



RESEARCH ARTICLE

ISOLATION, IDENTIFICATION OF *PSEUDOMONAS PSEUDOALICALIGENES GR1* FROM MARINE SEDIMENTS AND SCREENING OF ITS ANTIBACTERIAL POTENTIAL

*¹Gautam, K., ²Prasanna, J., ¹Mahadevan, G., ¹Mahendran, S and ¹Sahu, S.K

¹Faculty of Marine Sciences, CAS in Marine Biology, Annamalai University, Parangipettai, Tamilnadu, India

²School of Energy, Environment and Natural Resources, Madurai Kamaraj University, Madurai - 81

ARTICLE INFO

Article History:

Received 13th, March, 2013
Received in revised form 17th, April, 2013
Accepted 26th, May, 2013
Published online 28th May, 2013

Key words:

Pseudomonas sp, Marine Sediment,
Anti-bacterial activity, Molecular
identification

ABSTRACT

In the present study, 20 different bacterial species were isolated from offshore seawater and sediment of Koodankulam coast, Gulf of Mannar, South East coast of India. The isolated bacteria were subjected to screening by cross streaking method against ten different human pathogenic bacteria like *Staphylococcus aureus*, *Micrococcus luteus*, *Streptococcus pyogenes*, *Bacillus subtilis*, *Enterobacter faecalis*, *Escherichia coli*, *Klebsiella pneumonia*, *Shigella sonnei*, *Salmonella typhimurium*, *Vibrio cholerae* and the isolated bacteria. The results showed potential activity against three different human bacterial pathogen namely *Micrococcus luteus*, *Staphylococcus aureus* and *Klebsiella pneumonia*. The culture was extracted using ethyl acetate, n-butanol, hexane, chloroform and screened for antibacterial activity by disc diffusion method. Among the different solvents ethyl extract showed the broad spectrum of activity against all ten human pathogens (ST5). The strain ST5 was subjected for morphological and potential molecular identification based on 16s rDNA sequencing.

© Copy Right, IJRSR, 2013, Academic Journals. All rights reserved.

INTRODUCTION

Marine microorganisms represent the greatest percentage of described marine species (Pomponi, 1999). The bacterial populations typically range from 10³ to 10⁶ per milliliter with as many as 10⁹ per millilitre in marine sediments (Austin, 1988). Bacteria help regulate rates of organic matter mineralization, nutrient cycling and energy transfer in aquatic environments (Azam and Worden, 2004) and they produce a variety of metabolites, some of which can be used for drug development (Fenical, 1993; Grossart *et al.*, 2004). Bacteria living in complex relatives with animals are often proposed to be the factual producers of 'invertebrate' metabolites (Proksch *et al.*, 2002). But, the distributions of marine bacteria are poorly known.

Competition among microbes for spaces and nutrient in marine environment is a powerful selection pressure that bequeaths marine microorganisms to produce many natural products owning medical and industrial values (Armstrong *et al.*, 2001). The secondary metabolites are 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 (Carte, 1996; Rinehart, 2000; Schwartzmann *et al.*, 2001).

Marine sediments are predominantly occupied by bacterial community. Marine microbes, especially, bacteria and archae are directly influenced by the physicochemical processes in aquatic sediments (Cetecioglu *et al.*, 2009; Peroni and Rossi, 1986). Thus, understanding the bacterial community in

sediment is essential in order to relate with physicochemical properties of marine sediment. They have been distinguished the bacterial diversity between the sequences of culturable isolates in marine water and sequences of rDNA from direct marine water samples (Suzuki *et al.*, 1997). The bacterial growth from marine sediment takes place and it is well known (Kaerberlein *et al.*, 2002) and procedures based in the extraction of nucleic acid from environmental samples have permitted the identification of microorganisms through the isolation and sequencing of ribosomal RNA or rDNA (Handelsman *et al.*, 1998). Therefore, the presents study focused on isolation, identification and screening of antimicrobial potential of *Pseudomonas* sp.

MATERIALS AND METHODS

Sample collection

The seawater and sediment samples were collected from coast of Kundankulam (Lat 08^o10.886'N; Long 077^o45.102'E) south east coast of Tamilnadu, India. Surface water samples were collected in 30 ml sterile screw capped bottles. Enough space was left in the bottles to allow thorough mixing. Precautionary measures were taken to avoid contamination through handling. Sediment samples were collected at 1m depth from surface using the sterile polyvinyl corer (10cm) and these samples were transported to sterile vials and tightly sealed. The collected samples brought to the lab in an ice-box.

Isolation of bacteria

The isolation and enumeration of bacteria was carried out by using the method of Zheng *et al.*, 2005. The collected samples were serially diluted and plated on Zobell marine agar. The Petri dishes were then incubated at room temperature and the colonies were observed up to 3 days. The colonies were counted and expressed as colony forming units (CFU). Single strains of marine bacteria were picked out and purified by repeated streaking on ZMA medium. The pure cultures were transferred to ZMA slants and preserved at 4°C consistently 8 and 12 morphologically different bacterial strains were selected from seawater and sediment for screening antibacterial activity against human pathogens and were given labeled codes.

