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SCREENING AND CHARACTERIZATION OF BIOTECHNOLOGICALLY IMPORTANT MARINE BACTERIA FROM BALUCHISTAN COAST

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ABSTRACT

A total of 100 bacteria were isolated from crustaceans (shrimp, crabs, lobster), fish and water samples 9surface and deep sea) collected from Baluchistan coast. Identification of these isolates was performed by gram reaction, colonial and cellular morphology, pigment production and biochemical characters. Different characters such as resistance to antibiotic and heavy metals, pigment production, sugar fermentation, antibacterial and solubilizing activity were studied. It was found that 29% of the isolates showed production of different colour pigments which were affected by different concentration of heavy metals, 45% of the isolates showed multiple metal and antibiotic resistance, 40% showed solubilizing activity and 15% showed production of antibacterial substance, 16% strains were found to be lactose fementer where as 57% were non lactose fermenter.

Keywords: Solubilizing activity, antibacterial acivity, Bacterial flora of Baluchistan coast.

INTRODUCTION

The marine environment covers three quarters of the surface of our planet, is estimated to be home to be more than 80% of life forms and yet remains largely unexplored. The ocean contains huge diverse range of environmental niches characterized by extremes in temperature, pressure, exposure to radiation (UV) and chemical stress mechanisms. This environmental pressure has in turn led to the evolution of a range of animals and plant life that does not matches any terrestrial ecosystem (Kjellberg, 1993).

Bacteria have been evolving for the last 4 billions years and respond to selective pressures by developing elegant and rapid systems of adaptations and survival. Microorganisms represent an enormous and largely unexploited source for biotechnological applications. Microbial diversity can be regarded on one hand as a problem, for example due to the large variety of macrooganisms causing (new emerging) diseases and on other hand as a solution due to the rich biotechnological potential including disease control (Markus *et al*., 2002). Microorganisms have developed the basic types of metabolisms and a wide range of activities, which allow them to colonize all ecosystems and create biosphere. As they are the movers and shakers of global cycles of elements they contribute significantly to the stability and functioning of ecosystems, which can be threatened by fishery operations, chemical pollution and eutrophication, alteration of physical habitat, exotic species invasion and effects of other human activities. Effective solution will require an expanded understanding of the pattern and processes that control the diversity of life in sea (Colwell, 1996).

Pakistan spans a remarkable no of world's broad ecological regions ranging from coastal mangrove forest of Arabian Sea to spectacular mountain top. This variety of habitat supports a rich variety of different species which constitute the overall biological diversity of the country. Pakistan has a total marine water resource area of 527 nautical miles. Microbial form in these environment forms an integral part of this unique ecosystem (Ahmed and Yasmeen, 1998). The marine microorganisms, including bacteria, fungi and microalgea have received increasing attention over the past ten years (Davidson, 1995) but marine bacteria of this region have remained unexplored. Marine biodiversity plays a key role in biogeochemical processes that derives the global climate. Nowhere in the biosphere is biological diversity greater than in seas. An understanding of marine environment is proving to be a valuable source of novel bioactive compounds with antibacterial, antiviral, and anticancer properties (Colwell and Hill, 1992). Moreover the solubilization of insoluble heavy metals by bacteria has biotechnological application (White *et al.,* 1997)). The concentration and availability of Zinc in aquatic environment can affect the productivity and biodiversity of marine ecosystem (Simine *et al.,* 1998).

Because of so much waste is dumped into ocean daily, the marine environment becomes a potential biological reservoir of antibiotic and heavy metal resistant bacteria. Increased introduction of antimicrobial agents into the environment has resulted in new selective pressure (Lederberg *et al.,* 1992) Many genes conferring antibiotic and metals are located on genetic element (plasmid transposone, and integron), some of which are easily exchanged among phylogenetically distant bacteria. Many of these mobile genetic elements encode resistance to multiple antibiotics, heavy metals and other toxic compounds. (Davison, 1999).

