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RESEARCH ARTICLE

First Report on Marine Actinobacterial Diversity around Madras Atomic Power Station (MAPS), India

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ABSTRACT

Baseline assessments of marine microbial studies are very limited around ecologically sensitive areas of Nuclear Power Plant (NPP) site with respect to their occurrence, distribution, role in adaptation. Distribution and diversity of marine microbes are largely dependent on the physico-chemical parameters relating to specific area, especially spore producing marine actinobacteria are a source for different application. Marine actinobacterial diversity with conventional and 16S rRNA gene analysis was done around Madras Atomic Power Station. Totally, 60 different strains are identified in genera level and it's belongs to 10 genera with dominant by Streptomyces sp. (8 species) Nocardiopsis (8), Microbispora (7) and Rhodococcus (5). This is the first report on marine actinobacterial diversity and the results could be act as baseline inventory in terms of microbial diversity around NPP sites.

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Introduction

The marine environment is complex, dynamic and it's mainly characterized by the presence of saline and alkaline conditions that affect the way of life of organisms living in Sea. The environmental factors such as topography, water movement and stratification, temperature, pH, salinity, light availability, nutrients and sediment texture will be determined the composition of the biota around marine ecosystem (Karande, 1991). These marine areas are colonized with rich marine life owing to the nutrient sources which are necessary for the growth of the microbes that mediate the environmental factors, which of central importance in the ocean including microbes (ICMAM, 2002).

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Among all the marine microbes, the marine actinobacteria is unique and one of the most striking ecologically important group. These bacteria inhabit an extraordinary array of habitats, from those that offer an ideal condition for most living creatures to support most marine life forms. They found of relatively benign and nutrient-rich environments of oceans and also survive in extreme environments such as hot springs (Brock 1978), salt brines (Anton *et al.*, 2005), acid mine waters at pHs near zero (Baker and Banfield, 2003), deep in Antarctic ice (Christner *et al.*, 2001; Price, 2000) and kilometres below the Earth's surface (White *et al.*, 1995) coastal and mangrove environment (Sivakumar, 2005). Further, it could play an important role in nutrient cycle in marine environment by re-calcitrating the material, nitrogen fixation and breakdown of organic matter in to more readily assimilated nutrient (Jensen *et al.*, 1991).

Isolation and identification of actinobacteria has been difficult because of their slow growth as compared to other bacteria. When they were cultivated in nutrient rich media, some of them took double the normal time period; similarly few of them took half of time for their growth (Feller and Gerday, 2003). To obtain more and rare actinobacteria, a variety of pre-treatment techniques are used (Goodfellow and Haynes, 1984; Cho *et al.*, 1994; Kamala *et al.*, 2011&2013). Further, identification of actinobacteria also very difficult as compare with other bacteria. Yet, one characteristic alone would be inadequate especially in the identification of a genera as well as species (Burkholder et al., 1954; Krasilnikov, 1960). Application of molecular techniques has given us cutting edge knowledge for identification and phylogenetic determination of microorganisms (Edwards-Ingram *et al.*, 2004). In recent decades, application of rRNA gene sequence analysis facilitated to bring out some new order of this phylum in to the taxonomy (Edwards -Ingram *et al.*, 2004). However, marine microbial diversity around ecologically importance area is very limited. Hence, the present study was carried out to obtain baseline inventory of marine actionbacteria around MAPS.

Materials and Methods

Sampling Site

Marine sediment samples were collected around Madras Atomic Power Station, India. Five locations were selected around NPP site comprising of two different marine environment viz., inshore (intertidal) and offshore respectively. The offshore and inshore sediment samples were collected by Van Veen grab of 0.04 m^2 and corer in sterile polythene bags and transported to the laboratory.