Preliminary antibacterial screening

Antibacterial assay was used as a preliminary screening to isolate antagonistic bacteria against human pathogen such as *Staphylococcus aureus* (MTCC 3160), *Micrococcus luteus* (MTCC 4821), *Streptococcus pyogenes* (MTCC 1923), *Bacillus subtilis* (MTCC 1133), *Enterobacter faecalis* (MTCC 2729), *Escherichia coli* (MTCC 50), *Klebsiella pneumoniae* (MTCC 3384), *Shigella sonnei* (MTCC 2957), *Salmonella typhimurium* (MTCC 1357) and *Vibrio cholerae* (MTCC 3906) obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India.

The agar well method of Ahmed *et al.* (2008) was used to access the antibacterial activity of supernatant. The isolated and selected bacterial strains were inoculated into 50 ml of zobell marine broth and incubated on a shaker at 120 rpm for 48 hrs. At the end of incubation period the fermentation medium was centrifuged at 5000 rpm for 15 minutes to separate cell mass from fermentation medium. The collected supernatant was concentrated under vacuum conditions. The residue was diluted with one ml of distilled water and 100 µl of the supernatant was loaded on to the respective 5 mm wells of the medium seeded with test pathogens. The plates were incubated at room temperature for 18 to 24 hours, and then observed. The zone of inhibition was measured from the end of the well to the end of the clear zone. Based on the activity in supernatant, active strain was selected for extraction of secondary metabolites using different solvents.

Extraction of secondary metabolites

The selected antagonistic bacterial strains were inoculated into 100 ml ZMB broth, and incubated in a shaker at 120 rpm for 48 hrs. After incubation period the broth culture was centrifuged at 5000 rpm for 15 min. The supernatant was extracted twice with equal volume of Hexane, Ethyl acetate, Chloroform and n-Butanol (Zheng *et al.*, 2005). The solvent phases were then separated using separating funnel and concentrated by evaporation. The concentrate (crude extract) dissolved in 1 ml of respective solvents was then impregnated at 100 µg/disc concentration on to sterile Whatman no1. 6 mm dia disc and the antibacterial activity were assayed following the disc diffusion assay (Becerro *et al.*, 1994; Murugan and Santhannramasamy, 2003). The solvents alone in the disc were used as control. The inhibition zone was measured from the border of the disc to edge of the clear zone in mm. The active crude extract was selected and then subjected to minimum inhibitory activity against the same bacterial pathogens.

Isolation of genomic DNA

The strain was inoculated in LB broth supplemented with 3% NaCl and was kept in shaking incubator at 37°C for 24 hours. DNA isolation and purification was performed using QIAGEN DNA mini kit. DNA was quantified in 0.8% agarose gel. Bacterial DNA isolation and purification was performed using QIAGEN DNA mini kit (QIAGEN Company, Valencia, CA). The culture was centrifuged at 5000 X g for 10 min. The collected pellet was suspended in 1 ml of Buffer B1 (with RNase A) by vortexing at top speed followed by 20 µl of lysozyme (100 mg/ml), 45 µl of proteinase k were added and incubated at 37°C for 30 min. Finally 0.35 ml of buffer B2 was added and incubated at 50°C for 30 min. The lysed cells were transferred to equilibrate QIAGEN Genomictip and it allowed entering the resin by gravity flow. The QIAGEN Genomic-tip was washed with 3x1 ml of Buffer QC and the genomic DNA was eluted with 2x1 ml of Buffer QF. The eluted genomic DNA was precipitated with isopropanol. Finally the pelleted DNA was suspended the TE (pH 8.0) buffer and stored at 4°C for immediate use and at -200°C for long term storage.

DNA amplification

The PCR was carried out using Taq PCR Master Mix Kit (QIAGEN) in DNA thermocycler and PCR condition were 1µl of target DNA was added to the PCR mix, and PCR was then applied to the DNA thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). PCR conditions were fixed as initial denaturation for 10 min at 94°C for 10 min followed by 30 cycles of melting for 1 min at 94°C, annealing for 1 min at 57°C, extension for 1 min at 72°C. a final extension was carried out for 10 min at 72°C. the formation of PCR products were confirmed by running 5 µl on a 1% (w/v) agarose gel electrophoresis in 0.5X TBE buffer. The DNA bands were stained with ethidium promide and visualized by UV light photography.

PCR products purification

PCR products were purified using QIAquick PCR purification kit (QIAGEN, CA) according to manufacturer's instruction. The amplified PCR products were mixed with Buffer PB and it applied to bind QIAquick column. The DNA bind column was centrifuged for 1 min and discarded through flow. Followed by column was washed with 0.75 ml of Buffer PE and centrifuged for 1 min. Finally the DNA was eluted by 50 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) added to the QIAquick membrane and centrifuged for 1 min.

Sequencing of 16S rRNA genes

The purified PCR product sequencing reaction was performed with an Applied Biosystems BigDye terminator cycle sequencing version 2.0 kit as per the manufacturer's instructions (Applied BioSystems, Foster City, Calif.). After the cycle sequencing, excess dye terminators and primers were removed by purification with Centri-Sep spin columns (Princeton Separations, Adelphia, N.J.) and sequencing was resolved in Applied Biosystems model 3100 automated DNA sequencing system (Applied Biosystems).

Sequence analysis

The 16S rRNA gene sequence was compared with all other known rRNA sequences using a BLAST search

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). Similar DNA sequences were downloaded from the database and were subjected to phylogenetic analysis.

