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Phylogenetic analysis and biological characteristic tests of marine bacteria isolated from Southern Ocean (Indian sector) water

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Abstract

Fifty-seven bacteria were isolated from Southern Ocean (Indian sector) water samples which were collected from different latitude and longitude of the ocean. All the isolates were able to grow at 4°C, 20°C, 37°C and tolerable NaCl concentration up to 13.5% (w/v). 29 out of 57 isolates were identified using 16S rDNA amplification and the sequences were submitted to National Center for Biotechnology Information (NCBI). All the isolates were classified by using Ribosomal Database Project (RDP) and found that isolates belongs to Proteobacteria and Bacteriodes. The average G+C content was 56.4%. The isolates were screened for the presence of extracellular enzymes, *viz*. amylase, catalase, urease, esterase, lipase and protease. The disc diffusion method is used to screen antibiotic production by the isolates against four pathogenic bacteria, *viz*. *Salmonella typhimurium* (NCIM 2501), *Staphylococcus aureus* (NCIM 2122), *Bacillus subtilis* (NCIM 2193), and *Pseudomonas aeruginosa* (NCIM 2036). Nine out of 29 were found to be antibiotic producer.

Key words: Southern Ocean, marine bacteria, 16S rDNA, phylogenetic tree, antibacterial

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1 Introduction

Marine system is a less characterized environment regarding microbial diversity and ecology. It is estimated that there are 10^6–10^8 bacterial species present in marine system under wide variation of pressure and temperature, where as salinity is 3.5% (Murray and Grzymski, 2007). Marine organisms lives in complex habitat and exposed to extreme conditions such as pressure and temperature. Under these extreme conditions they produce a wide variety of secondary metabolites that are biologically active such as polysaccharides, polyunsaturated fatty acids (PUFAs), antioxidants, sterols, proteins, pigments and anticancerous activity (Rasmussen and Morrissey, 2007; Lordan et al., 2011; Gupta et al., 2014). Considering its immense taxonomic diversity, microbiologists are paying their attention to the search of new bioactive compounds from the marine environment that are utilised in cosmetics, neutraceutical, pharmaceutical companies, etc.

Bacterial productivity and metabolism are affected by certain factors such as hydrostatic pressure, salinity, temperature, dissolved oxygen, etc. (Yoshida et al., 2007). According to endimicity theory some microbes require special environment (hot springs, cryosphere and hyperhalophilic habitats) for the production of secondary metabolites. There is drastic change in marine environment in the month of December to March, which ultimately affects the productivity of organisms (Campbell and Claridge, 1987; Teixeira et al., 2010). Upwelling of nutrients supports more than 75% of primary productivity which is lower in winter season and high in summer (January–March). In Southern Ocean, circumpolar fronts separate several zones that form at different longitude (Whitworth, 1980; Orsi et al., 1995; Sokolov and Rintoul, 2009; Wilkins et al., 2013). Satellite altimetry data and several other data are used to determine the frontal structure of Southern Ocean. The different major fronts in Southern Ocean are Subtropical Fronts (STF), Polar Fronts (PF), Subantarctic Fronts (SAF) and Southern Antarctic Circumpolar Current Fronts (SACCF). Among them, Polar Fronts play an important role in distribution of both bacterioplankton (Giebel et al., 2009; Weber and Deutsch, 2010) and zooplankton (Ward et al., 2003). Marine environment are dominated by Archaea, Bacteria, Eukarya and

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other forms of life surviving in marine system such as invertebrates (Mollusc, Cnidaria, etc.). This unknown diversity of marine life is currently being explored through metagenomic study or culture dependent method (Schmalenberger et al., 2001; Petti et al., 2005; Chakravorty et al., 2007; Gupta et al., 2015).

The aim of the present study is to isolate bacteria from Southern Ocean particularly in Indian sector. The study includes water from different longitude, latitude and depths during austral summer (January–March, 2011). Culture-dependent method was used to isolate the bacteria. After PCR amplification and sequencing, sequences were analysed using BLAST and ribosomal database project (RDP). Phylogenetic analysis was done using MEGA 5.2 software. The isolates were also characterized in terms of the presence of extracellular enzymes and antibacterial activity against pathogenic bacteria.

2 Materials and methods

2.1 *Water sample collection*

Water samples were collected from four different stations (latitude and longitude) (Fig. 1) and their respective depths (Table 1) during 5th Southern Ocean Expedition (SOE) in austral summer (January–March, 2011). The expedition was organised by National Centre for Antarctic and Ocean Research (NCAOR), Government of India, GOA and Ministry of Earth Sciences (MoES), Government of India, New Delhi. Water samples were collected using rosette conductivity temperature depth (CTD) instrument (Sea-Bird Electronics, Inc. Florida, USA). The samples were shipped to India at 4°C in 500 mL polypropylene bottle.

