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Research Article

ANTIMICROBIAL PROPERTIES OF MARINE BACTERIA ISOLATED FROM THE BAY OF BENGAL AND THEIR PHARMACEUTICAL PROSPECTS AS ANTIBIOTICS AGAINST MULTI DRUG RESISTANT PATHOGENS

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ABSTRACT

In these experiments, in vitro antimicrobial studies of marine microbes collected from rhizoidal soil of coastal vegetation from Rameshwaram, Pichavaram and Thiruvanmayyur regions of Bay of Bengal have been done. Its study reveals the immense antibacterial and antifungal properties against human bacterial pathogens which are having potential application in pharmaceutical industry. Out of 18 isolates of 6 are characterized as *Pseudomonas* and 12 are Actinomycetes. In vitro antimicrobial test in both agar well and disc diffusion methods are employed to test against the selected pathogens having the resistance capacity towards antibiotics. The strain AC10 was found to highly active against both bacteria and fungus noticeably, whereas PS2 for bacteria and PS1 was found active against human fungal pathogens. On the contrary, the isolates AC3 and PS5 has less bactericidal activity and AC12 and PS5 did not so any significant fungicidal activity. Our future aspects are evaluation of potent antibiotic drug molecule from the best strains against human pathogen and purification, identification of the lead compound by using various sophisticated instruments like FPLC,GC-MS, FTIR, NMR and their pharmacokinetics studies.

Keywords: Marine environment, Rhizoidal soil, Antimicrobial activity, Pharmaceutical, Pharmacokinetics.

INTRODUCTION

70% of the planet surface area are ocean and almost 99% of the volume is known to sustain life. India is one among 12 mega-biodiversity countries and 25 hotspots of the richest and highly endangered eco-regions of the world. More and more researches have been interested on marine organisms as sustainable sources because of low content of known active compounds in marine animals and plants as well as limitation of bioresource supply. The number of potential compounds isolated from marine habitat has virtually soared and this number now exceeds 10,000 with hundreds of new compounds still being discovered every year[1]. Actinomycetes are an important group of bacteria, producing over 70% of naturally occurring antibiotics as well as other bioactive compounds [2]. Compare with terrestrial organisms, the secondary metabolites produced by marine organisms have more novel and unique structures owing to the complex living circumstance and diversity of species, and the bioactivities are much stronger [3,4].

Marine source for utilizable microbes active as anticancer, antidiabetic and antibiotic compound producer has been studied very less. Because of low content of known active compounds in marine animals and plants as well as limitation of bioresource supply, more and more researchers have been focused on marine microorganisms as sustainable resources. The number of potential compounds isolated from marine realm has virtually soared and this number now exceeds 10,000 with hundreds of new compounds still being discovered every year [10]. The focus on the physiology and the potential of bioactive substances of non-cultivable marine microorganisms is of current problem and pose a great challenge to researchers to cultivate and isolate novel secondary metabolites for therapeutic applications [21].The coastal water zone has high biological potential and some evidence indicates that many compounds previously found in sponges are biosynthesized through microorganisms associated with them[7]. Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products [8].

Why the marine compounds? It is noteworthy that ocean is the great sink of biodiversity from microscopic organisms to giant whale. The secondary metabolite produced by marine microbes has great impact in human society as having their pharmaceutically active molecule with biological moieties. From the past few decades, the advanced molecular biology and genetic engineering fulfills the gap between the marine resources and the technology. Out of the 22,500 total bioactive secondary metabolites, 10,100 (45%) are reported to be produced by actinomycetes [8]. Marine microbes show great promise for various biotechnological applications in the area of health, environment and agriculture [9].The bacteria isolated from rhizospheric regions of different marine coastal vegetation can be found antagonistic against different plant or animal pathogen which can be studied through testing of in vitro antibiosis or by bioassays through several methods[8]. Furthermore, along with the deep studies of marine natural products biosynthesis, some evidence indicates that many bioactive compounds previously found in marine animals and plants were in fact produced or metabolized by associated microorganisms [10, 11].The objectives of our study were to reveal the hidden prospects of marine bacteria of Indian marine environment in relation to the study of antimicrobial to evaluation of new potential drugs.