Phylogenetic analysis

The downloaded sequences were loaded into MEGA software (Kumar *et al.*, 2004). After the multiple sequence alignment, phylogenetic trees were constructed by Maximum Parsimony (MP) and Neighbour Joining (NJ). Maximum Parsimony analysis was carried out with a heuristic search (Swofford, 2004) under constraint of simple addition of sequences and tree bisection reconnection (TBR) branch swapping, with Max Trees set to 100. All characters were unordered and equally weighed, with gaps treated as missing data. The confidence of the branches was measured by bootstrap analysis with 1000 bootstrap replicates using heuristic search (Felsenstein, 1985). Neighbor-joining analysis was conducted with uncorrected distance (p) model. Branch swapping algorithm was TBR, steepest descent option not in effect, multrees option in effect. Support for internal nodes was estimated by 100 bootstrap replicated under the same model settings (Swofford, 2004). Only bootstrap values above 50% are shown, and only those above 70% were considered significant.

RESULT

In the present study bacterial density was found to be 45×10^4 CFU/ml and 68×10^5 in seawater and sediments respectively, the results obtained is represented in Table (1). A total of 20 individual bacterial strains were selected based on the different colony morphology and further used to screen antibacterial activity. Among these 8 and 12 strains were isolated and selected from seawater and sediment samples respectively.

Table 1 Total bacterial population in seawater and sediment sample

Sample	Colony count CFU/ml
Seawater	45×10^4
Sediment	68×10^5

Preliminary screening

A total of 20 distinct different strains were selected and isolated based on the colony morphology from the above mentioned sources (Table 1). The antibacterial activity of bacterial supernatant was considered as a good tool to preliminary screen active strains. In preliminary screening employing agar well method against ten human pathogens indicated moderate activity in supernatant of 13 strains and the results obtained is represented in Table 2 and 3.

Table 2 Screening of antibacterial activity of bacteria isolated from seawater

S.No	Isolated bacterial strains	Human pathogens									
		<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>	<i>Streptococcus pyogenes</i>	<i>Bacillus subtilis</i>	<i>Enterobacter faecalis</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Shigella sonnei</i>	<i>Salmonella typhimurium</i>	<i>Vibrio cholerae</i>
Zone of inhibition level (mm) at 100µl/disc											
1	SW1	-	-	1	-	1	1	-	-	-	-
2	SW2	1	2	1	1	1	2	1	3	1	1
3	SW3	-	-	-	-	-	-	-	-	-	-
4	SW4	3	2	1	-	-	-	2	1	-	-
5	SW5	2	2	2	1	1	2	1	3	1	2
6	SW6	1	3	-	-	-	-	1	-	-	-
7	SW7	-	-	-	-	-	-	-	-	-	-
8	SW8	-	-	-	-	-	-	-	-	-	-

(- = No activity)

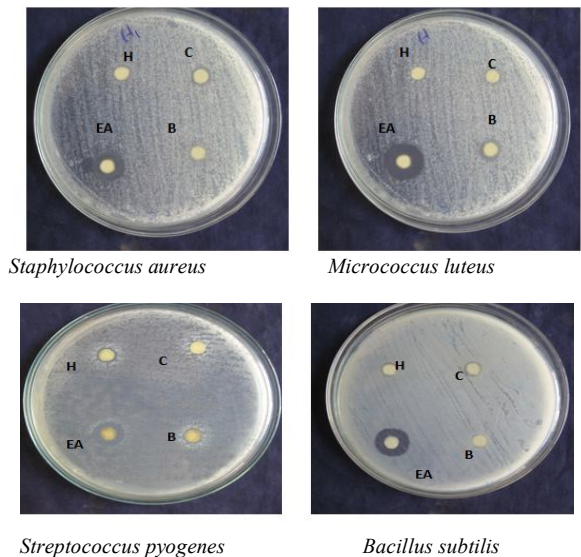
Table 3 Screening of antibacterial activity of bacteria isolated from sediment

S.No	Isolated bacterial strains	Human pathogens									
		<i>Staphylococcus aureus</i>	<i>Micrococcus luteus</i>	<i>Streptococcus pyogenes</i>	<i>Bacillus subtilis</i>	<i>Enterobacter faecalis</i>	<i>Escherichia coli</i>	<i>Klebsiella pneumoniae</i>	<i>Shigella sonnei</i>	<i>Salmonella typhimurium</i>	<i>Vibrio cholerae</i>
Zone of inhibition level (mm) at 100µl/disc											
1	ST1	2	1	1	1	-	-	-	1	-	-
2	ST2	-	-	-	-	-	-	-	-	-	-
3	ST3	1	1	3	2	1	1	1	1	1	1
4	ST4	-	-	-	-	-	-	-	-	-	-
5	ST5	4	5	6	5	5	4	4	3	4	4
6	ST6	1	1	-	-	-	-	2	-	1	-
7	ST7	3	2	1	2	1	1	2	2	2	3
8	ST8	-	-	2	-	-	3	-	-	-	-
9	ST9	-	-	-	-	-	-	-	-	-	-
10	ST10	1	1	-	-	1	-	1	-	-	-
11	ST11	1	1	2	-	-	-	-	-	-	2
12	ST12	-	-	-	-	-	-	-	-	-	-

Table 4 Antibacterial activity of crude extracts at various solvents from ST5 strain