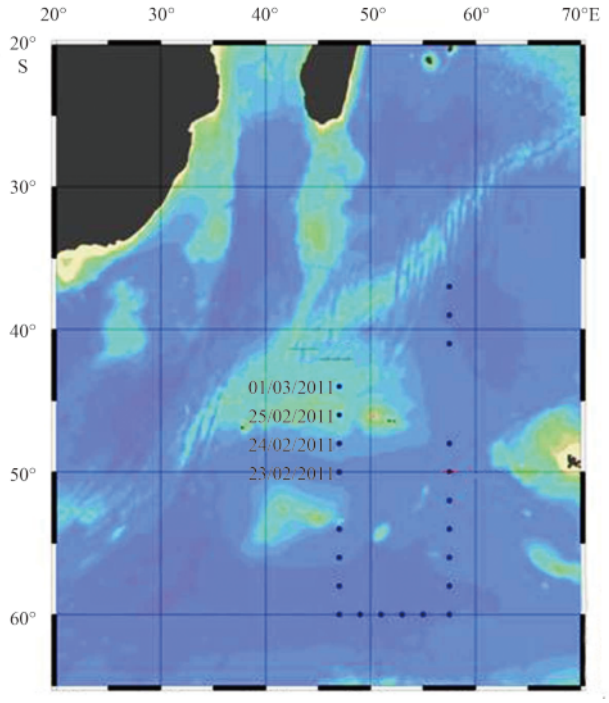

Fig. 1. Sampling site for collection of water sample, with date of collection, longitude and latitude (*x*-axis and *y*-axis). Blue dot represents the cruise track for expedition.

Table 1. Sampling location with latitude, longitude and environmental data

	Station No.			
	22	23	25	26
Date of sampling	23/02/2011	24/02/2011	25/02/2011	01/03/2011
South latitude/(°)	50	48	44	42
East longitude/(°)	47	47	47	47
SST/°C	5.5	6.0	9.0	17
Atmospheric pressure/Pa	101 900	102 670	102 700	101 800
Depths/m	1 000, 3 800	1 000, 2 000, 3 000, 3 400	1 000, 1 500, 2 000, 2 500	0, 30, 50, 75, 100, 200, 300, 500, 1 000, 1 500, 2 000, 3 000, 3 400

Notes: SST represents sea surface temperature.

2.2 *Isolation of bacteria*

250 mL of sea water sample was filtered through membrane of pore size 0.22 μm. After filtration, membrane was washed in 5 mL of autoclaved sea water. 50 μL from the above 5 mL seawater was used as inoculum and spreaded on Antarctic Bacterial Medium (ABM) (Shivaji et al., 2013) (peptone, 5 g; yeast extract, 2 g; and agar, 20 g; volume make up to 1 L with sea water) and incubated for 15–20 d at 20°C. Bacteria with different morphology and colour were obtained and tabulated in tabular form. Bacteria were purified by re-streaking and maintained in marine agar slants. The isolated bacteria were further incubated at 4°C and 37°C. The culture plates were regularly observed for their growth. NaCl tolerance was tested by inoculating bacteria at different NaCl concentration of 4%, 8%, 10%, 11%, 12%, 13%, 13.5%, 14%, 14.5% and 15% (w/v) in ABM. Gram characteristic of marine isolates were determined by Gram staining kit as per the instruction provided by the manufacturer (Hi-Media, India) and their structures were visualized under the microscope (CH20, Olympus Corp., Japan).

2.3 *DNA extraction, amplification and phylogenetic analysis*

DNA was isolated using PrepMan^{Tm} ultra DNA isolation kit (Applied Biosystem, USA) according to the manufacturer's instruction. 16S rDNA amplification was carried out in GeneAmp PCR 9700 (Applied Biosystem, USA) using 8f (5'–GAGTTTGATCATGGCTCAG-3') and 1459r (5'–CTACGGCTACCTTGTTACG-3') primer and amplification condition was: initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of 30 s denaturation at 95°C, annealing temperature at 55°C for 30 s, and 72°C for 2 min for extension and then 10 min final extension at 72°C. The PCR product was then purified, sequenced (3130× Genetic analyzer, Applied Biosystem) and analysed. The sequences were

blasted using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi.htm) to determine the homology. The sequence were submitted to Classifier program of Ribosomal Database Project (RDP) and classified according to phylum, class, order, family and genus. Bacterial sequences were aligned and phylogenetic tree was prepared using Neighbour-Joining method with Kimura-2-parameter having 1 000 bootstrap replication through MEGA 5.2 (Tamura et al., 2011).

2.4 *Biochemical characterization*

Isolates were screened for starch hydrolysis, in ABM containing 1% soluble starch (Antony et al., 2012). After the proper growth, the plates were flooded with Gram's iodine and appearance of clear zone around the colony was taken as positive result. Esterase activity was confirmed by streaking bacteria on ABM containing tributyrin as a substrate. Lipase and urease test was also performed (Shivaji et al., 2013). Catalase test was performed by adding hydrogen peroxide to loop of culture taken on a slide (Holding and Collee, 1971). Protease test was conducted in ABM supplemented with 0.4% gelatin. All the plates were incubated at 20°C for 2–3 d.

