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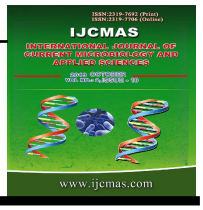

Original Research Article

Antimicrobial potential of marine bacterial isolates from different Coastal regions of Andhra Pradesh and Tamil Nadu, India

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A B S T R A C T

The current study characterizes marine bacteria isolated from the coastal regions (Chennai, Ongole & Nellore), and tests their antimicrobial activities. From these samples bacteria were isolated by spread plate technique using marine agar. About 24 bacterial strains were isolated and screened for antimicrobial activity through agar diffusion method, of which only two isolates exerted an inhibitory effect against target organisms (*Enterococcus feacalis*, *Staphylococcus aureus* and *Klebsiella pneumoniae*). These isolates were characterized phenotypically by means of morphological techniques and physiologically by conventional tests. One of the bacterial isolates was Gram positive rod, identified as *Bacillus licheniformis*, and other Gram negative rod conformed as *Pseudomonas aereuginosa*.

Keywords

Sea water; marine bacteria; antimicrobial activity; coastal regions; resistant pathogens.

Introduction

Currently emerging and reemerging infectious diseases are a major problem in public health and global economics. They are caused by different types of infections such as drug – resistant infections, mostly involving bacteria, and many emerging pathogens are increasing significantly over time because they are becoming progressively more resistant to conventional antibiotic compounds. For example, *Pseudomonas aeruginosa* has been reported as an opportunistic pathogen and one of the most common causes of nosocomial infections by the intrinsic resistance to many antimicrobial agents (Depardieu *et al.*, 2007 and Kollef, 2005). In *Staphylococcus aureus*, virulence and antibiotic resistance are contributing to its emergence as leading overall cause of nosocomial infections in both nosocomial and outside the hospital setting (Lowy, 2003 and Livermore, 2004). So, the need for the development of new antibiotics to counter drug resistance in bacterial pathogens has been stressed by various researchers worldwide.

The marine environment is becoming increasingly appreciated as an exceptional reservoir of bioactive natural compounds, which exhibit structural/chemical features not found in terrestrial natural products (Carter, 1996). Oceans cover 70% of the earth's surface and harbor most of the planet's biodiversity (Faulkner, 1995). Marine microorganisms have become an important point of study in developing for novel antibiotics.

This is consequent to the decrease in discoveries from terrestrial microbial sources, as well as, the emergence of antibiotic resistant clinical pathogens such as *Mycobacterium tuberculosis*, *Enterococcus*, *Pseudomonas sp.*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* which led to constant need to find new sources of effective antibiotics.

Marine bacteria are a rich source of potentially useful antimicrobial molecules. It was known to produce antimicrobial metabolites have been reported like macrolactin F, 7-*O*- succinylmacrolactin F and 7-*O*-succinylmacrolactin A, (*Bacillus sp.* Sc026) (Jaruchoktaweechai *et al.*, 2000), new thiopeptide compounds (*Bacillus cereus* QN03323) (Nagai *et al.*, 2003), and three bacteriocin-like peptides namely Lichenin, Bacillocin 490 and P40 (Cladera-Olivera *et al.*, 2004).

However, much of the microbial diversity in marine ecosystems with its potential for uncovering new antimicrobial compounds remains to be discovered. Hence, the present study was undertaken to isolate different strains of marine bacteria from sea water and to evaluate their antibiotic producing efficacy by minimum inhibitory concentration.

Materials and Methods

Sample collection

Marine water samples were collected from Chennai (Marina Beach) (35.04'E, 5.45'N) and Andhra Pradesh (Kotthapattanam) (59.12'E, 6.28'N) coastal regions, India at 10m depth. Samples were collected in a sterile glass bottle and stored at 4^{o}C. The marine water samples were stored under sterile conditions for preventing the bacterial cross contamination until use.

Physico chemical characteristics

pH, temperature, electrical conductivity, color, texture and salinity of samples were measured the sampling site.