S. No	Human pathogens	Zone of inhibition level (mm) at 100µg/disc			
		H	C	EA	B
1	<i>Staphylococcus aureus</i>	-	2	6	-
2	<i>Micrococcus luteus</i>	-	-	8	2
3	<i>Streptococcus pyogenes</i>	2	-	7	-
4	<i>Bacillus subtilis</i>	-	1	8	2
5	<i>Enterobacter faecalis</i>	-	-	10	-
6	<i>Escherichia coli</i>	1	-	6	-
7	<i>Klebsiella pneumoniae</i>	-	-	7	-
8	<i>Shigella sonnei</i>	-	-	5	1
9	<i>Salmonella typhimurium</i>	1	-	5	1
10	<i>Vibrio cholera</i>	-	-	8	1

Fig: 1 Antibacterial activity of crude extracts of ST5 strain



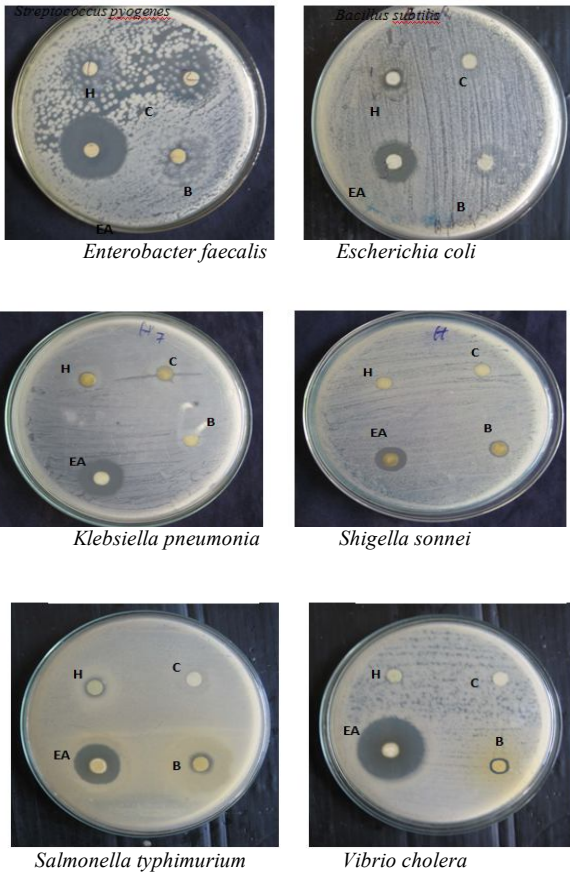
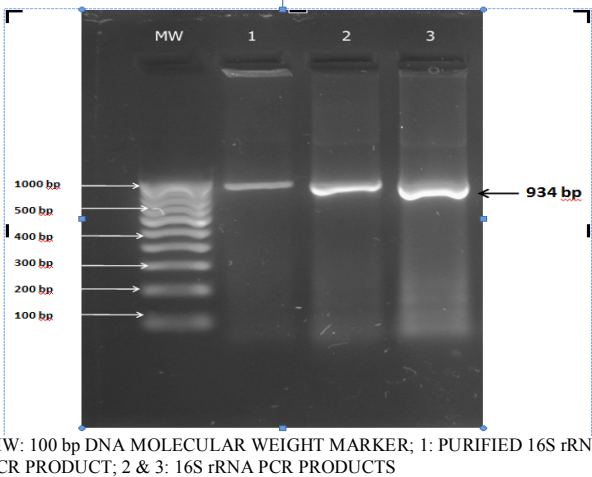


Fig.2 PCR amplification of 16s rRNA fragments from Bacterial sample.



MW: 100 bp DNA MOLECULAR WEIGHT MARKER; 1: PURIFIED 16S rRNA PCR PRODUCT; 2 & 3: 16S rRNA PCR PRODUCTS

The activity exhibited by supernatant is ranged from low to high level activity according to their inhibition against number of pathogen. Out of 13 active bacterial strains of seawater and sediments, the supernatant of strains SW2, SW5, ST3, ST5 and ST7 showed wide spectrum activity against all the human pathogens. The zone of inhibition was ranged from 1 mm to 6 mm.

