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Isolation and characterization of bioactive compounds from marine bacteria

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With a huge volume of unexplored wealth, marine environment dazzles out to be a very good source, capable of answering many unsolved questions and a treasure of unresolved mysteries. In this work coastal water samples were collected from Thiruchendur, Thoothukudi and Kanyakumari, Tamil Nadu. From these samples, organisms were isolated by spread plate technique using marine agar. About 21 bacterial isolates were isolated and were screened primarily through agar diffusion method, of which only 6 isolates exerted an inhibitory effect against the target organisms were selected for antimicrobial analysis. Their supernatants were used to study the antagonistic activity against target organisms (*Salmonella typhi, Enterobacter* sp., *Pseudomonas* sp., *Streptococcus mutans*, *Staphylococcus epidermidis* and *S. aureus*). Cell free extracts of two organisms *Alteromonas* sp. and *Rhodopseudomonas* sp. identified in the isolates were found antagonistic against all the test organisms. Both the supernatants were checked with Nuclear Magnetic Spectroscopy for characterizing the active factor. The cell free supernatant from the first one was suspected to contain the compound similar to Drechslerine A and the other supernatant was found suspected to contain the compound similar to that of cis-Sativenediol.

Keywords: Anti-bacterial, Antagonistic, Bioactive compounds, Drechslerine, Marine bacteria, Sativenediol.

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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. The marine environment is a rich source of both biological and chemical diversity. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, enzymes, fine chemicals and agrochemicals. Each of these classes of marine bio-products has a potential multi-billion dollar market value. Thousands of unique chemical compounds have been identified from a relatively small number of ocean's biological and chemical diversity[1]. The ocean represents virtually untapped resource for discovery of even more novel compounds with useful activity[2].

In the 19th and early 20th centuries, cod liver oil was used as food supplement. However, it was only in the middle of the 20th century that scientists began to systematically probe oceans for medicine. So far a number of bioactive molecules have been discovered from marine sources, with many more compounds still being discovered every year[3].

Antibiotics have been defined as substances produced by microorganisms that are antagonistic to the growth or life of other microbes. Thousands of marine organisms are known to contain antibiotic substances and only a very minimal quantity has been examined for their pharmacological activity[4].

Bacteria are known to produce bioactive substances in the marine environment, predominantly protecting themselves from their predators. Bacteria exhibiting antibacterial activities have been isolated from various water samples. In recent years marine microbes have become important in the study of novel microbial products exhibiting antibacterial, antiviral, antitumour as well as anticoagulant and cardioactive properties[5,6]. These active compounds may serve as model systems in the discovery of new drugs[7-9].

For the past 50 years antibiotics have revolutionized medicine by providing cures for formerly life threatening diseases. However, strains of bacteria have recently emerged that are virtually unresponsive to antibiotics. Such multi-drug resistance arising mainly through antibiotic misuse is now recognized as a global health problem. The situation is exacerbated by the fact that no novel

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chemical class of antibiotics has been discovered for 20 years. Although many pre-existing antibiotics have been modified to yield new derivatives, bacteria have the potential to mutate known resistance mechanisms to combat these[10,11].

Recently, the marine bacterium *Alteromonas rava* has been found to produce new antibiotic thiomarinol[12]. The studies made by the scientists at the Scripps Institution of Oceanography, La Jolla, California show that marine bacteria are capable of producing bioactive compounds that are not observed in terrestrial sources[8,13].

In 1936 at the Scripps institute a study based on antibiotic activity from marine microbes was found as a result of study to determine why sea water was bacteriostatic or bactericidal to some non-marine bacteria in culture[14]. They identified nine species of marine microbes that showed antibiotic activity. The sea may represent a reservoir of microbial antagonists of possible importance, they wrote.

The Department of Ocean Development has brought out a vision perspective plan for 2015 and it appears that 80% of drugs needed for human health care could be derived from natural sources. A great percentage of marine microbes have not been described[15], although marine microbes have been shown to have an increasing interest, as a source of new bioactive molecules[13]. Some marine bacteria secrete exo-polysaccharides[16] and many marine free-living bacteria produce secondary metabolites possessing antibacterial properties[17].