2.5 *Antibacterial activity of marine isolates*

2.5.1 *Extract preparation of marine isolates*

Isolated marine bacteria were inoculated in 100 mL of ABM in 250 mL of conical flask. Flasks were incubated at 20°C for 6 d in rotary shaker at 250 r/min. After 6 d of cultivation, broth was centrifuged at 5 000 *g* for 20 min to remove the cells. The supernatant was extracted three times with ethyl acetate (100×3). The ethyl acetate extract was evaporated in rotary evaporator and further dissolved in 1 mL of ethyl acetate. The ethyl acetate extract was further used for antimicrobial activity.

2.5.2 *Disc diffusion assay*

Antimicrobial assay were performed in triplicate using paper disc (Mearns-Spragg et al., 1998). 24 h grown test microorganisms *Salmonella typhimurium* (NCIM 2501), *Staphylococcus aureus* (NCIM 2122), *Bacillus subtilis* (NCIM 2193), and *Pseudomonas aeruginosa* (NCIM 2036) was swabbed on Muller Hinton agar and the disc containing 15 μL extract (50 mg/mL) was placed on the inoculated plate. Ethyl acetate was used as control for the experiment. The plate was incubated at 4°C for 5 h for diffusion of extract. After that, plate was kept at 37°C for overnight. The zone of inhibition was measured in millimetre (mm).

3 Results

3.1 *Water sample characteristic*

Water samples were transparent. Water salinity of the four stations was varied from 33 to 35. The atmospheric pressure was also varied at different latitude and longitude (Table 1). Sea surface temperature was varied in the ranges of 5.5°C to 17.0°C.

3.2 *Physiological characterization of isolates*

A total of 57 bacteria were isolated from four different stations (latitude and longitude) with 23 different depths. The morphology of the isolates was recorded in tabular form (Table 2). Initially all the isolates were grown at 20°C, after they grew at 20°C, the bacteria were inoculated and incubated further at 4°C to check their growth and viability. As the bacteria were isolated from cold environment, it will grow at low temperature (Srinivas et al., 2009). In our present study, isolated bacteria were well grown within 3 to 4 d at 4°C and it was observed that psychrotolerant bacteria were predominant in Southern Ocean (Indian sector). All the isolated bacteria from the present study can tolerate NaCl concentration up to 13% (w/v) and few of them can tolerate up to 13.5% (w/v) of salt. Microscopic examination of the isolated bacteria revealed that station number 26 were dominated by gram negative rods, whereas most of the gram positive rods were present at station number 25. Except the above two types, three samples, *viz*. PR-MB-1, PR-MB-14 and PR-MB-26 were gram positive cocci.

3.3 *Antibacterial activity of marine isolates*

The discoveries of new drugs are necessary to combat with the emerging evidence of multidrug resistant pathogenic microbes. The isolated marine isolates were screened for antibacterial activity against pathogenic bacteria. Nine out of 29 bacterial strains showed antibacterial activity (Table 3). PR-MB-4, and PR-MB-42 has activity against gram negative bacteria where as PR-MB-24, PR-MB-32 and PR-MB-44 has maximum activity against gram positive bacteria. PR-MB-16 (*Pseudoalteromonas espejiana*) among all the nine isolates showed potent activity against both gram positive and gram negative bacteria (Fig. 2).

3.4 *Sequence and phylogenetic analysis*

Marine system consist of microbial community that have both uncultured and cultured bacterial population. In the present study, culture based method is adopted to explore the part of bacterial community. Based on the 16S rRNA gene sequences, the nucleotide was subjected to BLAST analysis and prokaryotic phylogeny was analysed through RDP (Classifier) (Wang et al., 2007). Considering BLAST and RDP result, it was further confirmed that all the isolates were showing more than 98% sequence similarity with the corresponding sequences reported in the GenBank.

All the 29 sequences have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) at NCBI with accession number KF019643 to KF019670. Among 29 marine isolates Proteobacter were dominant. BLAST result revealed that 15 isolates belonged to uncultured bacterium (Table 4) with 100% similarity having *E* value 0.0. Phylogenetic tree (Fig. 3) shows that one clade consist of Alphaproteobacteria, Gammaproteobacteria and Flavobacteria where as another clade consist of only Gammaproteobacteria. According to phylogram interpretation Gammaproteobacteria was found as dominant (86%) (Fig. 4) in Southern Ocean water sample (Indian sector). All the sequences were aligned through MEGA 5.2. The average G+C content in all the 29 isolates was 54.6% and the substitution probabilities were given in Table 5. The overall transition/transversion bias (R) was 0.62. Abundance of bacteria in Southern Ocean was shown in Fig. 5.

3.5 *Biochemical characterization*

In the present study, isolated bacteria were tested for the presence of extracellular enzymes, *viz*. amylase, esterase, lipase, catalase, urease, and protease (Table 6). 14 isolates out of 29 have extracellular enzymes, *viz*. amylase, esterase, lipase, catalase, and urease, except protease enzyme. PR-MB-26 isolate has maximum zone of inhibition against protease enzymes followed by PR-MB-6, PR-MB-16, PR-MB-47 and PR-MB-42.