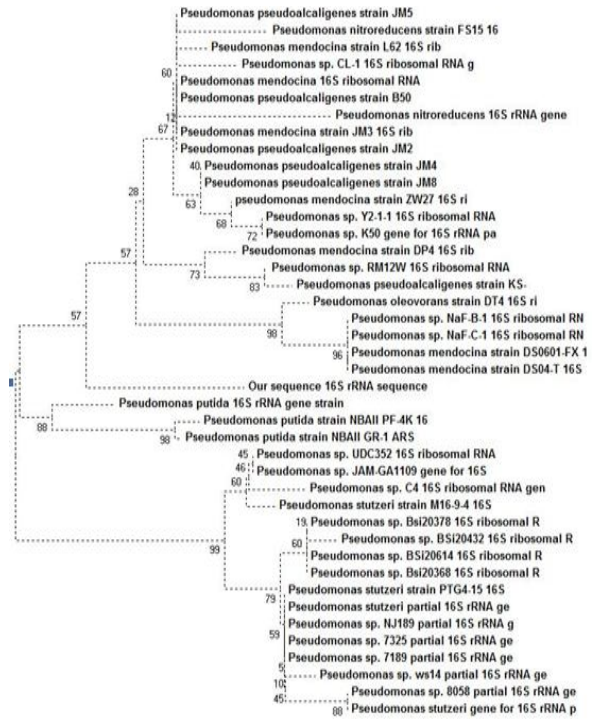


Fig.3 Phylogenetic tree of *Pseudomonas pseudoalcaligenes*

Among these strains the ST5 strain was exhibited higher level of zone of inhibition against all the human bacterial pathogens than other strains. The zone of inhibition was ranged from 3 mm to 6 mm. The maximum level of zone was observed against *Streptococcus pyogenes*. The minimum level of zone was exhibited against *Shigella sonnei* whereas other strains such as SW4 and ST1 showed broad spectrum activity against 5 bacterial pathogens. The zone of inhibition was ranged from 1 to 3 mm. The strains SW1, SW6, ST6, ST8, ST10 and ST11 were observed to have low level activity against one to three pathogens. Other strains did not inhibit any human pathogens considerably. The strain ST5 of sediment was further selected for screening.

Antibacterial activity of crude extracts

The active ST5 strain screening was subjected to secondary screening for the isolation of bacterial metabolites using various solvents and for their antibacterial activity, results are given in Table. 4. In this study the Ethyl acetate extracts were shown to have wide spectrum activity against all ten pathogens. The zone of inhibition level was ranged from 5 to 10 mm, the maximum zone (10mm) was observed against *Enterobacter faecalis* and minimum zone (5mm) was exhibited against *Shigella sonnei* and *Salmonella typhimurium* respectively. Butanol extract was observed to have broad spectrum activity against 5 bacterial pathogens. The extracts hexane and Chloroform exhibited moderate level activity against two to three pathogens but the zone of inhibition was considerably very low (Fig 1).

Molecular characterization of bacterial strains

The molecular characterization of high antibacterial active strain ST5 was identified to *Pseudomonas pseudoalcaligenes*. After the sequencing products were purified and run in agarose

gel electrophoresis with 100 – 1000 bp molecular marker. The purified sequencing 16S rRNA PCR products were indicated by the presence of single band having similar height to the 1000 bp of molecular marker. Although the unpurified sequencing 16S rRNA PCR products were indicated by the presence of single band showed the 934 bp, when compared to the 100- 1000 bp molecular marker (Fig.2)

An attempt was made to perform the 16S rRNA analysis to clarify the phylogenetic status of bacteria with high antibacterial activity. For phylogenetic characterization the aligned sequences appeared to be identical, redundant sequences were excluded based on a shorter sequence length or slight ambiguity in sequence data. Each of these sequences was then aligned to sequences available in the NCBI database to determine the identity of the sequences. Tree topologies were essentially the same for maximum parsimony and neighbour-joining analysis (Fig. 3). Molecular phylogenetic analysis demonstrated all bacterial isolates with antibacterial activity belonged to the genus *Pseudomonas* family Pseudomonadaceae. Neighbour-joining analysis of 16S rRNA sequences revealed that strains belonged to *Pseudomonas pseudoalcaligenes*GRI and were shown 97 % similarity. Based on their 16S rRNA genes, some isolates were closely related to each other or even identical.

DISCUSSION

Marine microorganisms emerge as a new field for the discovery of novel biologically active compounds from marine origin. Isolation of bacteria can originate mainly from sediments, but also from open oceans or marine surfaces including marine living organisms. Antibiotic production by marine bacteria has been documented for a long time. Therefore, it is of interest to develop different approaches for the discovery of marine bacterial species that produce biologically active secondary metabolites (Madhava Charyulu *et al.*, 2009).

In this study the average density of bacteria on seawater was 45×10^4 CFU/ml. The present observation of bacterial population was very high when compared with Zongjun *et al.* (2002) who have revealed that the 5.4×10^4 CFU/ml of bacteria was isolated from seawater of Qingdao, 1.2×10^4 CFU/ml in east china sea and 3.5×10^3 CFU/ml in eastern tropical north pacific ocean (Du *et al.*, 2005), 4×10^5 CFU/ml in seawater (Guerrero *et al.*, 2007). Besides the study bacterial density of 68×10^5 CFU/g was observed in sediments. The present results may be attributed to rich organic matter around the sediments, which provide the nutrients for bacteria (Guerrero *et al.*, 2007). It is to be indicated that the marine sediments represent some of the most complex microbial habitats on Earth, and benthic microbial communities play an important role in the marine biogeochemical cycles (Pringault *et al.*, 2007). The observed results was very low when compared to the Guerrero, *et al.* (2007) who has reported that 3.83×10^7 CFU/g was isolated from sediment.

In the next aspect of my study was to screen antibacterial activity of 20 individual strains which were selected based on the colony morphology. Out of 20 strains, the SW2, SW5, ST3, ST5 and ST7 have showed to have wide spectrum activity against the human bacterial pathogens, other strains were low to moderate level activity. Among these strain ST5 showed higher zone of inhibition than other wide spectrum

strains. In a similar study Prabha devi *et al.* (2010) who have revealed the antibacterial activity of *Bacillus licheniformis* supernatant against human pathogens. The present observation was coincide with Ahmed *et al.*, (2008) who have reported that the antibacterial activity of marine bacterial supernatant from Arabian sea of Pakistan. It is to be noted that the production of biologically active compounds of marine bacteria depending on the pressure, temperature, salinity, and depletion of micronutrients, with survival and proliferation (Williams, 2009; Palloma Rodrigues Marinho *et al.*, 2009; Nascimento *et al.*, 2004; Pringault *et al.*, 2007).