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Baluchistan is the largest province of Pakistan with an area of 347056 sq.kms. It's a land of contrasts. The Baluchistan coast line extends over 750 kms. From Hub near Karachi beyond Gawadar till Pakistan Iran boarder. The whole area is rich with long un spoilt golden sunny beaches and variety of sea fish. In spite of this great potential very little work has been done on marine bacteria of Baluchistan coast. This coast remains unexplored as a source of genetically diverse bacteria. The aim of the present study was to isolate and characterize the bacterial strains both attached and free living from Baluchistan coast and study the distribution of pigmentation, antibiotic and metal resistance, salt tolerance, and solubilization and antibacterial activity. This shall help in conserving the biodiversity of bacterial flora and provide base line data for further studies.

MATERIALS AND METHODS

Sampling was carried out from Crustaceans (shrimps, crabs, loabster) and water samples (surface and deep sea) of Baluchistan coast during peak season of fishing that is for Fish September to March and for Crustaceans sampling was carried out from August to December.

Both attached and free bacteria were isolated from Baluchistan coast, attached bacteria were isolated from different kinds of fish (ribbon fish, mackeral, pomfert, sole, eal etc) and from crustaceans (crabs, lobster and shrimps), free bacteria were isolated from water samples (surface and deep sea). These fish and crustaceans samples were caught by fisher man and kept in ice. Sampling was done in three stages in first stage sample viable count was determined. Samples were grinded and serially diluted in 1% peptone and spreaded on half strength nutrient agar plates containing 1% NaCl by the spread plate method (Koch, 1981) and were incubated at 30°C for 24-48 hrs. In second stage grinded samples were first enriched using enriched media Brain heart infusion broth(Oxoid), MacCoonkey broth(Oxoid), Selenite broth (Oxoid), Alkaline peptone water (Oxoid) and incubated at 30°C for 24 - 48 hrs.

In third stage, after enrichment bacteria were isolated on different kinds of enriched media TCBSagar (Oxoid), VRBDA agar, (Merck) S.S agar (Oxoid),Blood agar (Oxoid)), plates were then incubated at 30°C for 24 –48 hrs. Morphologically different colonies were picked and purified.

Water samples were serially diluted with 1% peptone and spread on nutrient agar plates containing 1% NaCl. The plates were incubated at 30°C for 24-48 hrs. Morphologically different colonies were picked and restreaked on nutrient agar plates for purification. Isolates were identified on the basis of biochemical tests and API kits (20E and 20NE).

Pigment production ability of all strains was studied by growing them on nutrient agar supplemented with1% NaCl. Pigment production ability was noted at 30°C after 24 to 48 hrs. Pigment production were also noted when isolates were checked for antibiotic and metal resistance marker.

Resistance to various heavy metal salts $CuSO_4$, $(CH3C00)_2$ Pb, $NiCl_2$, $ZnCl_2$ $CrCl_2$(V1),$CoCl_2$ $CdCl_2$, $CrCl_2$(III) were determined by using Tris minimal media having following combinations gm/L (Tris HCl 6.06, NaCl 4.68, KCl 1.49, NH_4Cl 1.07, $NaSo_4$ 0.43, $MgCl_2$ 0.20, $CaCl_2$ 0.20 carbon source0.02% pH 7 with HCl supplemented with various concentration of heavy metals (mM). Plates were incubated at 30°C for 24- 48 hrs. Stock solutions of metal salts were prepared at concentration of 1M.

Resistance to selected antibiotics Erythromycin (Sigma), Kanamycin monosulphate (Sigma), Tetracyclin-hydrochloride (Fluka), Streptomycin sulphate (Sigma) and Chloramphenicol (Park Davis) were checked using nutrient agar plates containing 1% NaCl with various concentration of antibiotics (μg/ml). Plates were incubated at 30°C for 24- 48 hrs. Antibiotic stock solutions were prepared (Maniatis *et al.,* 1982).

Lactose fermenting ability of all the strains was checked using MacConkey agar (Oxoid). Culture was streaked on MacConkey agar plates, and results were noted after the incubation at 30°C for 24 hrs.

Solubilization of heavy metal salts were tested using Tris glucose Minimal media (TGM) having following combinations gm/L (Tris HCl 6.06, NaCl 4.68, KCl 1.49, NH_4Cl 1.07, $NaSo_4$ 0.43, $MgCl_2$ 0.20, $CaCl_2$ 0.20 carbon source glucose monohydrate 1%. pH 7 with HCl). Following insoluble heavy metals were studied, ZnO (14mM), ZnPO4 (5mM), Solubilizing activity was detected by the disappearance of added mineral particles producing clear zones around the growth as described by (Fasim *et al* .,2002).

