positive
- Indicates Negative

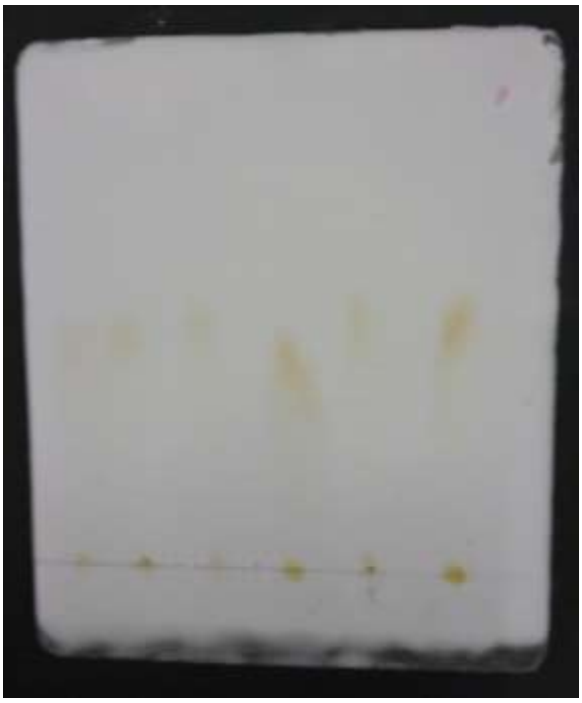
Table – 3: **Biochemical Characteristics**

S.No	Organisms	Oxidase	Catalase	IMVIC			
				I	M	V	C
1	MB_{11}	+	+	+	-	+	-
2	MB_{17}	+	+	-	-	-	-

+ Indicates Positive
- Indicates Negative

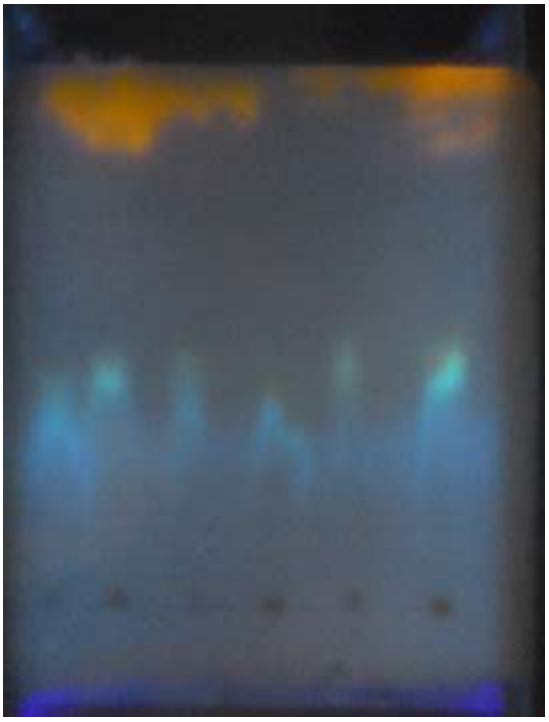
Table – 4: Antimicrobial activity of isolated Organisms

S.NO	Isolated Organisms	Zone of Inhibition in mm				
		Salmonella typhi	***Staphylococcus aureus***	***Escherichia coli***	***Enterobacter aerogenes***	***Streptococcus mutans***
1	MB_{11}	18	14	12	14	21
2	MB_{17}	20	22	14	15	15



MB_1 MB_{11} MB_{14} MB_{15} MB_{16} MB_{17}
Figure 1 – TLC autobiographic overlay assay

TLC autobiographic overlay assay for wide antimicrobial spectrum strains against *Staphylococcus aureus* . The samples are extracted from the strains: MB_1, MB_{11}, MB_{14}, MB_{15}, MB_{16} and MB_{17}. The inhibition spots were observed.



MB_1 MB_{11} MB_{14} MB_{15} MB_{16} MB_{17}
Figure 2 – TLC autobiographic overlay assay

CONCLUSION

It can be concluded that isolation of Marine bacterial samples can offer a numbers of microbial strains for screening of new biomolecules from Marine sources. Bacteria are known to produce bioactive substances in the Marine environment of they are specifically antibiotic principles. This study indicated that produce antibiotics for treat varieties of human diseases.

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