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Study on Bioactive Property of Marine Bacteria Isolated from Soil Sediment along Vasai Coast

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In the present investigation 9 marine bacteria were isolated and found to be positive during primary screening. Initially the isolates were named as SS1to SS9(soil sediment isolates1 to 9). Among them two organisms SS5 and SS9 were found to be exhibiting broad spectrum antibiotic activity and were subjected to secondary screening. In secondary screening it is found that this organism demonstrated antimicrobial activity against Gram positive,Gram negative, a fungi *Candida albicans* along with the MRSA (methicillin resistant *staphylococcus aureus.)*. Production of antibacterial and antifungal metabolite were extracted by solvents (chloroform, ethyl acetate, acetone and methanol).Ethyl acetate and methanol and acetone extract of SS5 gave activity against all the test organisms while SS9 gave prominent zone of inhibition in ethyl acetate extract only. The isolates growth parameters were studied and it was found that they grow well at 28±2°C and pH 8. After characterization it was found that the active marine bacteria SS5 and SS9 belongs to *Vibrio neocaledonicus.*

Key words: SS5, SS9, broad spectrum, MRSA, zone of inhibition.

Antibiotic resistance by human pathogens is great threat to public health in general as many human pathogens are resistance to multiple drugs because of various reasons; mutation is one of them. For nearly 30 years, pharmaceutical companies have been searching for new antibiotics to counter increasing bacterial resistance.

Marine bacteria showing antibiotic activities have been isolated from various biotopes. In some marine ecosystems, such as the deep sea floor and coral reefs, experts estimate that the biological diversity is higher than in the tropical rainforests (Imada *et al.*, 2007). It is estimated that marine habitat is nowadays one of the richest sources of new bioactive compounds due to the diversity of metabolically complex microorganisms (Schloss & Handelsman 2003).

The bacteria showing such activities belong to *Bacillus*, *Micrococcus*, *Pseudomonas*, *Vibrio*, *Flavobacterium*, *Alcaligenes*, *Xanthomonas* and *Achromobacter.* (Berman *et al* 1997, Gauthier *et al.*, 1975 and Austin 1989). Vibrionaceae family, Gram-negative Gammaproteobacteria ubiquitous in marine and brackish environments (Thompson *et al.*, 2004), harbors strains with antagonistic activity (Gram *et al.*, 2010).

In spite of such successes in drug discovery from microorganisms, marine microorganisms have received very little attention. The difficulty in the search of metabolites from marine bacteria is mainly due to the non-culturability of the majority (over 99%) [Hugenholtz 1996].

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The metabolic and physiological capacity that allows marine organism to survive in extreme conditions provides an enormous potential for the production of unique compounds that are not present in the terrestrial organisms.

That is way marine organisms are an attractive source of compounds with pharmaceutical activity, (Faulkner, 2002).Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites (Baskaran *et al.*, 2011).

The present research work is an effort to exploit the Vasai coast for the isolation and characterization of marine bacteria from soil sediment to evaluate the antimicrobial properties as this coast is not explored for similar studies.

MATERIALS AND METHODS

Sample Collection

Soil sediment were collected from Vasai costal area (latitude 19.315°N longitudes 72.875°E) forms the northern boundary of Salsette Island, and empties west into the

Arabian Sea soil sediments were collected from near shore about 2 to 3 m depth. The samples were collected in sterile plastic bags and kept cool until transported to laboratory for further processing.

Isolation of Bacteria from soil sediment

Sediment soil sample (1 g) was transferred to sterile test tube with 1 ml autoclave-sterilized seawater aseptically. The bacteria were suspended in sterilized seawater by vigorous vortexing for 5 min. Representatives of each colony morphotype were isolated using standard serial dilution and plating techniques in triplicate on Zobells Marine Agar 2216 (Hi-Media Laboratories, India), a medium for isolation and enumeration of marine heterotrophic bacteria, and the pH was adjusted to 8.5 in accordance with the sediment pH. All plates were incubated at 28 °C, for 3–7 days. Pure cultures were isolated and subcultured in the same medium at 28 °C. Glycerol stocks were prepared with 30% glycerol in Zobells Marine medium and stored at -4°C for future work. Bacterial counts were represented as CFU/g for each sediment sample.

Bacterial characterization

The well isolated colonies of marine bacteria were studied for their colony characterization including the pigmentation produce by them.