Isolation of marine bacteria

For isolation of marine bacteria from each water sample, 1.0 ml of the sample was mixed with 9.0 ml of distilled water and serial dilution was performed up to 10^{-5}. From this about 0.1ml of the aliquots was spread on the surface of the Marine agar (Hi-media 2216); triplicates were maintained for each dilution. The inoculated plates were incubated at 37^{o}C for 7-10 days. Colonies developed after incubation were purified by repeated sub culturing on marine agar and finally, bacterial colonies with distinct characteristics such as pigmentation, size, opacity, elevation, margin and surface appearance (Yeon *et al.*, 2005) were chosen for further characterization.

Physiological and biochemical characters

The characters of the organisms were studied following the standard

microbiological methods. Morphology, vegetative cell and spore characters were observed under microscope (100X objective) from 12 h old culture at 37 ± 1 °C. Growth characteristics were observed using different types of media, such as nutrient agar, Macconkey Agar and Muller Hinton Agar (Atlas, 1993). The physiological and biochemical characters viz. indole production, oxidase, catalase, urease hydrolysis, acid from glucose, mannitol, xylose, citrate, and propionate utilization and tyrosine hydrolysis were performed.

Crude extract preparation

Marine bacterial cells were separated from the spent broth by centrifugation (at 3000 *g* for 15 min at 4° C) and washed twice with sterile natural seawater. Two grams of biomass were resuspended in 10 mL of PBS and sonicated. Spent broth and crude extract was tested for antimicrobial activity.

Determination of antibacterial activity of marine bacterial isolates

The crude extracts were screened for antibacterial activity using the disc diffusion method as described by Pandey *et al.,* (2004). Freshly grown colonies of tested bacterial pathogens like *Bacillus subtilis, Enterococcus feacalis, Staphylococcus aureus, Streptococcus thermophilus, E. coli, Proteus mirabilis* and *Klebsiella pneumoniae* were used to inoculate Muller Hinton Broth (Himedia) medium at 37°C for 5-8 hours until turbidity could be observed (Asthana *et al.,* 2009). The bacterial suspension (0.1 mL) was poured on each plate containing Muller Hinton Agar (MHA, Merck). The lawn culture was prepared by sterile cotton swab. The dried crude extract was dissolved in methanol to a concentration of 1.0 mg/ mL. The sterile filter paper discs (6 mm diameter) (Kanoh *et al.,* 2008) were saturated with 20µL of this concentration of each extract and then were placed on lawn cultures. The Petri dishes were subsequently incubated at 37°C for 24 hours and the inhibition zone around each disc was measured in mm. Four different antibiotics including Ciprofloxacin 5µg, Amikacin 25µg, Vancomycin 30µg, and Clindamycin 25 µg were used as control.

Determination of Minimum Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of bacterial isolates was determined against the bacteria like *Bacillus subtilis, Enterococcus feacalis, Staphylococcus aureus, Streptococcus thermophilus, E. coli, Proteus mirabilis* and *Klebsiella pneumoniae* by the micro broth dilution assay method (Motamedi *et al.,* 2010). In the tube dilution assay, standard bacterial suspension and of crude extracts (125, 250, 500 and 2000 µg/mL) were added to tubes containing 1 mL Muller Hinton Broth. These tubes were incubated at 37°C for 24 hours. The first tube in the above series without sign of visible growth was considered as the MIC.

Result and Discussion

A total of 24 isolates were isolated from the different coastal regions of sea water samples. Physico chemical properties were measured for all the samples and they were represented in Table 1. Out of the 24 isolates, two isolates were CMB-3 (Chennai Marine Bacteria) and KMB-1 (Kothapattanam Marine Bacteria) was selected for further analysis, since they showed significant antibacterial activity against test organisms. The two isolates were identified and confirmed by

microscopic and macroscopic examination. CMB-3 strains were a gram positive, rod shaped with spiral spore chain bacteria. KMB-1 strain was gram negative, cocci in nature, long spore chain, and filamentous bacteria (Fig.1). The macroscopic appearance of the isolates KMB-1 showed leathery, white powdery colonies in Muller Hinton agar whereas CMB-3 were showed creamy, pin point, powder colonies (Table. 2).