Table 2. Morphological characteristic of bacteria isolated from Southern Ocean (Indian sector)

Station No.	Isolates	Depth/m	Colony morphology					
			Size/cm	Form	Margin	Colour	Elevation	Opacity
22	PR-MB-1	1 000	0.15	circular	entire	whitish	flat	opaque
	PR-MB-3	3 800	1	irregular	undulate	yellowish	convex	translucent
	PR-MB-B	3 800	0.9	circular	entire	brown	pulvinate	opaque
23	PR-MB-4	1 000	0.2	irregular	undulate	whitish,	convex	opaque
	PR-MB-5	1 000	>0.1	circular	entire	whitish	flat	opaque
	PR-MB-6	1 000	0.15	highly irregular	erose	whitish	flat	translucent
	PR-MB-7	1 000	0.3	circular, glistening	entire	whitish	whitish	opaque
	PR-MB-8	2 000	0.3	circular , glistening	entire	whitish	raised	opaque
	PR-MB-9	2 000	0.17	circular	entire	whitish	flat	translucent
	PR-MB-10	3 000	>0.1	circular	entire	creamish	flat	translucent
	PR-MB-11	3 000	0.15	circular	entire	creamish	flat	translucent
	PR-MB-12	3 400	>0.1	circular	entire	creamish	flat	translucent
	PR-MB-13	3 400	0.15	circular	entire	creamish	flat	translucent
25	PR-MB-14	1 000	>0.1	punctiform	entire	whitish	raised	opaque
	PR-MB-15	1 000	0.25	circular	entire	yellowish	raised	opaque
	PR-MB-16	1 500	>0.1	circular	entire	whitish	flat	opaque
	PR-MB-17	2 000	>0.1	circular	entire	whitish	flat	translucent
	PR-MB-18	2 000	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-19	2 500	0.2	irregular	undulate	pinkish	flat	opaque
	PR-MB-20	2 500	0.4	highly irregular	erose	creamish	flat	translucent
	PR-MB-21	2 500	0.2	circular	entire	creamish	convex	opaque26
26	PR-MB-22	0	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-23	0	0.4	irregular	undulate	whitish, yellow	flat	opaque
	PR-MB-24	30	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-25	30	0.2	irregular, glistening	undulate	creamish,	flat	opaque
	PR-MB-26	30	1	circular	undulate	whitish	flat	round
	PR-MB-27	50	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-28	50	0.2	irregular, glistening	undulate	creamish	raised	opaque
	PR-MB-29	50	0.25	circular	entire	whitish	flat	opaque
	PR-MB-30	50	>0.1	irregular	undulate	whitish	flat	translucent
	PR-MB-31	75	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-32	75	0.25	irregular	undulate	creamish	flat	opaque
	PR-MB-33	75	0.2–0.3	irregular	undulate	whitish	flat	slightly opaque
	PR-MB-34	100	>0.1	circular	entire	whitish	flat	translucent
	PR-MB-35	100	0.3	highly irregular	erose	creamish	raised	opaque
	PR-MB-36	100	0.25	circular	entire	creamish	raised	opaque
	PR-MB-37	200	0.25	circular	entire	whitish	raised	opaque
	PR-MB-38	300	>0.1	circular	entire	yellow	convex	opaque
	PR-MB-39	300	>0.1	circular	entire	whitish	flat	translucent
	PR-MB-40	500	>0.1	circular	entire	yellow	convex	opaque
	PR-MB-41	500	0.3	circular	entire	yellow	convex	opaque
	PR-MB-42	500	0.2	circular	entire	whitish	raised	opaque
	PR-MB-43	500	0.1	circular	entire	orange	convex	opaque
	PR-MB-44	500	0.3	highly irregular	erose	yellowish creamish	raised	translucent
	PR-MB-45	500	0.2–0.3	irregular, faintly visible	undulate	whitish	flat	translucent
	PR-MB-46	1 000	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-47	1 000	0.2	circular	entire	yellow	convex	opaque
	PR-MB-48	1 000	0.3	circular	entire	whitish	raised	opaque
	PR-MB-49	1 500	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-50	1 500	0.2	irregular	undulate	creamish	convex	opaque
	PR-MB-51	1 500	>0.1	irregular	undulate	whitish	flat	translucent
	PR-MB-52	2 000	0.2	circular	entire	whitish	raised	opaque
	PR-MB-53	3 000	>0.1	punctiform	entire	whitish	raised	translucent
	PR-MB-54	3 000	>0.1	circular	entire	whitish	raised	opaque
	PR-MB-55	3 000	0.2	circular	entire	creamish	raised	opaque
	PR-MB-56	3 400	0.2	circular	entire	creamish	flat	opaque
	PR-MB-57	3 400	0.2–0.3	irregular	undulate	whitish	flat	translucent
				faintly appear				

Table 3. Antibacterial activity of marine isolates against pathogenic bacteria using disc diffusion method

Marine isolates	Gram characteristic	Antimicrobial activity			
		ST	SA	BS	PA
PR-MB-4	G^-	++	–	–	+++
PR-MB-6	G^-	–	–	++	+
PR-MB-7	G^-	–	–	+	–
PR-MB-16	G^-	+++	+	–	+
PR-MB-24	G^+	–	++	+++	–
PR-MB-32	G^+	–	–	++	–
PR-MB-42	G^-	+	+	–	–
PR-MB-44	G^-	+++	+++	+++	–
PR-MB-B	G^-	++	+	–	+++

Notes: ST represents *Salmonella typhimurium* (NCIM 2501), SA *Staphylococcus aureus* (NCIM 2122), BS *Bacillus subtilis* (NCIM 2193), and PA *Pseudomonas aeruginosa* (NCIM 2036). +++ means zone of inhi bitionwas more than 6 mm, ++ zone of inhibition was 4–6 mm, + zone of inhibition was 1–4 mm, and – no zone of inhibition. G^- represents gram negative and G^+ gram positive.