This study is focused on the isolation of different species of marine bacteria on marine agar from different coastal water sources, production of natural marine secondary metabolites through broth inoculation from all the isolates screened through agar well diffusion method, qualitative estimation of antagonistic activity of the secondary metabolites on the test organisms used by Kirby-Bauer well diffusion method and identification of the active factor eliciting antagonistic activity through Nuclear Magnetic Resonance (NMR) spectroscopy.

Materials and Methods

Sources of organisms

Coastal sea water from Thiruchendur (8.08′N, 78.11′E), Thoothukudi (8.45′N, 78.13′E) and Kanyakumari (8.35′N, 77.05′E), Tamil Nadu, India were collected in clean, sanitized and sterilized glass bottles. The samples were serially diluted and spread plated on to marine agar plates. Individual isolates from the dilution plates were then sub-cultured and used for further studies.

Marine broth with a high percentage of salt was used in the production and characterization of the active factor of the isolates. All the organisms were identified to the generic level based on their biochemical characteristics[18].

Agar diffusion method

All the 21 isolates were screened for the production of bacteriocin. To demonstrate their production, individual isolates were stab inoculated on separate nutrient agar Petri dishes, incubated at 30°C for 24 h. Overlay each plate with one test organism (in soft agar), and same process repeated with the other test organisms as well. Plates were incubated again at 30°C for 24 h. After incubation, the presence of bacteriocins was observed by the presence of zone around the stabbed area of inoculation.

Sample preparation for antimicrobial activity

The cultures which were found to possess any antibacterial activity were grown in marine broth media for two weeks. After incubation the culture supernatants were obtained by centrifuging the whole content at 10,000 g. The supernatants obtained were screened for the presence of bacteriocins against all the six standard type cultures chosen. The supernatants which were found to possess the maximum antagonistic activity were also characterized through NMR.

Antagonistic activity[19]

The supernatants obtained were screened for their antimicrobial activity against *Salmonella typhi, Enterobacter* sp*., Pseudomonas* sp., *Streptococcus mutans, Staphylococcus epidermidis* and *S. aureus* (Type cultures procured from MTCC, Chandigarh). Marine agar was prepared, sterilized and poured into Petri plates up to a depth of 3 mm. The organisms were suspended in saline and 0.1 ml of organisms (10^{10} colony forming units per ml) were spread on these plates on which wells were made using an 8 mm cork borer. To each well, 50 µl of each supernatant were added and plates were incubated at 37°C for 24 h. After incubation, the results were recorded by measuring the diameter of zone of inhibition surrounding the well. The experiments were done in triplicate.

NMR

The crude supernatants of the sample showing antagonistic effect were analyzed using nuclear magnetic resonance (NMR) spectroscopy to investigate the chemical structural properties. The NMR experiment was conducted on a 400 MHz JOEL NMR spectrophotometer with trimethyl sulphoxide as the standard and deuterated water as the solvent.

Results

The coastal seawater sample collected from Thiruchendur, Thoothukudi and Kanyakumari were diluted up to 10^{-8} and twenty one organisms were isolated by spread plating it on marine agar. On screening them with agar diffusion methods only 6 organisms had antagonistic activity against at least one test organism used, which was qualitatively noted (Table 1). Not all the organisms were found antagonistic against all of the test organisms. There might be a number of reasons that could answer this and one among them is the incubation time.

According to morphology and biochemical characteristics, these organisms were identified up to the generic level in accordance with the Cowan and Steel model (Table 2). The culture supernatants of these six cultures were selected for further studies. Isolate one and fourth were found to belong to the genera *Bacillus*, the third and the sixth one were found to be *Acinetobacter* and *Marinococcus*, respectively. The second one was found to be *Alteromonas* and the fifth one gave the impression to be *Rhodopseudomonas*, according to the nature of the results observed.

Out of the six isolated organisms the supernatants of *Alteromonas* sp., and *Rhodopseudomonas* sp., were antagonistic against all the test organisms studied (Table 3). Extracts from the other isolates were of less interest, as they were not inhibiting the growth of the test organisms.