Out of the 24 isolates, CMB-3 and KMB-1 showed antibacterial activity. CMB-3 is active against the gram positive cocci and gram negative bacilli i.e. *E. faecalis, S. aureus* and *K. pneumoniae*). The inhibition zone diameter ranges 10.0 mm to 18.0 mm. But inhibition was not observed in *Proteus mirabilis, B. subtilis, Streptococcus thermophilus* and *E.coli*. KMB-1 showed inhibition against both gram positive and gram negative bacteria (Fig.3). Inhibition zone ranges 11.2, 12.0 and 16.0 mm. i.e. for *E. faecalis, S. aureus* and *K. pneumoniae.* Whereas in CMB-1, CMB-2, KMB-2, NMB-1 and NMB-2 inhibition was not observed in all the tested bacteria (Table.3). Antimicrobial activity of CMB-3 was more significant in *K. pneumonia* than that of KMB-1. In case of *S.aureus* and *E. faecalis* the results were not significantly different in both the isolates.

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Minimum inhibitory concentration (MIC) was tested for the marine Isolates i.e.CMB-1, CMB-2, CMB-3, KMB-1 and KMB-2**.** MIC values for CMB-3 on *E. faecalis* and *S. aureus* were found to be at 1000µg/ml and for *K. pneumoniae* at 2000µg/ml concentration. MIC of KMB-1 on *E. faecalis* was found to be at 1000µg/ml concentration, whereas for *S. aureus* and *K. pneumoniae* at 2000µg/ml concentration. But there was no inhibition of bacteria at the tested concentrations in CMB-1, CMB-2, KMB-2, NMB-1 and NMB-2 (Table.4).

Biochemical tests of the isolate CMB-3 isolate showed a positive reaction on catalase, oxidase, urease and citrate utilization, nitrate reduction and a negative reaction of indole, methyl red and VP. CMB-3 showed negative reaction for sugar fermentation with glucose and sucrose and positive reaction for lactose. KMB-1 isolate showed positive reaction for nitrate reduction, catalase test and citrate utilization. Negative reaction in indole, methyl red, VP, urease and oxidase. Lactose fermentation was also observed in KMB-1. Both acid and gas production was observed. In contrast to KMB-1, CMB-3 showed positive results for urease (Fig.2). Biochemical characteristics of the isolates were listed in Table.5.

Based on the cultural, morphological and biochemical nature of the isolates and with reference to Bergey's manual, the organisms were tentatively identified as species of *Bacillus sps* and *Pseudomonas sps.*

Antibiotic resistance among pathogenic bacteria is usually of greater magnitude in developing countries that is very important in national and international economical, political and scientific aspects. For instance, MRSA particularly is responsible for the largest outbreak of hospital acquired infection (HAI) that the world has ever seen, so there is a continued need to develop new antibiotics. Unfortunately, because of the ability of bacteria in exchanging of genetic information and new mutations this problem has been more complicated (Tenover, 2006). With respect to enormous biodiversity in the marine

Table.1 Physico chemical properties of the marine samples.

S.N^{o}	Sampling site	pH	Eh	Temp (^{o}C)	Color	Salinity	Texture
1.	Kotthapattanam (Ongole)	8.4	32.4	26.2	White	3.4	Clear
2.	Nellore	8.5	64.2	24.4	White	3.3	Turbid
3.	Chennai	8.2	52.4	23.8	White	3.8	Turbid

Table.2 Morphological characteristics of marine isolates

Morphological characteristics	Isolates						
	CMB-1	CMB-2	CMB-3	KMB-1	KMB-2	NMB-1	NMB-2
Gram stain	+	+	+	-	-	-	-
Straight rods	+	+	+	+	+	+	+
Arrangements	Single	Single	Clusters	Single	Clusters	Single	Single
Spore	+	+	+	+	+	+	+
Motility	+	+	+	+	+	-	-

Table.3 Antimicrobial activity of marine isolates on different pathogenic microorganisms