Isolation Method

The sediment samples were dried in room temperature and grind aseptically with mortar and pestle. To reduce the contaminants and support the actinobacterial growth, several methods of pre-treatments have been proposed which includes dry heat, phenol, calcium carbonate, phenol with heat, and calcium carbonate and heat with calcium carbonate (Baskaran *et al.*, 2011; Kamala et al., 2011). After pre-treatment the samples were serially diluted with sterile sea water up to 10^{-4} to 10^{-6} dilution. One ml of diluted suspension was spread on Starch Casein Agar (SCA), Kuster's Agar (KUA) and Actinomycete Isolation Agar (AIA) without antibiotics supplement. The actinobacterial colonies were counted from 7thday onwards up to 28 days and the colonies were picked up and grown separately by streaking in petriplates.

Identification of Marine Actinobacteria

The colour of mature sporulation aerial mycelium, soluble pigment (Tresner et al., 1961), formation of melanoid pigment, colour of substrate mycelium or reverse side pigment and spore chain morphology (Shirling and Gottlieb, 1966) using Cell wall amino-acid (Cummins and Harris 1958) and whole cell sugar patterns (Lechevalier and Lechevalier, 1970) were studied. Molecular identification of marine actinobacteria was done and phylogenetic analysis and evolutionary relationship were done by MEGA 6 software (Tamura et al., 2011).

Results and Discussion

Isolation of Marine Actinobacteria

Physico chemical parameters of the coastal environment depend on the regional environmental condition such as rainfall, fresh water inflow, tidal movement and other biological activities (Satpathy *et al.*, 2010). Depending on human activities, consumption pattern and industrial area like power plant etc., water and sediment quality criteria have been specified to determine its suitability for particular purpose. Hence, in the present study, the analyse of different physico chemical parameters such as atmospheric and surface temperature, water and sediment pH, salinity, dissolved oxygen, nutrients in water like, nitrite, nitrate and inorganic phosphate, macronutrient *viz.*, nitrogen, phosphorous and potassium in sediment sample, organic carbon in sediment and sediment texture were performed. In addition, the actinobacterial population densities from various locations in different NPP sites were also studied. Actinobacterial populations in the estuarine and marine sediments vary in density with varying regions and even among sites within an ecosystem and actinobacteria are being reported from the marine sub habitats such as marine sediments (Ellaiah *et al.,* 2002; Okazaki, 2006) of almost all parts of the world. Thus, they have worldwide distribution which indicates their plasticity and adaptability to extremely varied environmental conditions. In the present study, marine actinobacterial population density of onshore and offshore sediment samples varied from 10^1 to 10^9 $CFUg^{-1}$.

Conventional and molecular Identification of marine actinobacteria from TAPS

A total of 60 marine actinobacterial strains were isolated from inshore and offshore sediment samples of MAPS which belonged to 8 genera *viz.*, *Streptomyces* spp. (23), *Nocardiopsis* sp. (17), *Micromonospora* sp. (7), *Nocardia* sp. (4), *Rhodococcus* sp. (4), *Saccharomonospora* sp. (2), *Actinopolyspora* sp. (2) and *Pseudonocardia* sp. (1). From onshore sediment sample twelve strains were isolated and it produce aerial mycelia were grey (5), yellow (3), pink (1), red (1), white (1) and cream (1) and that of substrate mycelia were yellow (4), red (2), brown (2) and green (1) colour respectively. Similarly, five isolates were produced melanoid pigment and soluble pigments, which were brown, yellow and red in colour (Table 1). All twelve isolates were identified as *Streptomyces* through cell wall aminoacid, sugar pattern and three types of spore chain morphology namely spiral (9), spiral with rectiflexibiles (2) and spiral with rectinaculiaperti (1). The phylogenetic analysis involved 17 nucleotide sequences. All twelve isolates belonged to *Streptomyces* sp. and the phylogentic tree had IV clusters; cluster I had 5 isolates with three reference

sequences (NR043504, AB184397 and AB184430) at 89% bootstrap level; cluster II had five isolates and reference strain NR115673 at 93% bootstrap level; One isolate M1S6 at 92% bootstrap level was in cluster III while nother isolate M1S2 had 73% bootstrap level was in cluster IV with reference strain (NR115673) (Fig.1).