The subsequent study was to extract bacterial metabolites from active strain ST5 with solvents such as hexane, ethylacetate, chloroform and n-butanol. Ethyl acetate extracts showed wide spectrum activity against all the human bacterial pathogens. The present observation was corroborated with Darabpour *et al.*, 2010, who have exposed that the ethyl acetate extracts of *pseudomonas aeruginosa* isolated from Persian Gulf, which have a good activity against the human bacterial pathogens. These results observed antagonistic activity could be attributed to the production of some common antibacterial compound which was reported such as siderophores, antibiotics, volatile compounds HCN, enzymes and phytohormones, that the compound could be extracted from the growth medium by portioning with organic solvents (Uzair *et al.*, 2009). This observation was substantiated by Ravikumar *et al.*, 2010 who have reported that ethyl acetate extracts of marine bacteria were antibiotic activity against human bacterial pathogens. Thus, the present study indicates the antibacterial activity of marine bacteria. This observation was supported by Wratten *et al.*, (1977) isolated antibacterial compounds: 4-hydroxybenzaldehyde, 2-n-heptyl-4-quinolinol, and 2-n-pentyl-4-quinolinol from *Pseudomonas* sp. in a similar study with Isnansetyo and Kamei (2003) who have isolated bactericidal antibiotic, MC21-A (3,3', 5,5'-tetrabromo-2, 2'-biphenyldiol, 26) from the *Pseudoalteromonas phenolica*. This study indicated that the sediment isolated marine bacteria are potential source for antibacterial activity against human bacterial pathogens.

Marine environment has proven to be a chemical repository for many bioactive compounds. Many of these compounds show structural similarity with compounds from bacteria of terrestrial origin. To ensure that these bioactive compounds are of bacterial origin and to tap this valuable source, bacterial identification is of utmost importance. Molecular identification of sediment bacteria might prove valuable in identification of these bacteria. In this study, we have identified antibacterial active marine sediment associated bacteria by amplifying the 16S rRNA gene from the genome of marine bacteria.

In the present study, the characterization of antibacterial active strain from sediment was phylogenetically analyzed and indicated the isolated active bacterial strain belonged to Pseudomonadaceae family. In 16S rRNA sequences revealed that isolated strain was belonged to *Pseudomonas pseudoalcaligenes* and have 97% close similarity. In a similar study with Radjasa *et al.* (2007) who have revealed that the PCR based identification of softcoral bacterium *Pseudomonas* sp. TASC.16 showed strong growth inhibition against *Streptococcus equi subsp. Zooepidemicus*. The present observation may be attributed to the marine *Pseudomonas* sp are producing antimicrobial substances such as pyroles,

pseudopeptide pyrrolidinedione, phloroglucinol, phenazine, benzaldehyde, quinoline, quinolone, phenanthren, phthalate, andrimid, moiramides, zafrin and bushrin (Isnansetyo and Kamei, 2009). It is to be noted that the genus *Pseudomonas* are well known to various peptide natural products including antibacterial (Mossialos *et al.*, 2002), biosurfactants (Morikawa *et al.*, 1993; Roongsawang *et al.*, 2003) and antifungals (Ramette *et al.*, 2001; Nielsen *et al.*, 2002; Sorensen *et al.*, 2002). The present observation was supported with Ivanova *et al.* (2003) who have demonstrated that the identification of marine bacteria in seawater, four genera of the family *Vibrionaceae*, the genus *Aeromonas* of the family *Aeromonadaceae*, and the genera *Alteromonas*, *Marinomonas*, *Shewanella*, *Pseudomonas*, and *Deleya*. These sequences were aligned, the similarity values and evolutionary distance values were determined, and a phylogenetic tree was constructed by using the neighbor-joining method (Kita-Tsukamoto *et al.*, 1993). It is to be noted that the microorganisms evolving in habitats with low temperatures need to be studied to understand their adaptation, the distribution within the ocean and the role of in the biogeochemical processes as well as their biotechnological potentials. Rajadsa *et al.*, 2007 revealed the isolation and identification of marine bacteria of *pseudomonas* sp and *vibrio* using the PCR based amplification and 16s rRNA sequencing. The present results was corroborated with (Mohapatra *et al.*, 2002; Norimasa Tamehiro *et al.*, 2002) who have reported that the PCR based identified *Bacillus* sp in the coral tissues promises much useful enzymes/bioactive compounds for exploration. Many bioactive compounds such as bacilysoicin, subtilosin, sublancin, surfactin and bacilysin have already been derived from *Bacillus subtilis* which can be used as antibacterial and antifungal agents.

PCR amplification and sequencing lead to the proper identification and the true antibacterial active bacterial genera present in marine sediment. This in a way overcomes the disadvantages of conventional phonetic system which is time consuming and might also result in classifying the Genera in the wrong group. This study once again emphasizes the importance of employing molecular techniques for assessing bacterial diversity and to gain knowledge about the microbial world beneath the sea.

References

Ahmed, N., Uzair, B., Ayaz, S and Ahmed, V.U. 2008. Antibacterial activity of marine bacteria from Arabian Sea of Pakistan. *The Internet Journal of Microbiology*. 4(2):1-10.