Antimicrobial test (agar cup method)

All sediment bacteria were screened for antimicrobial activity by primary screening method. Freshly grown culture of human pathogen *E. coli* (ATCC 10536), *Salmonella typhi* (ATCC 23564), *Staphylococcus aureus* (NCIM 2602), *Klebsiella pnuemoniae,* (NCIM 2957) *Streptococcus pyogen* (NCIM 2608),*and Candida albicans* (ATCC 10231),were procured from National chemical Laboratory(NCL) ,Pune Maharashtra ,India**,** applied to each agar plate and uniformly spread with a sterilized cotton swab over the surface. Absorption of excess moisture was allowed to occur for 10 min. Then soil sediment isolates (60 µL) were added to 6 mm diameter wells punched with a stainless steel, and plates were incubated at 37 °C up to 72 h. Zones of clear inhibition were measured from the edge of the well and recorded in millimeter (Nissimov *et al*., 2009).

Extraction of solvent fraction of potent soil sediment isolate

The potent strains were inoculated in Brain heart infusion Broth (Hi-media) at 6×10^5 and incubated at 28±2 °C for about 72 h.followed by the incubation, the cell were freed from medium by centrifugation at 10000 rpm at 4 °C,(Remi cooling centrifuge CM8 plus, India) mixed with equal volume of solvents for extraction (Acetone ,chloroform, ethyl acetate and methanol).After centrifugation with solvents the extract were collected and transferred in sterile petriplate and complete evaporation of solvent was carried out at 40-50 °C in oven to give crude solvent extract of antibiotic substance.

Antimicrobial test(Agar disc diffusion method) of potent soil sediment solvent fraction

Bioassay of crude solvent extract was performed by agar disc method using solvent as control.

Presterilsed plates of Muller-Hinton agar plates were seeded with test culture used for earlier bioassay along with MRSA a clinical isolate and were used for agar diffusion test. Six millimeter sterile filter paper disc impregnated with 300 ug/

disk of of crude solvent extract of isolate SS5 and SS9 and allowed it to dry in aseptic condition and then placed onto the surface of the plates .All the plates were incubated at room temperature for 24-48 h. Antimicrobial activity was calculated by measuring the diameter of the inhibition zones

Bacterial sequencing

For identification of potent marine bacteria the sequencing studied were carried out at geneOmbio technologies, Pune; India

Bacterial 16S region gene was amplified using standard PCR reaction. The primer pair 27F and 1492R was used in a PCR reaction with an annealing temperature of 57°C. After amplification, products were purified by using a geneO-spin PCR product Purification kit (geneOmbio technologies, Pune; India) and were directly sequenced using an ABI PRISM BigDye Terminator V3.1 kit (Applied Biosystems, USA). The sequences were analyzed using Sequencing Analysis 5.2 software. BLAST analysis was performed at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). DNA sequencing was performed using one of the PCR primers.

BLAST analysis of the 16S rRNA sequence indicated that SS5 and SS9 belonged to the gamma subdivision of the *Proteobacteria* phylum and was included a member of the *Vibrionaceae* family, order *Vibrionales*. The 16S rRNA sequence was aligned with and compared to 96 type *Vibrio* strains

RESULTS AND DISCUSSION

In view of studying antimicrobial activity of marine bacterial isolated from Vasai coast been tested for primary screening against human pathogen. The results of primary screening by agar diffusion method showed that isolate SS5 and SS9 showed broad spectrum antibiotic activity by inhibiting all the test organisms. Moderate to low antibacterial activity was shown by isolate SS6 and SS8.Higehest zone of inhibition was exhibited by SS5 and SS9 against *S.typhi* while against *E.coli* it was recorded 15mm and 18mm respectively (Table-1). The majority of antagonistic bacteria were found to be Gram negative .The antagonistic bacteria isolated from the soil sediment from the marine environment exhibited variable inhibitory activity against test organisms.