Antibacterial activity of all the isolates was tested by two activity-monitoring procedures. (a)Cross streak method. Producer strain was inoculated as a streak across the surface of an agar plate using blunt ended tooth pick or by allowing a drop of culture to run across the plate. Plate was incubated at 30°C for 16-24hrs after 24 hrs of incubations plate was exposed to chloroform and test strains were cross streaked to producing strains and incubated at 30°C for 24 hrs and observed for inhibition of growth at each side of the producing strain (Fig**1**).

(b)Agar well diffusion method (Torreblanca *et al*., 1989) .On nutrient agar plates aseptically punch holes (4mm) using corck borer and surface of the plate with was swabbed with indicator organism, 100µL of cell free culture supernatant of producer strain was dropped in appropriate wells incubated at 30°C for 16-24hrs, after 24 hrs of incubations plates were observed for zone of inhibition (FIg.**2**). Antifungal activity was determined by petri plate method (Jayswal, 1990).

RESULTS

Isolation and Identification.

A total of 100 bacterial strains were isolated using simple and enriched media, 60 strains were isolated from fish and crustaceans and 40 strains were isolated from water samples these strains were preserved and purified, identification was done by studying gram reaction, colonial and cellular morphology, biochemical reactions and API kits (20E and 20NE). About 32 strains were isolated from crustaceans, 28 from fish and 23 from surface water and 17 from deep sea water. Most predominant flora among all the isolates was *Pseudomonas* sp., and *Vibrio*.sp., followed by S*taph.*sp., *Shewanella.*sp., *Serratia* sp. and *Klebsiella* sp. Out of 100 isolates 81% were identified as gram negative and 19 were gram positive this result correspond to Ahmed and Yasmeen (1988) and out of 100 strains 29% strains showed production of different Pigments (yellow, Green, Orange, Red) pigment production was also effected by certain heavy metals.

Table1. Feature Frequencies (%) For Complete Data Set.

Data set	Total no n=100	water n=40	Fish n=28	Crustaceans n=32
Basic characteristics				
Gram negative	81	85	71	84.3
Gram positive	19	15	28.5	15.6
Oxidase positive	66	59	53	57
Oxidase negative	21	15	55	27.5
Lactose Fermenter	16	17.5	14.2	15.8
Non lactose fermenter	57	67.5	50	50
Pigmentation	29	22	25	40.6
Antibiotic Resistance				
Streptomycin	77	30	78.5	84.37
Kanamycin	65	37.5	60.7	78.12
Erythromycin	52	45	67.8	65.62
Tetracyclin	31	20	32.14	43.75
Chloramphenicol	20	Nil	28.57	37.5
Heavy Metal Resistance				
CrO_3	74	35	71.42	84.37
$CuSO_4$	56	45	67.85	78.12
$ZnCl_2$	48	45	60.7	62.5
$Pb(CH_3COO)_2$	53	50	89.28	65.62
$NiCl_2$	27	22.5	28.57	37.5
$CoCl_2$	11	0	35.71	28.12
$CdCl_2$	0	0	0	0
$CrCl_3.6H_2O$	0	0	0	0
Solubilization Activity				
ZnO	20	10	21.4	31.25
$Zn_{3(}PO_4)_2$	40	20	25	78.12
Antibacterial activity				
Producer strains	15	2.5	17.85	28.12

Antibiotic Resistance

Determination of resistance pattern to five antibiotics among free and attached bacteria showed that in attached bacteria 81.66% isolates showed resistance against Streptomycin, 70% against Kanamycin, 66.66% to Erythromycin, 38.33% to Tetracyclin, 33.33% to Chloramphenicol (Table 1).

Whereas in free living bacteria 45% of the isolates showed resistance to erythromycin, 37.5%to kanamycin30% to streptomycin and 20% to Tetracyclin. It is important to remark that all of the strains isolated from water samples were susceptible to Chloramphenicol.

Resistance to streptomycin (81.66%) was most common in attached bacteria, whereas resistance to Erythromycin (45%) was most common in free living bacteria and the most important resistance pattern among all the isolates were streptomycin, kanamycin, erythromycin (30%) followed by Streptomycin, Kanamycin (19%) and Streptomycin, Erythromycin, Tetracyclin 8 %.