Table 1. Morphological and cell wall analysis of marine actinobacteria from MAPS inshore sediment samples.

Isolates	A.m.color	M pigment	Spigment	Rpigment	Spore chain morphology	Cell wall aminoacids					Whole cell sugars					Cell wall type & sugar pattern	Index
						LL-A2P	DL-A2P	Glycine	Alanine	Lycine	Arabinose	Galactose	xylose	Mannose	Ribose		
M1S1	WY	0	1(Y)	0	SRF	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M1S2	P	1	1(R)	1(R)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M1S3	Gy	0	0	0	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M1S4	Gy	1	1(Br)	1 (Br)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M1S5	Gy R	0	0	0	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M1S6	WY	0	0	1 (Y)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M1S7	Gy	1	1(Br)	1 (br)	SRA	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M2S1	W	1	1(Br)	1(R)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M2S2	W gy	1	0	1(Y)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M2S3	Cr	0	0	1(Y)	SRF	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M2S4	Wy	0	0	1(Y)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.
M2S5	Gy	0	0	1(Gr)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	Streptomycessp.

Morphological analysis: 1 –Present; 0 – absent; W – white; R – red; Cr-cream; Br – brown; Y-yellow; P – pink; Blk – black; Or – orange; Iy- ivory; Gy – gray; Bl – blue; Gr – green; Bg – beige.

Spore chain morphology: RF –rectiflexibiles; RA – rectinaculiaperti; S – spiral; St- straight; Vs – Verticillate; RARF - rectinaculiaperti and rectiflexibiles; SRF - Spiral and rectiflexibiles; SRA - spiral and rectinaculiaperti.

Cell wall analysis: + Present; - Absent; DLA^{2}P- meso diaminopimelic acid; $+^m$ – minor amount was detected; N.C – Non characteristic.

A.m.color- Aerial mass color, Mel. Pigment- Melanine pigment, Sol. pigment- Soluble pigment, Rev. pigment- reverseside pigment.

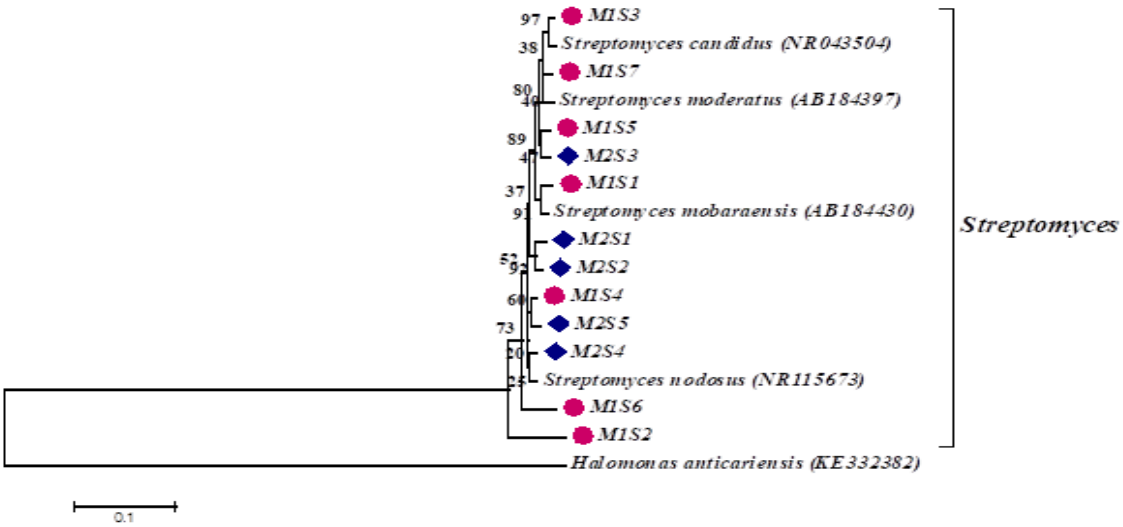

Fig. 1 Neighbor-joining dendrogram showing the phylogenetic relationship of 16S rDNA sequences from the stations M1 and M.