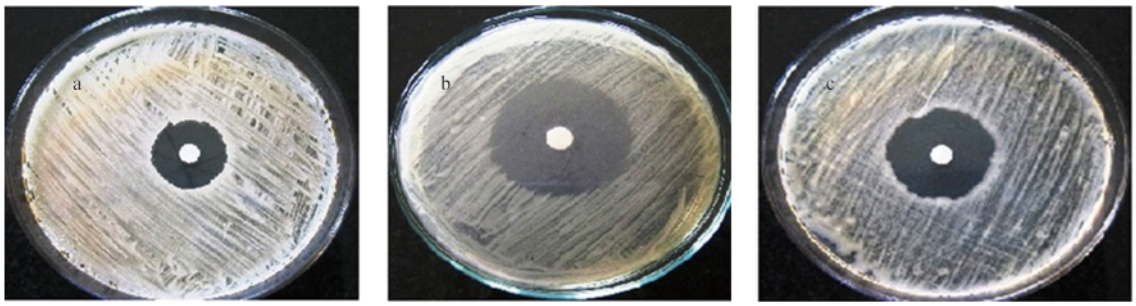

Fig. 2. Antibacterial activity of PR-MB-16 against *Staphylococcus aureus* (a), *Salmonella typhimurium* (b) and *Bacillus subtilis* (c) using disc diffusion method.

Table 4. Bacterial sequence identification on the basis of BLASTn search and their percentage similarity of bacteria isolated from Southern Ocean

Sl. No.	Assigned code for isolates	Sequence length/bp	Closest relative	Similarity/%	GenBank accession ID	GC content/%
1	PR-MB-1	861	*Marinobacter* sp. Ice-oil81	100	KF019654	54.7
2	PR-MB-3	854	*Marinobacter* sp. Ice-oil-81	100	KF019655	55.0
3	PR-MB-4	751	uncultured bacterium	100	KF019656	55.3
4	PR-MB-6	751	*Pseudoalteromonas tetraodonis*	100	KF019657	52.4
5	PR-MB-7	774	uncultured bacterium	100	KF019658	52.6
6	PR-MB-8	843	*Marinobacter algicola*	100	KF019659	54.2
7	PR-MB-9	671	*Marinobacter* sp. Ice-oil-81	100	KF019660	55.3
8	PR-MB-10	755	uncultured bacterium	100	KF019661	56.2
9	PR-MB-11	844	uncultured bacterium	100	KF019662	57.2
10	PR-MB-12	883	uncultured bacterium	100	KF019663	52.4
11	PR-MB-14	673	*Marinobacter algicola*	100	KF019664	54.5
12	PR-MB-15	700	*Marinobacter algicola*	100	KF019665	54.2
13	PR-MB-16	561	*Pseudoalteromonas espejiana*	99	KF019666	53.5
14	PR-MB-18	512	*Marinobacter algicola*	100	KF019667	55.3
15	PR-MB-19	839	*Marinobacter* sp.	100	KF019668	55.2
16	PR-MB-24	618	*Zunongwangia profunda*	99	KF019669	49.1
17	PR-MB-26	771	uncultured bacterium	99	KF019670	57.2
18	PR-MB-32	830	uncultured bacterium	99	KF019643	55.2
19	PR-MB-34	532	uncultured bacterium	99	KF019644	55.5
20	PR-MB-35	878	uncultured bacterium	100	KF019645	55.1
21	PR-MB-36	802	*Marinobacter* sp.	100	KF019646	55.1
22	PR-MB-39	915	*Marinobacter* sp.	99	KF019647	54.9
23	PR-MB-42	743	*Erythrobacter citrus*	100	KF019648	52.7
24	PR-MB-44	828	*Marinobacter algicola*	99	KF019649	54.2
25	PR-MB-45	290	*Halomonas hydrothermalis*	99	KF019650	58.3
26	PR-MB-46	862	*Marinobacter* sp. Ice-oil-81	99	KF019651	54.6
27	PR-MB-47	801	*Pseudoalteromonas* sp.	98	KF019671	53.6
28	PR-MB-54	834	uncultured bacterium	100	KF019652	55.1
29	PR-MB-B	871	uncultured bacterium	100	KF019653	56.9

Notes: Sl. No. means serial number.