MATERIALS AND METHODS

Isolation, Identification and Characterization of Microorganisms

The different samples have been collected randomly from the three different beaches; Thiruvanmayyur (12°59′08″N and 80°15′41″E), Rameshwaram (9°16' 60N and79°17' 60E) and Pichavaram (11°27'N and 79°48′E) of bay of Bengal of Tamilnadu region from the rhizospheric soil of different coastal vegetations as this portion contains most potential microbes[12] which shows the microbial diversity.The selective media plates have been prepared as YEME agar (HiMedia, Mumbai), Potato Dextrose agar (HiMedia) and Starch Casein agar (HiMedia) media for isolating Actinomycetes [13]; Kings B media (HiMedia) for the marine pseudomonas and Nutrient Agar media(HiMedia) for the other bacteria. The rhizospheric soil of coastal vegetation have been serially diluted as 1g of soil was suspended in 100 ml of respective beach's sterilized sea water [11], then incubated at 28^0 C with 200 rpm shaking for 30 min [4]. Serial dilution was done up to 10^{-6} dilution. The diluted isolates have been spread and pour plated to the all selective media. For isolation of Actinomycetes and *pseudomonas* the plates were kept at 28°C and 35°C respectively in incubator. The particular dilution of 10^{-2} and 10^{-3} heated in 60°C for 10 minutes to spread the marine sample in SCA and PDA media plates which shows typical morphology of the actinomycetes and King's B media plates of 10^{-5} dilution shows pigmentous *pseudomonas* morphological isolates[10].Morphological study of spore and mycelia was performed under high power magnification in Phase Contrast microscope by lactophenol cotton

blue staining (Photomicroscope, Nikon, Japan) [14,15,16,17]. Again the expected *pseudomonas* isolates in King's B media has been taken for the identification being in 35°C. For preliminary characterization of the bacterial strains isolated Grams staining was performed[18, 6]. Moreover the catalase, oxidase and other biochemical tests are performed for characterizing the microorganisms which have been confirmed by the help of Bergey's Manual of Determinative Bacteriology, 2009. Growth curve was designed by taking O.D.at 560nm from each grown *Pseudomonas* broth culture taken by Beckman UV spectrophotometer from the time of inoculation to 96 hours of incubation [19].For the preparation of growth curve of Actinomycetes isolates the dry biomass (mg/ml) method was applied [20].

In vitro antibiosis

Bioassay and antibiotic susceptibility test

The preliminary identification of the antibiotic producer strains is done by making subculture slants of SCA and King's B medium at 25°C [9]and 35°C. Antifungal activity was checked by streaking isolates against the pathogens in PDA plates, zone of inhibition was counted[19] and percentage inhibitions of mycelial growth were calculated using the formula:

% Inhibition of mycelial growth = D_C-D_T /Dc × 100. Where, D_C = diameter of control and

D_T = diameter of test. Test pathogens (bacterial: B. *subtilis*, E. *coli*, K. *pneumoniae*, P. *mirablis*, S. *aureus* and Fungal: H. *oryzae*, F. *oxysporum*, F. *udum*, P. *oryzae*, C. *falcatum*, A. *alterneta*, M. *phaseolina*, R. *solani*, A. *niger*) were collected from MTCC, Chandigarh, India. For the culturing and maintaining of the pathogens nutrient broth and potato dextrose broth and agar medium were used.

Agar well diffusion

The antibiotic susceptibility test was done by agar well diffusion method as referred by Grammer [21]. Agar wells were filled with 100µls of the culture filtrate of selected marine strains against test pathogen spread. The plates were then incubated at 30°C for 24 hrs for the growth of bacteria in NA and 48 hrs for fungi in PDA plates and zone of inhibition developed were measured. 50 ml of SCA and King's B Broths were inoculated with the selected strains for antibiotic bioassay. For antibiotic bioassay the 5 days grown culture broth was centrifuged at 10000 rpm for 10 min [22]. 200 µl of the culture filtrate was loaded in the agar wells followed by incubation at 25^0, 30^0 and 35^0C for 24 to 48 hrs for the development of inhibition zone [23]. Selected actinomycetes and *pseudomonas* isolates grown in typical broth were subjected for crude extraction with ethyl acetate (1:1) [12]. The crude extract was mixed with 1ml of Dimethyl sulfoxide (DMSO, 10%) and same agar diffusion cup method was used by 100 µl of crude extract diluted as 25mg/ml was loaded in cup. Zone of inhibition were observed after incubation [24, 25].

RESULTS AND DISCUSSION

In the analysis of the various activities shown by the microbes from the three types of marine soils has come to arise a view of character based variety in them in manner of metabolite etc. production as the selection was done for the actinomycetes and *Pseudomonas* spp. from the isolates because of their high potentiality. The antibacterial and antifungal activity of marine actinomycetes revealed that it contains potential compound against potent drug resistant bacteria. Due to the bacterial disease, the mortality rate of human and aquaculture are highest[26]. The isolates of *Pseudomonas* and Actinomycetes are differing in their pigmentation as well as their location of collection. However, the Thiruvanmayyur sample has shown presence of red and green pigmented *Pseudomonas* in 10^{-5} dilution in King's B medium. The microbes, *Pseudomonas* isolates found to be Gram negative and actinomycetes as Gram positive.