A total of 48 strains were isolated from the offshore sediment samples of MAPS sites. The colours of spore masses of the isolates were grey (13), white (9), red (5), yellow (7), ivory (21), orange (3), green (3), blue (2), pink (2), cream (1), brown (1) and beige (1) colour on aerial mycelia and yellow (10), orange (9), pink (3), red (6), brown (9), beige (3), ivory (1) and green (1) on substrate mycelia. However out of 48 isolates, fourteen isolates showed melanoid pigments and 11 isolates produced soluble pigments of red, brown, yellow, blue, grey and orange colour. All 48 isolates showed four (I, II, III & IV) of cell wall types and three (A, D & N.C) of sugar patterns (Table 2). The isolates belonged to eight genera *viz.*, *Streptomyces* (11), *Nocardiopsis* (7), *Micromonospora* (7), *Nocardia* (4), *Rhodococcus* (4), *Saccharomonospora* (2), *Actinopolyspora* (2) and *Pseudonocardia* (1). The evolutionary history was inferred

using the Neighbor-Joining method and the optimal tree with the sum of branch length was shown to be 1.7981 (Fig. 2). The phylogentic analysis involved 57 nucleotide sequences and there were a total of 818 nucleotide positions in the final dataset. The phylogentic tree had six clusters at 50 to 90% bootstrap level. Cluser I had *Nocardiopsis*, cluster II had *Streptomyces*, cluster III has *Psuedonocardia* and *Saccharomonospora*, cluster IV had *Actinopolyspora*, cluster V had *Nocardia* and *Rhodococcus* and cluster VI had *Micromonospora*.

Table 2. Morphological and cell wall analysis of marine actinobacteria from MAPS offshore sediment samples

Isolates	A.m.color	M pigment	S pigment	Rpigment	Spore chain morphology	Cell wall amino acids					Whole cell sugars					Cell wall type & sugar pattern	Index
						LL-A^2P	DL-A^2P	Glycine	Alanine	Lycine	Arabinose	Galactose	xylose	Mannose	Ribose		
M3S1	WGy	1	0	1(Y)	Mono spores	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M3S2	W	0	0	0	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M3S3	Gy	1	0	1(Or)	Mono spores	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M3S4	W	1	0	1(Y)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M3S5	R	1	0	1(P)	Short chains	-	+	-	-	-	+	+	-	-	-	IV (A)	*Nocardia* sp.
M3S6	Y	0	0	1 (or)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M3S7	Iy	1	1 (R)	1 (Blk R)	RA	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M3S8	WGy	0	1 (Y)	1 (Y)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M3S9	Or	0	0	1 (Or)	Mono spores	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M3S10	R	1	0	1(R)	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M3S11	GyY	0	0	1 (Y)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M3S12	Y	0	0	1 (Br)	SRA	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M3S13	W	0	0	1 (Br)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M4S1	W	0	0	1(Bg)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M4S2	Or	0	0	1 (Or)	Short rods	-	+	-	-	-	+	+	-	-	-	IV (A)	*Rhodococcus* sp.
M4S3	Gy	1	0	1 (Or Br)	RF	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M4S4	Gr	0	1 (Br)	0	Single spore	-	+	-	-	-	+	+	-	-	-	IV (A)	*Saccharomonospora*sp.
M4S5	Gy	0	0	1 (Y)	Short cocci	-	+	-	-	-	+	+	-	-	-	IV (A)	*Rhodococcus* sp.
M4S6	Y	1	0	1(Bg)	Short chain	-	+	-	-	-	+	+	-	-	-	IV (A)	*Nocardia* sp.
M4S7	Y R	0	0	1 (or)	Mono spore	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M4S8	Gy	0	0	0	S	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M4S9	W	0	0	1 (Bg)	Single spore	-	+	-	-	-	+	+	-	-	-	IV (A)	*Saccharomonospora*sp.
M4S10	Bl	0	0	1 (Or)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M4S11	Gy	0	0	0	RF	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M4S12	R	0	0	1 (Or)	Short chain	-	+	-	-	-	+	+	-	-	-	IV (A)	*Nocardia* sp.
M4S13	Gy	0	0	1(R)	Mono spore	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M4S14	R	0	0	1 (R)	Short cocci	-	+	-	-	-	+	+	-	-	-	IV (A)	*Rhodococcus* sp.
M4S15	P	1	1(Br)	1 (P)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M4S16	Gy	0	0	1 (Or Br)	Mono spore	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M4S17	W	1	0	1 (Y)	Short chain	-	+	-	-	-	+	+	-	-	-	IV (A)	*Nocardia* sp.
M4S18	Gy	1	0	1 (Br R)	Spiral	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M4S19	P	0	0	1 (OR)	Mono spore	-	+	-	+	-	+	-	+	+m	+m	II (D)	*Micromonospora*sp.
M5S1	W	0	0	1(Y)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S2	Gy	1	0	0	Long spore	-	+	-	-	-	+	+	-	-	-	IV (A)	*Actinopolyspora* sp.