Armstrong, E., Yan, L., Boyd, K.G., Wright, P.C and Burgess. J.G. 2001. The symbiotic role of marine microbes on living surfaces. *Hydrobiologia*, 461: 37-40.

Austin, B. 1988. *Marine Microbiology*, Cambridge Univ. Press, Melbourne.

Azam, F and Worden, A.Z. 2004. Microbes, molecules and marine ecosystems. *Science*, 303: 1622-1624.

Becerro, M.A., Lopez, N.L., Turon, X and Uniz, M.J. 1994. Antimicrobial activity and surface bacterial film in marine sponges. *J. Exp. Mar. Ecol.*, 179:195-205.

Uzair, B., Ahmed, N., Mohammad F.V., Ahmad, V.U. and Edwards, D. 2009. Screening of marine bacteria of pakistan coast for drug discovery potential. *Proc. Pakistan Acad. Sci.*, 46(3):137-144.

Ravikumar, S., Gnanadesigan, M., Suganthi, P and Ramalakshmi, A. 2010. Antibacterial potential of chosen mangrove plants against isolated urinary tract infections bacterial pathogens. *International Journal of Medicine and Medical Sciences*, 2(3): 94 – 99.

Carte, B.K. 1996. Biomedical potential of marine natural products. *Bioscience*, 46: 271-286.

Cetecioglu, Z., Ince, B.K., Kolukirik, M., and Ince, O. 2009. Bio geographical distribution and diversity of bacterial and archaeal communities within highly polluted anoxic marine sediments from the Marmara Sea, *Mar. Pollut. Bull.*, 2009, vol. 58, pp. 384–395.

Darabpour, E., Ardakani, M.R., Motamedi, H., Ghezelbash, G., Ronagh, M.T., Darabpour, E., Ardakani, M.R., Motamedi, H., Ghezelbash, G. and Ronagh, M.T. 2010. Isolation of an antibiotic producer *Pseudomonas* sp. from the Persian Gulf. *Asian Pacific Journal of Tropical Medicine*, 4: 318-321.

Du, H., Jiao, N., Hu, Y and Zeng, Y. 2005. Diversity and distribution of pigmented heterotrophic bacteria in marine environments. *FEMS Microbiol Ecol.*, 57: 92–105.

Felsenstein, J. 1985. Phylogenies and comparative method. *The American Naturalist*, 125(1):1-15.

Fenical, W and Jensen, P.R. 2006. Developing a new resource for drug discovery: marine actinomycete bacteria. *Nat Chem Biol* 2:666– 673

Fenical, W. 1993. Chemical studies of marine bacteria: Developing a new resource. *Chem Rev.*, 93: 1673-1683.

Grossart H.P., Schlingloff, A., Bernhard, M., Simon, M and Brinkhoff, T. 2004. Antagonistic activity of bacteria isolated from organic aggregates of the German Wadden Sea. *FEMS Microb. Ecol.*, 47: 387-396.

Guerrero, M.J.F., Paez, M.E.C and Mourino, G.G. 2007. Variation of a benthic heterotrophic bacteria community with different respiratory metabolisms in Coyuca de Benitez coastal lagoon (Guerrero), Mexico. *Rev. Biol. Trop.*, 55(1): 157-169.

Handelsman J., Rondon, M.R., Brady, S.F., Clardy, J. and Goodman, R.T. 1998. Molecular biological access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chem. Biol.* 5: 245- 249.

Isnansetyo, A and Kamei, Y. 2003. MC21-A, a bactericidal antibiotic produced by a new marine bacterium, *Pseudoalteromonas phenolic* *asp.* Nov. O-BC30^T, against Methicillin-Resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, 47(2): 480-488.

Ivanova, E.P., Nicolau, D.V., Yumoto, N., Taguchi, T., Okamoto, K., Tatsu, Y and Yoshikawa, S. 1998. Impact of conditions of cultivation and adsorption on antimicrobial activity of marine bacteria. *Marine Biology*, 130: 545-551.

Kaeberlein T., Lewis, K and Epstein, S.S. 2002. Isolating “uncultivable” microorganisms in pure culture in a stimulated natural environment. *Science*. 296: 1127-1129.

Kita-Tsukamoto, K., Oyaizu, H., Nanba, K and Simidu, U. 1993. Phylogenetic relationships of marine bacteria, mainly members of the family *Vibrionaceae*, determined on the basis of 16s rRNA sequences. *Int J Syst Bacteriol*, 43, 8-19.

Kumar, S., Tamura, K and Nei, M. 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Oxford Journal*, 5: 150-163.