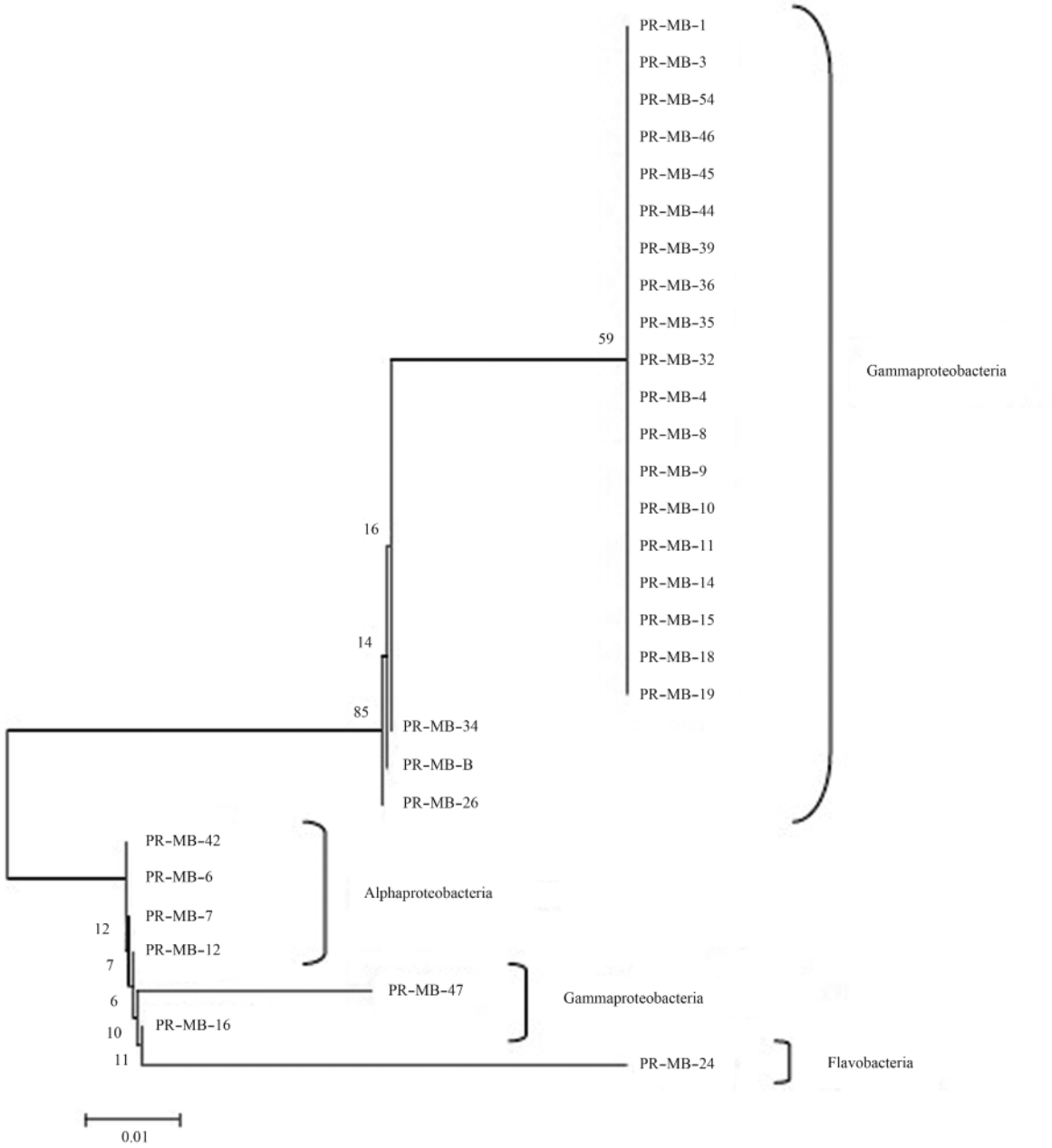

Fig. 3. Phylogenetic tree constructed by using neighbour-joining method with Kimura 2 distance parameter, having bootstrap value of 1 000 replicates. The bar represents 0.01 substitutions per alignment.

4 Discussion

Natural compounds from marine source play an important role in pharmaceutical industries (Zhang et al., 2008). These natural compounds have various biological potentials such as antimicrobial, anticancerous, antidiabetic, etc. (Demain and Sanchez, 2009; Gupta et al., 2014). Natural products from marine microorganisms are entirely different from terrestrial microorganisms as marine microbes are living in harsh and highly competitive environment. Hence compounds from marine source are most prominent source for discovery and production of new drugs.

Culture dependent approach have advances in microbiology, in spite of its well known, serious limitations (Amann et al., 1995; Jannasch and Jones, 1959), mainly related to the selectivity of the nutrient media and culture conditions which leads to favouring only a fraction of the inhabiting bacterial community. The major advantage of culture dependent approach over the modern molecular techniques is providing researcher with the microbial material that can be used for further studies. In the present study, we tested the antibacterial potential of isolates against pathogenic bacteria and thus adopted the culture dependent technique for isolating bacteria from Southern ocean water sample.