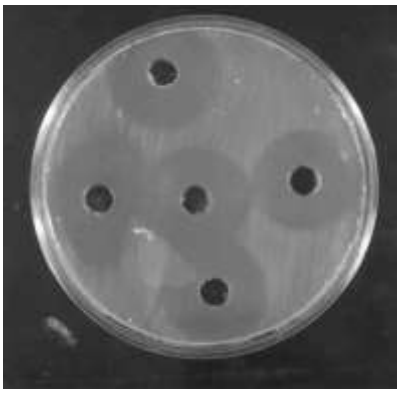

Fig1. Inhibition of **A**. *E.coli*, **B** *Staph*.sp,**C** *Enterobacter*.sp, **D** *Klebsiella* sp by marine *Pseudomonas* (Cross Streak Method).

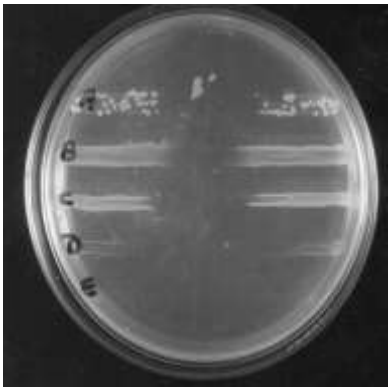

Fig 2. Inhibition of *Staphaylococcus* by Different Strains of *Pseudomonas* (Agar Well Diffusion Method).

Fig 3. Inhibition of Fungus By *Pseudomonas*.sp. (Petri plate Assay).

Table 2. Identification and Phenotypic Characters of Marine Isolates.

Strain code	Origin	Strain Identification	Strain phenotypic characters
CMG1011	Ribbon Fish	*Pseudomonas.sp*	Cu,,Pb, Cr,Sm,metal solubilizer,antibacterial and antifungal activity
CMG1013	Mackeral Fish	*Pseudomonas.sp*	Cu,Pb,Km,Er,,metal solubilizer
CMG1017	Shrimp	*Pseudomonas.sp*	Cu,Km,metal solubilizer, antibacterial and antifungal activity
CMG1019	Crab	*Serratia.sp*	Km,S,Er,metal solubilizer
CMG1024	Crab	*Klebsiella.sp*	Sm,metal solubilizer
CMG1036	Water	*Klebsiella.sp*	Sm,Km,Er,metal solubilizer
CMG1038	Water	*Vibrio.sp*	Cu,Pb,
CMG1045	Lobster	*Shewanella.sp*	Zn , Sm,Km,metal solubilizer
CMG1054	Squid	*Pseudomonas.sp*	Metal solubilizer
CMG1056	Crab	*Vibrio.sp*	Sm,Er
CMG1057	Shrimp	*Shewanella.sp*	Metal Solubilizer
CMG1067	Shrimp	*E.coli*	Pb,Zn
CMG1068	Sole Fish	*Klebsiella.sp*	Zn , Sm,,metal solubilizer
CMG1069	Sole Fish	*Pseudomonas.sp*	Metal solubilizer
CMG1070	Water	*Pseudomonas.sp*	Cu,Ni,Sm,Er
CMG1074	Ribbion Fish	*Serratia.sp*	Metal Solubilizer
CMG1076	Loabster	*Pseudomonas.sp*	Pb,Zn,antibacterial activity
CMG1079	Squid	*Vibrio.sp*	sensitive
CMG1083	Water	*Shewanella.sp*	Sm,Zn,Pb,metal solubilizer
CMG1087	Shrimp	*Shewanella.sp*	Sm,Er,Ch
CMG1091	Ribbion Fish	*Pseudomonas.sp*	Zn,Ni,metal solubilizer
CMG1096	Shrimp	*Vibrio.sp*	Sm,Ch

Key: Sm (streptomycin), Km (kanamycin), Er (erythromycin), Zn ($ZnCl_2$), Cu ($CuSO_4$), Ni($NiCl_2$), Pb(Pb ($CH_3COO)_2$

Table 3. Percentage of Bacterial Strains Exhibiting Antibacterial and Antifungal Activity and their spectrum of activity.