- Madhava Charyulu, E., Sekaran, G., Suseela Rajakumar, G and Gnanamani, A. 2009. Antibacterial activity of secondary metabolite from marine isolate, *Pseudomonas* sp against gram positive and negative bacteria including MRSA. Indian journal of experimental biology, 47: 964-968.
- Mohapatra, B.R., Bapuji, M and Sree, A. 2002. Antifungal efficacy of bacteria isolated from marine sedentary organisms, Folia. Microbiol (Praha), 47(1):51-53.
- Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y and Imanaka, T. 1993. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. J. Bacteriol., 175: 6459-6466.
- Mossialos, E., Dixon, A., Figueras, J and Kutzin, J. 2002. Funding Health Care: Options for Europe. Open University Press: Buckingham.
- Murugan, A and Santhana Ramasamy, M. 2003. Biofouling deterrent activity of the natural product from ascidian *Distaplia nathensis* (Chordata). Ind. J. Mar. Sci., 32(2): 162-164.
- Nascimento, J.S., Abrantes, J., Giambiagi-deMarval, M and Bastos, M.C.F. 2004. Growth conditions required for bacteriocin production by strains of *Staphylococcus aureus*. World J Microbiol Biotechnol., 20: 941-947.
- Nielsen, T., Kristensen, N.H and Mikkelsen, B.E. 2002. Anvendelse af økologiske fødevarer i kommuner og amter [The use of organic foods in Danish municipalities and counties], Publication from Veterinary and Food Administration, Denmark.
- Norimasa Tamehiro, Yoshiko Okamoto-Hosoya, Susumu Okamoto, Makoto Ubukata, Masa Hamada, Hiroshi Naganawa and Kozo Ochi. 2002. Bacilysoicin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. Antimicrob agents Chemother, 46(2): 315-320.
- Palloma Rodrigues Marinho, Ana Paula Barbosa Moreira, Flavia Lucia Piffano Costa Pellegrino, Guilherme Muricy, Maria do Carmo de Freire Bastos, Katia Regina Netto dos Santos, Marcia Giambiagi-deMarval, Marinella Silva Laport. 2009. Marine *Pseudomonas putida*: a potential source of antimicrobial substances against antibiotic-resistant bacteria. Mem Inst Oswaldo Cruz, Rio de Janeiro, 104(5): 678-682.
- Peroni, C. and Rossi, G. 1986. Determination of Microbial Activity in Marine Sediments by Resazurin Reduction. Chem. Ecol., 2: 205-209.
- Pomponi, S.A. 1999. The bioprocess-technological potential of the sea. J. Biotechnol., 70: 5-13.
- Prabha Devi, Solimabi Wahidullah, Cheryl Rodrigues and Lisette D. Souza. 2010. The sponge-associated bacterium *Bacillus licheniformis* SAB1: A source of antimicrobial compounds. Marine drugs. 8(4): 1203-1212.
- Pringault, O., Tassas, V and Rochelle-Newall. 2007. Consequences of respiration in the light on the determination of production in pelagic systems. Biogeosciences, 4: 105-114.
- Proksch, P., Edrada, R.A and Ebel, R. 2002. Drugs from the sea-current status and microbiological implications. Appl. Microbiol. Biotech., 59: 125-134.
- Radjasa, O.K., Kencana, D.S., Sabdono, A., Hutagalung, R.A and Sri Lestari, E. 2007. Antibacterial Activity of Marine Bacteria Associated with sponge *Aaptos* sp. against Multi Drugs Resistant (MDR) strains. Jurnal Matematika Dan Sains, Desember, 12: 523-532.
- Ramette, A., Moenne-Loccoz, Y and Defago, G. 2001. Genetic diversity and biocontrol potential of fluorescent pseudomonads producing phloroglucinols and hydrogen cyanide from Swiss soils naturally suppressive or conducive to *Thielaviopsis basicola*-mediated black root rot of tobacco. FEMS Microbiol Ecol, 55, 369-381.
- Rinehart, K.L. 2000. Antitumor compounds from tunicates. Med. Res. Rev., 20: 1-27.
- Roongsawang, N., Hase, K., Haruki, M., Imanaka, T., Morikawa, M and Kanaya, S. 2003. Cloning and characterization of the gene cluster encoding arthrofactin synthetase from *Pseudomonas* sp. MIS38. Chem. Biol. 10: 869-880.
- Schwartzmann, G., Rocha, A.B., Berlinck, R.G.S and Jimeno. J. 2001. Marine organisms as a source of new anticancer agents. Lancet Oncol., 2: 221-225.
- Sorensen, D., Nielsen, T.H., Sorensen, J. and Christophersen, C. 2002. Cyclic lipoundecapeptide lokisin from *Pseudomonas* sp. Strain DSS41. Tetrahedron. Lett., 43: 4421-4423.
- Suzuki, M.T., Rappe, M.S., Haimberger, Z.W., Winfield, H., Adair, N., Strobel, J., and Giovannoni, S.J. 1997. Bacterial Diversity among Small Subunit rRNA Gene Clones and Cellular Isolates from the Same Seawater Sample, Appl. Environ. Microbiol., 63: 983-989.
- Williams, P.G. 2009. Panning for chemical gold: marine bacteria as a source of new therapeutics. Trends Biotechnol. 27: 45-52.
- Wratten, S.J., Wolfe, S.M., Andersen, R.J and John Faulkner, D. 1977. Antibiotic metabolites from a marine *Pseudomonad*. Antimicrobial Agents and Chemotherapy. 11 (3): 411-414.
- Zheng, L., Chen, H., Han, X., Lin, W and Yan, X. 2005. Antimicrobial screening and active compound isolation from marine bacterium NJ6-3-1 associated with the sponge *Hymeniacidon perleve*. World Journal of Microbiology & Biotechnology, 21: 201-206.
- Zongjun, D., Yun, L., Dehua, Y.U., Xianghong, W and Jixiang, C. 2002. Heterotrophic Bacterial Floras in Industrial Area and Unpolluted Marine Environments around Qingdao. Journal of Ocean University of Qingdao, 1(1): 59-62.