A total of 57 bacteria were isolated using ABM, among them some are pigmented. Bacteria grew between 4°C and 37°C are reported to be psychrotolerant (Reddy et al., 2009), and in our present study all the isolates were grown at 4°C, 20°C and 37°C. Similar study, was done from Antarctic land sample and the authors reported that psychrotolerant species (80%) are dominant than psychrophiles (20%) in Antarctic ice core region (Shivaji et al., 2013; Oh et al., 1991; Kogure, 1998). Isolates were able to grow

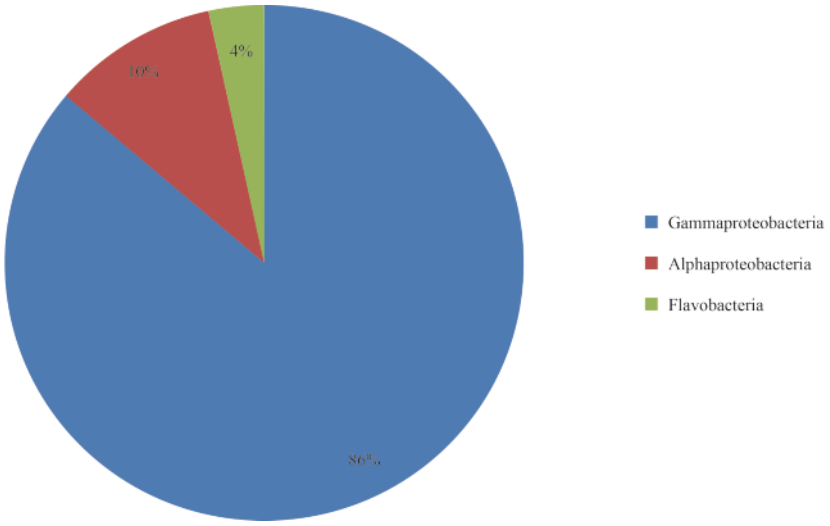

Fig. 4. Pie chart represents the percentage of bacterial class (of 29 isolated bacteria) in Southern Ocean water sample on the basis of 16S rDNA.

Table 5. Maximum composite likelihood estimate of the pattern of nucleotide substitution

	A	T	C	G
A	–	*4.96*	*6.16*	**14.74**
T	*6.26*	–	**12.35**	*8.92*
C	*6.26*	**9.95**	–	*8.92*
G	**10.35**	*4.96*	*6.16*	–

Notes: Rate of different transitional substitution are shown in bold and those of transversional substitution are shown in italics. – represents no translational/transversional substitution.

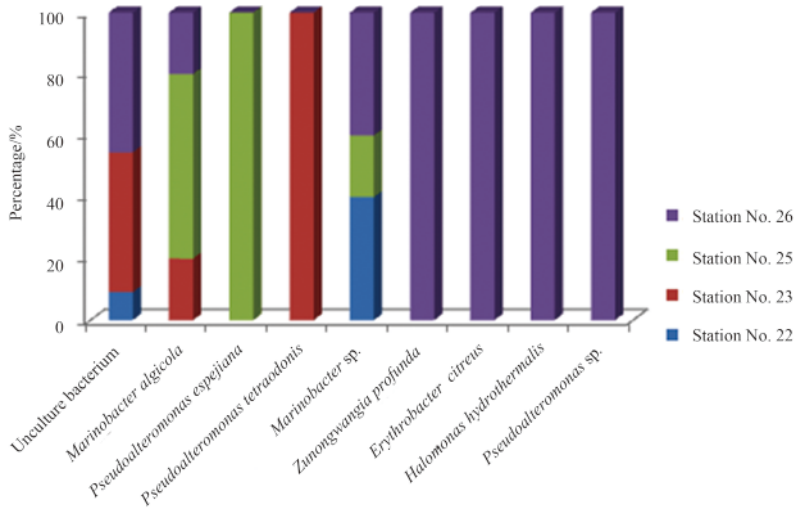

Fig. 5. Abundance of 29 bacteria present in different stations in Southern Ocean.

without NaCl and can tolerate NaCl up to 13% (w/v) and a few of them even tolerate at 13.5% (w/v).

Solvent extraction method is usually used for extraction of bioactive compounds. Organic solvent with different polarity were reported for the extraction of metabolite from marine source (Vijayakumar et al., 2012). In current study, ethyl acetate was used for compound extraction. Present studies support the general observation that gram negative bacteria were not susceptible for antibacterial compound present in marine bacteria, but gram positive bacteria were susceptible towards antibacterial compound present in marine bacteria (Saadoun et al., 1999, Basilio et al., 2003; Wong et al., 2011). PR-MB-16 belongs to *Pseudoalteromonas espejiana* and its ethyl acetate extract has potent activity against pathogenic gram positive and gram negative bacteria. Because of the importance of marine microorganisms for bioactive compound isolation, bacteria were isolated from Southern Ocean and screened for bioactive compound that have antibacterial property. Isolation and identification of drug molecule from *Pseudoalteromonas espejiana* (PR-MB-16) are needed to determine the active metabolite.