The peak obtained through NMR spectroscopy for the cell free supernatant of *Alteromonas* sp., (Fig. 1) was found to contain the compound Drechslerine A

Table 1 — Screening of antagonistic activity of isolated marine bacterial strains

Bacterial isolates/Test Organisms	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Salmonella typhi	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
Enterobacter aerogenes,	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
Pseudomonas aeruginosa	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus mutans	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-
Staphylococcus epidermidis	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+Antagonistic effect, – No antagonistic effect

Table 2 — Morphological and biochemical characterization for the identification of selected marine bacterial strains

Bacterial isolates	Grams Reaction	Shape	Indole	Methyl red	Voges proskeaur	Citrate utilization	Urease	Nitrate	Aerobic growth	Lactose Fermentation	Endospore	Motility	Catalase	Oxidase	Identity
1	+	Rod	-	-	-	-	-	+	+	+	+	+	+	-	*Bacillus* sp.
2	-	Rod	-	-	-	+	-	+	+	+	-	+	-	+	*Alteromonas* sp.
3	-	Cocci	-	-	-	+	-	-	+	-	-	-	+	-	*Acinetobacter* sp.
4	+	Rod	-	-	-	-	-	+	+	+	+	+	+	-	*Bacillus* sp.
5	-	Rod	-	-	-	-	-	-	+	-	-	+	+	+	*Rhodopseudomonas* sp.
6	+	Cocci	-	-	-	-	-	+	+	+	-	-	+	-	*Marinococcus* sp.

Table 3 — Antagonistic effect of cell free supernatants from selected bacterial isolates against the test organisms

Identified bacterial isolates	Antagonistic effect (Zone of inhibition, diam. in mm)					
	Test Organisms					
	Salmonella typhi	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*	*Streptococcus mutans*	*Staphylococcus epidermidis*	*Staphylococcus aureus*
Bacillus sp.	0	0	30	0	0	0
Alteromonas sp.	10	12	15	17	20	12
Acinetobacter sp.	0	0	0	0	15	0
Bacillus sp.	0	0	25	0	0	0
Rhodopseudomonas sp.	11	12	30	23	21	18
Marinococcus sp.	0	12	0	0	0	0

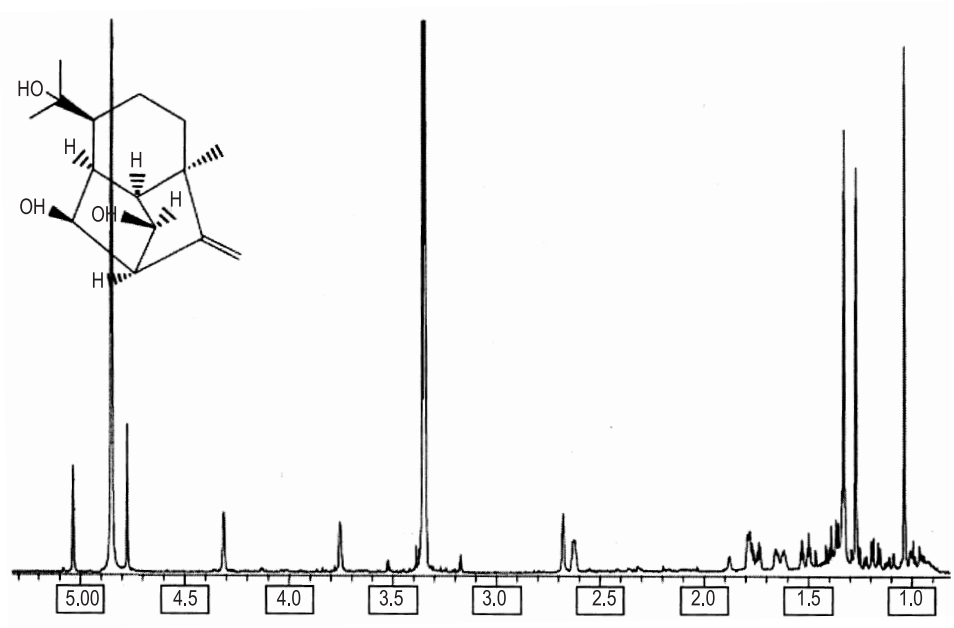

Fig. 1 — NMR Spectra for the supernatants of *Alteromonas* sp., showing the presence of Drechslerine A.

$(C_{15}H_{24}O_3)$[20] and the peak obtained for the cell free supernatant of the *Rhodopseudomonas* sp. (Fig. 2) was found to contain the compound cis-Sativenediol $(C_{15}H_{24}O_2)$[20]. The peaks obtained were compared with the standard peaks from the work of Osterhage[21] and were confirmed to be the compounds Drechslerine A and cis-Sativenediol present in the supernatants of *Alteromonas* sp. and *Rhodopseudomonas* sp., respectively.