Table 1. Bioassay results of soil sediment isolates. Zone of inhibition in millimeter(mm)

	(Assay was done in triplicate and mean and Standard deviation)					
	E.coli	*S.aureus*	*S.typhi*	*S.pyogen*	*Kl.pnumoniae*	*C.albicans*
SS 1	-	09±0.4	08±0.4	-	10±	08±0.4
SS 2	-	08±0.5	07±0.5	-	-	07±0.5
SS 3	-	08±0.4	07±0.5	-	-	-
SS 4	-	09±0.6	08±0.4	-	08±0.4	08±0.4
SS 5	15±0.5	16±0.5	20±0.5	12±0.5	12±0.5	10±0.5
SS 6	07±0.8	09±0.5	08±0.4	08±0.4	08±0.5	-
SS 7	-	07±0.6	-	-	07±0.4	-
SS 8	07±0.4	10±0.4	10±0.5	-	07±0.4	08±0.4
SS 9	18±0.6	12±0.5	20±0.5	16±0.4	12±0.5	12±0.5

SS-soil sediment bacterial isolates - No inhibition activity

The results of antibacterial and antifungal activity of SS5 and SS9 were also checked using crude extract of these isolates in different solvents which revealed that ethyl acetate extract of both the strains gave inhibition against all the test culture including *MRSA*(Table-2).Comparatively SS5 was found be more potent as acetone, chloroform and methanol fraction demonstrated high inhibition while SS9 solvent fraction could not show the inhibition. Optimization of the parameter for the growth of potent strains in present study shows that both SS5 and SS9 gave profuse growth at 28±2°C and optimum pH 8.(Table-3)

On the basis of morphological, biochemical characterization along with sequence analysis the potent marine isolates SS5 and SS9

Table 2. Bioassay of SS-5 & SS-9 different solvent extract

Isolates/Test organisms	*MRSA*	*E.coli*	*S.aureus*	*S.typhi*	*S.pyogen*	*Kl.pnumoniae*	*C.albicans*
SS 5							
Acetone fraction	-	+	+	+	+	+	+
Chloroform	+	-	+++	+	+	++	+
Ethyl acetate	+	+	++	++	+	+	+
methanol	+	+	++	+	+	++	+
SS 9							
Acetone fraction	-	-	+	-	-	-	++
Chloroform	-	-	+	-	-	-	-
Ethyl acetate	++	+	+	+	++	+	++
methanol	++	-	-	-	+	-	+

\+ inhibition zone upto 8mm, ++ inhibition zone upto 10mm
+++ inhibition zone more than 10 mm - no zone of inhibition

Table 3. Effect of temperature on Growth of SS5 and SS9

	Incubation Temperature	Remark	Incubation PH	Remark
SS5	22	-	6	-
	28	+++	7	+
	37	++	8	+++
	50	-	9	+
SS9	22	-	6	-
	28	+++	7	+
	37	++	8	+++
	50	-	9	+

_ N o growth + Low growth ++ Moderate growth +++ Luxuriant growth

belongs to *Vibrio neocaledonicus* .This Vibrio species was to be claimed new by Chalkiadakis *et al*, 2013.

BLAST analysis of the 16S rRNA sequence indicated that SS5 and SS9 belonged to the gamma subdivision of the *Proteobacteria* phylum and was included a member of the *Vibrionaceae* family, order *Vibrionales*. The 16S rRNA sequence was aligned with and compared to 96 type *Vibrio* strains

Throughout the world researchers are trying to isolate potent microbes from marine environment may be from free sea water, soil sediment, associated with animals and plants from ocean itself. To cite few recently from New Caledonia coast *Pseudomonas spp*. with potential antibacterial activity against the reference pathogenic stain and *Vibrio spp* for industrially valuable molecules .(Chalkiadakis *et al.,* 2013), Antimicrobial activity of *V.ruber* exhibited 100% inhibition of Gram postitive test organisms, 75%Gram positive and 88.8% yeast in the study Norhana (2005) along Peninsular Malysia east coast in comparision to present study the *V.ruber* could not show inhibition against *S.typhimurium* but the *Vibrio neocaledonicus* isolated by us exhibited activity against it.

All Vibrio strains showed antimicrobial activity in respect to *E. coli C. albicans S. aureus* and *P. aeruginosa* in the study by Balena *et al* 2013 from marine sample collected at Vietnam . *V. coralliilyticus* and *P. halotolerans* inhibited both *V. anguillarum* and *S. aureus*, whereas *V. neptunius* only inhibited *V. anguillarum* (Gram *et al.*, 2010) in global expedition study on antibacterial compounds from Marine Vibrionaceae .

Soil microorganisms provide an excellent resource for the isolation and identification of

therapeutically important products (Berdy, 2005).

In conclusion, natural products are very important resources for elaboration of medicine. Although the number of plants, animals and microbes from marine resources have been evaluated in the search of new bioactive compounds. The Vasai coast is not explored for such studies so the Vibrio species isolated from the soil sediments needs further purification and characterization of active compound for advance studies to know the details of bioactive compound using modern techniques.

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