Samples	percentage of producer Strains	Anti Vibrio	Anti Staphylococcus	Anti Enterobacteriaceae	Anti Fungal
Water	2.5	Nil	2.5	2.5	Nil
Crustaceans	28.12	15.62	21.87	12.5	6.25
Fish	17.85	10.71	14.2	7.14	Nil

Resistance to heavy metals

Determination of resistance pattern of 8 heavy metals showed that attached bacteria are more resistance to heavy metals (76.66%) as compare to free living bacteria 50%. In attached bacteria the most resistant heavy metal was Cr(111)78.33%, followed by $Pb(CH_3COO)_2$(76.66%), $CuSO_4$(73.33%), $ZnCl_2$(61.66%), $NiCl_2$ (33.3%), $CoCl_2$(31.66%)(table2)All of the strains were susceptible to Cr(vi) and $CdCl_2$. In free living bacteria the most resistant heavy metal was $Pb(CH_3COO)_2$ (50%)followed by $CuSO_4$ and $ZnCl_2$(45%), Cr(iii)35%, $NiCl_2$(22.5%).All of the strains were susceptible to $CoCl_2$, Cr(vi) and $CdCl_2$. It was also observed that pigment production of CMG1030, 1021, 1055 were effected by $ZnCl_2$ and $Pb(CH_3COO)_2$.There was enhance pigment production when grow in the presence of 1mM Pb(CH3COO)2 and 1mM $CuSo_4$. The most common resistance pattern was Cr,Cu,Pb(20%) followed by Cr,Cu,Zn(14%),Cu,Pb(11.6%),Cr,Pb(5%).

Solubilization of Insoluble Heavy Metals

Solubilization of insoluble heavy metals ZnO (14mM) and $Zn(PO_4)_2$ (5mM) were checked for both attached and free living bacteria. It was observed that 53.33% of attached bacteria solubilized ZnO and 26.6% solubilized Zn $(PO_4)_2$. Where as 16% among the attached solubilized both insoluble heavy metals. Among the free living bacteria 20% of the isolates solubilized ZnO and 10% of the isolates solubilized $ZnPO_4$ (5mM), where as none of the isolates solubilized both insoluble heavy metals checked.

Antibacterial and Antifugal Activity.

Antibacterial and antifungal activity was checked and it was observed that 23% of the attached isolates exhibited antibacterial activity whereas 2.5% of free bacteria showed antibacterial activity. Attached bacteria also showed antifungal activity (6.3%)(Fig. **3**) where as none of the free living bacteria of Baluchistan coast exhibited antifungal activity. All the producer strains were identified as *Pseudomonas* sp., but spectrum of their activity vary from strain to strain. The percentage was highest for anti *Staphylococcus* (Table 3) in all isolates (attached and free).

DISCUSSION

The oceans offer abundant resources for research and development, yet the potential of this domain, as the basis for new biotechnologies remains largely unexplored. Oceanic organisms are of enormous scientific interest, for two major reasons. First, they constitute a major share of the Earth's biological resources. Second, marine organisms often possess unique structures, metabolic pathways, reproductive systems, and sensory and defense mechanisms because they have adapted to extreme environments ranging from the cold polar seas at -2° C to the great pressures of the ocean floor, where hydrothermal fluids spew forth. Thus Oceans represent a source of unique genetic information.

Little is known about marine biodiversity especially when compared with the information we have on terrestrial ecosystem. Much of the success of marine biotechnology relies on investigating; characterizing and maintaining biodiversity. Similarly it will only be possible to accurately determine the structure and function of ecosystem, once the type of microorganisms that colonize particular habitat and metabolic processes they perform are accurately determined. To date no significant work has been done with regard of marine bacterial biodiversity in Baluchistan coast of Pakistan, present study was conducted to identify, and characterize bacteria of Baluchistan coast. It was observed that bacteria of Baluchistan coast exhibit diverse characters such as multiple metal and antibiotic resistances, solubilization of insoluble heavy metals, production of antibacterial and antifungal substance, production of pigment, fermentation of lactose (Table **2**). A total of 100 bacteria were isolated from wild catch and water samples. These samples were analyzed quantitatively and qualitatively for the presence of bacteria (Table**1**) and they were classified into attached and free living bacteria according to their origin.