Most of the isolated bacteria belong to uncultured bacterium and it could be they were not cultured or reported earlier. Among 19 gram negative bacteria, 3 belong to α-proteobacteria whereas rest of them are γ-proteobacteria. The gram negative bacteria *Pseudoalteromonas* sp. was previously isolated from Antarctic water and its best growth was observed at 20°C (Nam and Ahn, 2011). Current data supports earlier reports (Neufeld et al., 2004; Steven et al., 2007; Shivaji et al., 2013) that phylum proteobacter are common in cold habitats. In general, G+C content of the bacteria ranges from 16% to more than 75%, which is due to difference in mutation pattern in bacteria (Lightfield et al., 2011). In the present study, the G+C content of bacteria is 54.6% which supports the above statement.

The marine environment ranges from nutrient-rich regions to nutritionally bare areas where only a few organisms can survive. The complexity of the marine environment may contribute to the significant differences between the enzymes generated by marine microorganisms and enzymes isolated from terrestrial microorganisms (Zhang and Kim, 2010). This difference supports enzyme technology and results in production of valuable products. Marine bacteria have diverse extracellular enzymes and are source of therapeutic products that are applicable in pharmaceutical industries, food additives and fine chemicals (Struvay and Feller, 2012). In the present study, isolates were producing extracellular enzymes such as esterase, protease, urease, amylase, etc. Similar study was done by Antony et al. (2012), showed that bacteria isolated from Antarctic ice core region are also producing cold adaptive enzymes. These extracellular or cold-adaptive enzymes can be an important source of new catalysts possessing useful enzymological characteristics, e.g., *Candida antarctica* produces lipase A and B. Because of its stereospecificity, Lipase B has been involved in food and diet industry, cosmetics and pharmaceuticals (Joseph et al., 2008).

Table 6. Physiological characteristic and presence of extracellular enzyme of bacteria isolated from Southern Ocean

Isolates	Gram characteristic	Growth temperature range/°C	NaCl tolerance/%	Amylase test	Esterase test	Lipase test	Catalase test	Urease test	Protease test
PR-MB-1	+ve cocci	4–37	4–13	+	+	+	+	++	–
PR-MB-3	+ve rod	4–37	4–13.5	+	+	+	+	++	–
PR-MB-4	–ve rod	4–37	4–13	+	+	–	+	+	–
PR-MB-6	–ve rod	4–37	4–13	–	–	+	+	+	++
PR-MB-7	–ve rod	4–37	4–13	–	–	–	–	+	–
PR-MB-8	–ve rod	4–37	4–10	+	+	+	+	+	–
PR-MB-9	+ve rod	4–37	4–13.5	+	+	+	+	+	–
PR-MB-10	+ve rod	4–37	4–13.5	–	–	+	+	+	–
PR-MB-11	+ve rod	4–37	4–12	–	+	+	+	+	–
PR-MB-12	–ve rod	4–37	4–13	–	+	–	–	+	–
PR-MB-14	+ve cocci	4–37	4–13	+	+	+	–	+	–
PR-MB-15	–ve rod	4–37	4–13	+	+	+	+	+	–
PR-MB-16	–ve rod	4–37	4–13	–	+	+	+	+	++
PR-MB-18	–ve rod	4–37	4–13.5	+	+	+	+	++	–
PR-MB-19	–ve rod	4–37	4–13	+	+	+	+	+	–
PR-MB-24	+ve rod	4–37	4–13	–	+	–	+	+	+++
PR-MB-26	+ve cocci	4–37	4–13	–	+	+	–	+	–
PR-MB-32	+ve rod	4–37	4–13	+	+	+	+	++	–
PR-MB-34	–ve rod	4–37	4–13	–	+	+	+	+	–
PR-MB-35	–ve rod	4–37	4-10	+	+	+	+	++	–
PR-MB-36	+ve rod	4–37	4–13	+	+	+	+	++	–
PR-MB-39	–ve rod	4–37	4-10	+	+	+	+	++	–
PR-MB-42	–ve rod	4–37	4–10	–	+	+	+	+	+
PR-MB-44	–ve rod	4–37	4–13	+	+	+	+	++	–
PR-MB-45	–ve rod	4–37	4–13	–	+	–	+	++	–
PR-MB-46	–ve rod	4–37	4–13.5	+	+	+	+	+	–
PR-MB-47	–ve rod	4–37	4–13	–	–	+	–	+	++
PR-MB-54	–ve rod	4–37	4–13	+	+	+	+	+	–
PR-MB-B	–ve rod	4–20	4–13	–	+	+	+	–	–

Notes: +ve represents Gram positive, –ve Gram negative, +++ strong activity, ++ moderate activity, + less activity, and – no activity.

5 Conclusions

The current study emphasizes on small portion of microbiota of Southern Ocean that were explored through culture-dependent method. Based on the 16S rDNA sequencing, most of the cultured bacteria belong to the uncultured bacteria that are not cultured and characterised earlier. Bacterial isolated from Southern Ocean belong to the *Marinobacter* sp., *Pseudoalteromonas espejiana*, *Halomonas hydrothermalis*, *Erythrobacter citrus*, *Zunongwangia profunda*, *Pseudoalteromonas tetraodonis* and uncultured bacteria. The capibility of extracellular enzyme and antibacterial compound production may lead to the way of commercialization in the marine isolates.

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