M5S3	W bl	0	0	1(Br)	Zig zag	-	+	-	-	-	+	+	-	-	-	IV (A)	*Pseudonocardia* sp.
M5S4	Cr	0	1(Or)	1(Y)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S5	Gy	0	1 (Br)	1(Br)	Spiral	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M5S6	Y	0	0	1 (Iy)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S7	Gy	0	0	1 (Br)	SRA	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M5S8	W	0	0	1(Y)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S9	Y	0	0	1(Or)	RF	+	-	+	-	-	-	-	-	-	-	I (N.C)	*Streptomyces* sp.
M5S10	Gr	0	1(Bl)	1 (P)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S11	Br	1	1(red)	1(Br)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S12	Y	0	1(Gy)	1(Y)	Short cocci	-	+	-	-	-	+	+	-	-	-	IV (A)	*Rhodococcus* sp.
M5S13	Bg	0	1(Or)	0	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S14	Gr	0	0	1(Gr)	Long chain	-	+	-	-	-	+	+	-	-	-	IV (A)	*Actinopolyspora* sp.
M5S15	Or	1	0	1(R)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.
M5S16	W	0	1(Y)	1(Y br)	Long chain	-	+	-	-	-	-	+m	-	-	+m	III (N.C)	*Nocardiopsis* sp.

Morphological analysis: 1 –Present; 0 – absent; W – white; R – red; Cr-cream; Br – brown; Y-yellow; P – pink; Blk – black; Or – orange; Iy- ivory; Gy – gray; Bl – blue; Gr – green; Bg – beige.

Spore chain morphology: RF –rectiflexibiles; RA – rectinaculiaperti; S – spiral; St- straight; Vs – Verticillate; RARF - rectinaculiaperti and rectiflexibiles; SRF - Spiral and rectiflexibiles; SRA - spiral and rectinaculiaperti. Cell wall analysis: + Present; - Absent; DLA2P- meso diaminopimelic acid; +m – minor amount was detected; N.C – Non characteristic. A.m.color- Aerial mass color, Mel. Pigment- Melanin pigment, Sol. pigment- Soluble pigment, Rev. pigment-reverse side pigment.