The Actinomycetes strains are visually confirmed by the phase contrast microscopic observation. The isolates are cultured in respective media and selected for their growth rate study as well as screening against pathogen. The use of antibiotics increases significantly due to heavy bacterial infections, which cause challenging disease of human and plants. The indiscriminate use of antibiotics has resulted in the accumulation of more resistant pathogenic bacterial strains and also caused some side effects to human [27]. Actinomycetes are well known as secondary metabolite producers and hence of high pharmacological and commercial interest. The discovery of new classes of antibiotics is necessary due to the increased incidence of multiple resistance of among pathogenic microorganisms to drugs that are currently in clinical use [28]. In *Pseudomonas* (6 isolates) and in Actinomycetes (12 isolates) were checked antagonism against different pathogens. The *Pseudomonas* isolates showing antagonism against bacterial pathogens and antagonism against the fungal pathogens. Actinomycetes and *Pseudomonas* isolates showing antagonism against bacterial pathogens is shown in Table 1.

Table1: In vitro antimicrobial activity of ethyl acetate crude extract of marine isolates.

Test Organism	Marine isolates (ZOI in cm.)											
	AC1	**AC2**	**AC3**	**AC4**	**AC5**	**AC6**	**AC7**	**AC8**	**AC9**	**AC10**	**AC11**	**AC12**
	PS1	PS2	PS3	PS4	PS5	PS6						
B. subtilis	0.94	0.73	0.82	-	-	-	-	-	-	1.01	-	-
	0.94	0.81	0.78	-	-	0.83						
S. typhi	-	-	-	-	-	-	0.22	0.82	0.86	0.95	-	-
	0.83	0.62	0.75	0.60	0.80	-						
E. coli	0.83	0.49	0.45	0.66	-	0.68	0.43	0.58	-	0.80	0.55	0.66
	0.79	0.59	0.70	0.65	0.62	0.76						
K. pneumoniae	-	-	-	-	-	0.20	-	-	-	0.38	-	-
	0.88	0.82	-	0.69	-	-						
S. aureus	-	-	0.61	-	-	0.34	-	0.88	0.79	0.92	0.61	0.16
	0.98	0.75	0.64	0.78	0.73	0.71						
P. mirabilis	0.78	0.88	0.64	0.73	0.75	0.56	0.56	0.61	0.65	0.97	0.76	0.28
	-	0.29	0.48	0.52	0.27	0.39						

Standard Deviation at ± 0.021 cm

Moreover the antimicrobial activity is tested upon the prominent fungal plant pathogens. The marine bacteria has shown antimicrobial activity against them and the marine source given high activity against all bacterial and fungal pathogens. Marine microorganisms like actinomycetes are very potential sources of bioactive metabolites which inhibits the growth of various human pathogen. The 12 active strains of actinomycetes isolated from three different coastal areas, out of which one shows very active results against tested bacteria and fungi both. The strain AC10 was found to be highly active against both bacterial and fungal pathogens whereas PS2 for bacteria and PS1was found against human fungal pathogens. The isolates AC3 and PS5 has less bactericidal activity and AC12 and PS5 did not so any significant fungicidal activity.

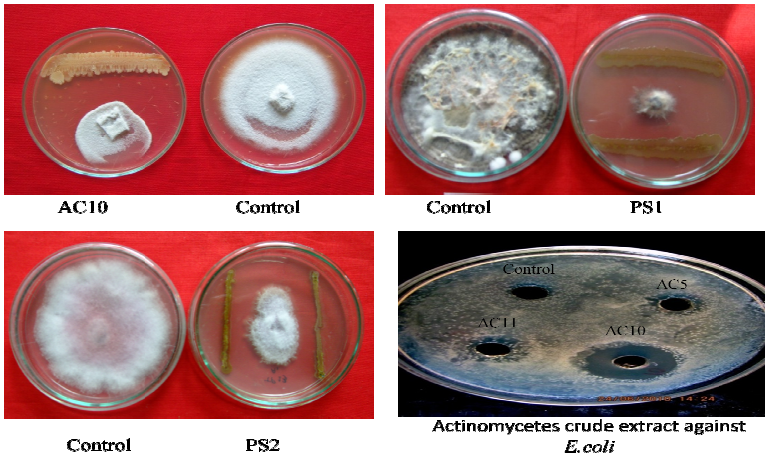

Fig. 1: Inhibitory effect against fungal and bacterial pathogens by marine isolates.

For the limitation of drug resistance of bacteria, demands to improve the pharmacokinetic properties, which necessitate continued research for development of new antimicrobial drug compounds [29]. The marine environment representing approximately half of the global biodiversity, is an enormous resource for new compounds. The marine actinomycetes is diverse in genomic and metabolic, a sink of novel secondary metabolites. In 1969, Weyland carried out an extensive survey on the distribution on marine actinobacteria in the sediments of North Sea and Atlantic Ocean and suggested that the marine actinobacteria are the best sources for isolation of unique bioactive compounds compared to terrestrial ones [30]. The exploitation of marine actinomycetes for its pharmaceutically valuable products is a future top notch research area and it has immense biological prospects. Further research going on for making a 16S rRNA based phylogenetic relationship between the isolates; targeting, determining and purification of the drug molecule having activity against MDR bacteria and plant pathogen.

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