Discussion

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from microbes, many based on their use in traditional medicine. However, an increasing role has been played by microbes in the production of antibiotics and other drugs for the treatment of some serious diseases.

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[8,13]. Nair and Saimidu[22] have also reported that the absence or reduction in number of bacteria with antibacterial activity in Tokyo bay is due to its eutrophic nature, which may tend to modulate the production of antibacterial compounds. The absence of reduction in number of bacteria with antibacterial

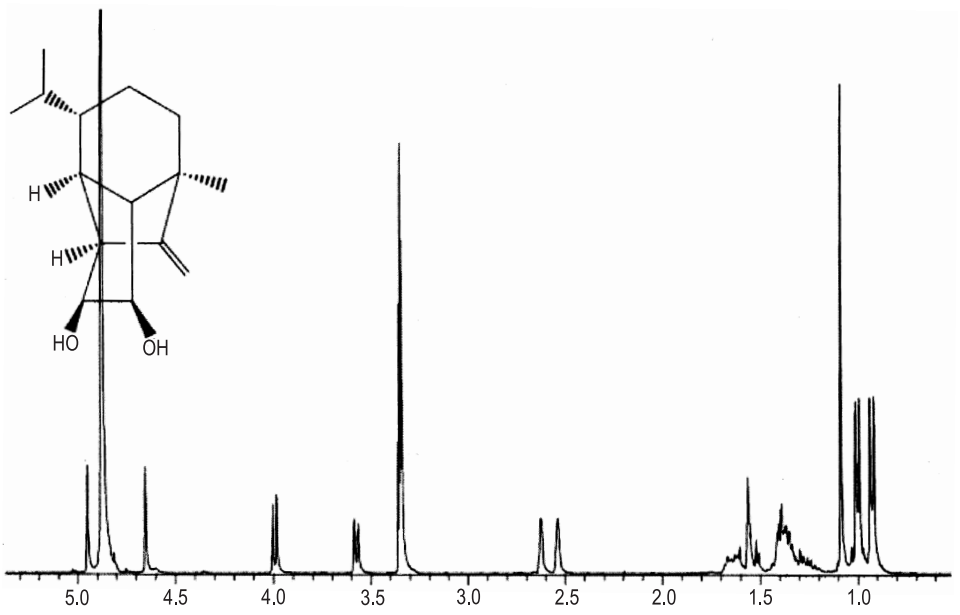

Fig. 2 — NMR Spectra for the supernatants of *Rhodopseudomonas* sp., showing the presence of cis-Sativenediol.

activity in Tokyo bay is due to its eutrophic nature, which may tend to modulate the production of antibacterial compounds.

Several workers have also recorded high incidence of *Alteromonas* and their inhibitory activity against many bacteria[6,20]. Recently, the marine bacterium *Alteromonas rava* was found to produce new antibiotic Thiomarinol. *Pseudomonas* is another genus which is also reported to have involved in the production of active factors which has also been under the scanner.

Stierile[23] stated that a microbial metabolite from *Alteromonas* has been developed with anti-HIV potential as reverse transcriptase inhibitor, from marine microbes isolated from the tissues of Bermudian marine sponge.

In the present study Gram negative bacteria showed antibacterial activity as compared to Gram positive bacteria. Okazaki and Okami[24] also reported a similar finding while screening actinomycetes isolated from marine sediments. These observation indicated that the antibacterial component of Gram negative marine bacteria produce bioactive substances.

The biological evaluation of marine derived extracts and pure compounds for pharmaceutical development have been based on assays development from the libraries of the already developed synthetic compounds[1]. Marine microbes as model systems offer the potential to understand and develop treatments for disease based on the normal physiological role of their secondary metabolites. For example, the mechanisms of Conus toxins action are well-known and are currently being applied to the development of new drugs.

Conclusion

From the current study it could be noted down that a vast stretch of bioactive compounds present in the marine environment is still unexplored. The two bioactive compounds Drechslerine A and cis-Sativenediol obtained from *Alteromonas* sp. and *Rhodopseudomonas* sp. which were found to be active against a wide range of pathogenic microorganisms were not explored as a potent bactericidal agent till now. If these two active compounds could be isolated in pure form they could be used as a drug in controlling the growth of the pathogenic strains. Also these active factors could be used to develop antimicrobial wound care finishes which could replace the commercial wound care agents, being cheaper in cost and effective in action.

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