Fig. 2 Neighbor-joining dendrogram showing the phylogenetic relationships of 16S rDNA sequences from the station M3, M4 and M5

Streptomyces are the dominant genera of actinobacteria in marine environment. From little Andaman and Nicobar group island, 32 and 52 actinobacterial strains were isolated and all of them are assigned to *Streptomyces*, respectively (Swarnakumar, 2010). In the present study *Streptomyces* was the dominant genus represented by a total of 98 strains which were isolated from inshore and offshore sediment samples. The previous study, 124 marine actinobacteria were isolated from the sediment samples collected from the intertidal zone in the Republic of Palau. These isolates are belonged to the family *Brevibacteriaceae*, *Corynebacterium*, *Dermacoccaceae*, *Dietziaceae*, *Geodermatophilaceae*, *Gordoniaceae*, *Intrasporangiaceae*, *Microbacteriaceae*, *Micrococcaceae*, *Micromonosporaceae*, *Mycobacteriaceae*, *Nocardiaceae*, *Nocardioidaceae*, *Nocardiopsaceae*, *romicromonosporaceae*, *Pseudonocardiaceae* and *Streptomycetaceae* (Gontang *et al*. 2007). Moreover, 300 isolates from six marine sediment samples collected from Gulf of Mexico and they belonged to the following genera *Actinomadura*, *Dietzia*, *Gordonia*, *Micromonospora*, *Nonomuraea*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinospora*, *Streptomyces*, *Solwaraspora* and *Verrucosispora* (Maldonado *et al*. 2008). Additionally, 64 isolates were identified from eight different marine sediment samples from Kerala and those were allocated to the genus of *Streptomyces*, *Glycomyces*, *Nocardiopsis*, *Nocardiodes*, *Actinopolyspora*, *Nocardia*, *Kibdelosporangium*, *Actinosynnema*, *Actinomadura*, *Thermoactinomycetes*, *Kineospora* and *Saccharopolyspora* (Remya and Vijayakumar, 2008). Furthermore, 20 actinobacterial strains which belonged to *Streptomyces* and *Nocardiopsis* were isolated from Mediterranean Sea (Oner et al., 2014). In the present study, *Streptomyces*, *Nocardiopsis*, *Rhodococcus*, *Nocardia* and *Saccharopolyspora* showed wide distribution in coastal environment especially in inshore sediments. Additionally, the predominant number of *Streptomyces* (38%) (Fig.3) is in agreement with earlier reported by Swarnakumar, 2010; Karthikeyan et al., 2014.

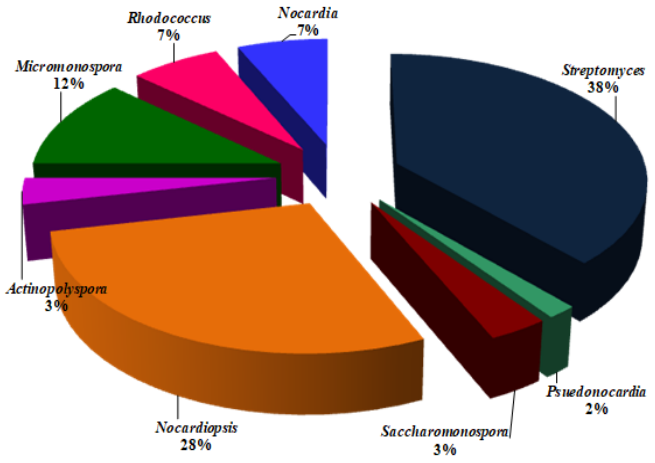

Fig. 3 Percentage composition of marine actinobacterial genera from MAPS

Conclusions

The baseline assessment of marine actinobacterial diversity were done around the proposed and running MAPS, India. This is a first kind of study around ecologically important area, besides, *Streptomyces* sp. *Nocardiopsis* sp., *Microbispora* and *Rhodococcus* kind of novel genus were isolated. These appear to be an indigenous part of microbial communities in the respective marine environments. This primary data will be useful in future ecological assessment and might be useful to analysis of diversity differ in future.

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