Our results and some previous reports showed that gram negative bacteria are abundant in marine environment (Ahmed and Yasmeen, 1988). 81% of the isolated strains were identified as gram negative whereas 19% were identified as gram positive. Most predominant flora among all the isolates was *Pseudomonas* sp., and *Vibrio* sp., followed by S*taph* sp., *Shewanella* sp., *Serratia* sp, and *Klebsiella* sp., Pigment producing strains were isolated from attached and free bacteria but proportion was higher in attached bacteria it is reported that bacteria in the surface region are pigment producer this character is helpful for protection from lethal portion of solar radiation (Pelaczar1986). Relatively higher no of pigment producing bacteria were isolated from wild catch as compare to water samples.

The incidence of antibiotic resistant bacteria in aquatic environment has increased dramatically as a consequence of the wide spread use of antibiotics (Aarestrup, 1996). Significantly higher proportion of antibiotic and heavy resistance bacteria were isolated from attached bacteria than free living bacteria (Table 1) This high frequency of antibiotic and metal resistance in bacteria of Baluchistan coast could be explained that there is possibility of horizontal spread of resistance genes, or presence of selective pressure in that environment, Many bacteria containing R plasmid exhibit higher rates of survival in aquatic environment (Tsubokura *et al.*, 1995) R plasmids may play a major role in the dissemination of high level of antibiotic resistance among attached bacteria of Baluchistan. It is also reported that bacteria can transfer plasmids *in situ* to indigenous microflora. The molecular mechanisms responsible for the emergence of multiple-antibiotic-resistant may be due to acquisition of antibiotic resistance genes through horizontal and lateral gene transfer (Davison, 1999). Plasmids, conjugative transposons, and integrons are all vehicles for the acquisition of resistance genes (Hall, 1995).

The significance of antibiotic resistant environmental bacteria has been much disputed. Significantly higher proportion of antibiotic and metal resistance bacteria isolated from Baluchistan coast might provide a pool of resistance genes capable of transfer to other bacteria. A better understanding of such processes in natural environment is crucial in order to assess the risk of antibiotic resistance among ubiquitous aquatic bacteria.

Bacteria isolated from wild catch and water samples were also screened for secondary metabolites of commercial importance and it was observed that marine bacteria of Baluchistan coast produced compound of commercial importance such as antibacterial compound and antifungal compound but the proportion of production of antibacterial substance was higher in attached bacteria then free living bacteria this result correspond to Long and Azam (2001) who reported that particle attached isolates produced more inhibitory compound than free living bacteria or produced compound that inhibited more species then the compound produced by free living bacteria. Moreover it is not uncommon for single specie to produce multiple inhibitory compounds. It was found out that all antibacterial and antifungal producer were belong to genus *Pseudomonas* which inhibit both gram negative and gram positive bacteria but gram positive bacteria (*Staphylococus*)were more susceptible than gram negative bacteria . It was also observed that all of antibacterial producing bacteria were pigment producer. A high proportion of pigmented bacteria with antibacterial activity were reported by (Shiba and Tega, 1980).It was observed that strains isolated from Crustaceans exhibit both antibacterial and antifungal activity whereas strains isolated from fish and water samples exhibited activity only against bacteria. The Solubilization of insoluble metal compounds has biotechnological applications e.g., reclamation of metals from low grade ores and recovery of metals from industrial by products (Burgstaller *et al.*, 1993). All of the isolated strains were also checked for their ability to solubilize insoluble heavy metals Zinc oxide and Zinc orthophosphate. Simple and Minimal medium were used to check the activity it was observed that activity was only detected on tris glucose minimal medium. Both attached and free living bacteria can solubilize insoluble heavy metals but proportion of solubilization was higher in attached bacteria then free living bacteria. Analysis of variation in pH activity in liquid media showed good correlation of drop of pH and metal solubilization both of them were directly proportional to each other same results have been reported by (Burgstaller *et al*., 1994).

Conclusion

Characterization of bacterial strains reveled that bacteria with diverse genetic characters are present in Baluchistan coast of Pakistan. Multiple antibiotic and heavy metal resistant bacteria were also isolated from samples of Baluchistan coast. Bacteria of Baluchistan coast also produced compound of commercial importance, it was also found out that attached bacteria were more active in producing compounds of commercial importance such as antibacterial, antifungal substance, and pigments. Thus study of marine microorganisms can provide important biotechnological information's with significance for the production of bioactive secondary metabolites, and heavy metal solubilizing strains.

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