Diameter of Inhibition Zone (mm)							
Name of the isolate	Name of the organism						
	E. faecalis	*S. aureus*	*P. mirabilis*	*K. pneumoniae*	*B. subtilis*	*S. thermophilus*	*E. coli*
CMB-1	-	-	-	-	-	-	-
CMB-2	-		-	-	-	-	-
CMB-3	10.4	15.0	-	18.0	-	-	-
KMB-1	11.2	16.0	-	12.0	-	-	-
KMB-2	-	-	-	-	-	-	-
NMB-1	-	-	-	-	-	-	-
NMB-2	-	-	-	-	-	-	-

mm- millimeter ; '-'indicates no zone of inhibition

Table.4 MIC values of the marine bacterial isolates. **ND**- not detected

Name of the microorganism	Minimum inhibitory concentration (MIC) in µg/ml						
	CMB-1	CMB-2	CMB-3	KMB-1	KMB-2	NMB-1	NMB-2
E. faecalis	ND	ND	1000	1000	ND	ND	ND
S. aureus	ND	ND	1000	2000	ND	ND	ND
P. mirabilis	ND	ND	ND	ND	ND	ND	ND
K. pneumoniae	ND	ND	2000	2000	ND	ND	ND
B. subtilis	ND	ND	ND	ND	ND	ND	ND
S. thermophilus	ND	ND	ND	ND	ND	ND	ND
E. coli	ND	ND	ND	ND	ND	ND	ND

Table.5 Biochemical characterization of selected marine bacterial isolates.

Biochemical tests	**CMB-3**	**KMB-1**
Indole production	-	-
Methyl red test	-	-
Voges Proskeaur	-	-
Citrate utilization test	+	+
Urease production	+	-
Nitrate reduction	+	+
Aerobic growth	+	+
Lactose fermentation	+	+
Glucose, Sucrose fermentation	-	-
Catalase test	+	+
Oxidase test	+	-
Production of fluorescent pigment	-	-
Identity	***Bacillus sps***	***Pseudomonas sps***

+: indicates positive reaction ; - : means negative reaction

environment so that in the case of microorganisms, sea water is composed of 78 million microscopic cell per ounce or the bottom, which mimic the soil, contain more than one billion cells in the volume of an ordinary cube of sugar this environment as a rich resource can be exploited for developing new natural pharmaceutical products especially antibiotics (Darabpour *et al.,* 2010).

In 1947, Rosenfeld and Zobel Rosenfeld *et al.,* (Deve and Desai, 2006) had carried out the first detailed study of antibiotic-producing marine bacteria (Kazan *et al.,* 2005). Since then, there are several reports of antibiotic-producing marine bacteria showing the antagonistic effect against human pathogens, (Mincer *et al.,* 2002) as strains of pathogenic bacteria that recently emerged are unresponsive or multi drug resistant to the already discovered antibiotics that are in use. The emergence of resistant strains to commonly used antibiotics. Among human microbial pathogens has necessitate the researches to discover the new antimicrobial agents that are produced in natural way. The oceans, which cover almost 70% of the earth‘s surface (Ganesh *et al.,* 2004) contain a variety of species, many of which have no terrestrial counterparts. So, marine bacteria serve as largely untapped source of secondary metabolites.

In the present study, the marine bacteria from the coastal region of Chennai and Ongole were isolated and these isolates i.e. CMB-3 and KMB-1 were identified as *Pseudomonas sps.* and *Bacillus sps*. showed good antimicrobial activity and was selected for further investigation. The production of inhibitory antibiotic compound by fluorescent *Pseudomonas* is well documented (Fravel, 1988 and Fenton *et al.,* 1992). This strain is aerobic and failed to grow under anaerobic and acetic conditions. It did not require organic growth factors and oxidase, catalase and citrate positive but indole negative and found to be resistant to several antibiotics (Ampicillin, kanamycin, erythromycin,

and tetracycline) and sensitive to chloramphenicol. A bioassay based on clearing zone on agar was used to determine the antibiotic activity of the metabolite produced by CMB-3 and KMB-1 against different pathogenic bacteria. Many members of the *Bacillus* group continue to be dominant bacterial workhorses in microbial fermentation for the production of novel proteins (Barbosa *et al.*, 2005).

From this study we find out that antibiotic producing microorganisms are present in Coastal regions of Chennai and Ongole Sea water. Out of these twenty four isolates, two produced antibacterial metabolites against the tested organisms.

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