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BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF ACTINOMYCETES ISOLATED FROM MARINE SOIL SAMPLE OF KANYAKUMARI COAST

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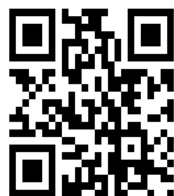


ABSTRACT

The main focus of this study was to isolate and identify the predominant actinomycetes from coastal area of Kanyakumari, Tamilnadu by cultural, microscopic, biochemical characterization and by 16S rDNA sequencing. About 52 actinomycete colonies were isolated by systematic serial dilution and plating technique and the size, shape and margin of the two selected predominant actinomycete colonies were observed on starch casein agar after 7 to 14 days of incubation. Based on experimental studies and genetic DNA sequence analysis, it was concluded that present strains A1 and A2 were identified as *Streptomyces* sp. and *Nocardiopsis dassonvillei* respectively and the 16s rRNA sequences of A1 and A2 were submitted to GenBank under accession numbers KU174216 and AB896798. The strains were submitted as *Streptomyces* sp. N56 and *Nocardiopsis dassonvillei* and the data are simultaneously made available to EMBL in Europe and the DNA Data Bank of Japan.

INTRODUCTION:

The morphological, physiological, ecological and molecular characteristics are the major characteristics used in taxonomy for the classification and identification of micro-organisms [1]. Actinomycetes form a distinct evolutionary line of organisms since they show marked chemical and morphological diversity [2]. They are gram positive, free living, saprophytic bacteria, which was characterized by the formation of substances and aerial mycelium on solid media, presence of spores and a high GC content of DNA (60-70 mol%) [3]. The application of new and reliable biochemical, chemical, genetical, numerical and molecular biology techniques have been responsible for rapidly changing views on how bacteria could be classified and identified [4,5]. Actinomycetes are highly valued for their unparalleled ability to produce biologically-active secondary metabolites and they perform significant biogeochemical roles in terrestrial soils. At least 4607 patents have been issued on actinomycete related products and processes [6] and there are approximately 32,500 natural products reported from microbial sources (Antibase data base) including about

1000 derived from marine microbes [7]. Many actinomycetes grow on the common bacteriological media used in the laboratory, such as nutrient agar, trypticase soy agar, blood agar, and even brain-heart infusion agar. Morphological characters of actinomycetes are still widely used for characterizing genera, for example, the presence or absence of spores on the substrate mycelium or the formation of zoospores in specialized spore vesicles or sporangia. The ability to produce motile spores is more wide spread in the actinomycetes. The colonies of actinomycetes have pastel colors, soil-like odor, hard and stick into agar [8].

The isolation and characterization of *Actinomycetes* were performed in different biochemical methods [9]. The tests generally used are starch hydrolysis, Triple Sugar Iron (TSI) agar test, citrate utilization test, indole test, methyl red test, vogus-proskauer (Acetone Production) test, Catalase test [10]. Physiological and biochemical characteristics were analysed as described by Thornley [11]; Barrow and Feltham [12]. Identification of actionmycetes to genus level was made possible in a fast and accurate manner. Even though, using only microscopic, cultural and biochemical techniques is not enough to ascertain the organism. To overcome the severe limitations of culture- dependent methods in discovering bacterial diversity [13, 14, 15, 16], molecular biological techniques have become increasingly popular [17]. Knowledge of the phylogenetic structure within the genus Actinomyces has improved in recent years because of the advent of 16s rRNA sequencing [18, 19, 20]. Comparative 16s rRNA sequence analysis has been applied to studies of natural microbial communities and has resulted in the discovery of unexpectedly high levels of biodiversity [21, 22, 13, 23, 24, 25]. The phylogenetic analysis of microbes further helps in selecting potential candidates for biotechnological applications. Hence, the present study was focused to identify the actinomycetes isolated from coastal area of Kanyakumari, Tamilnadu by cultural, microscopic, biochemical characterization and by 16S rDNA sequencing.

MATERIAL AND METHODS

Screening of actinomycetes from marine soil sample

The organisms which were predominantly present in the coastal area of Kanyakumari, Tamilnadu throughout the year withstanding the physicochemical variations were screened and used for further study. As the physicochemical parameter varies, the number and type of bacterial and fungal colonies were also varied but few actinomycetes colonies were isolated throughout the year. Hence this study was focused with actinomycetes. The predominant actinomycetes were separately streaked, subcultured, ensured for their axenicity and maintained in starch caesine agar slant (Himedia, Mumbai, India).

Cultural characterization

Colony characterization

The selected actinomycetes colonies were streaked on starch casein agar and incubated for 7 to 14 days. The isolated colonies were noted for their size, shape and margin.

Aerial mass color

To check the growth condition and aerial mass color of the selected isolates, the pure actinomycetes colonies were streaked on starch casein agar (Himedia, Mumbai, India), actinomyctes isolation agar (Himedia, Mumbai, India) and yeast extract agar (Himedia, Mumbai, India) and incubated at room temperature for 7 to 14 days. Filtered sea water was used for media preparation. After incubation, the aerial mass color was noted [26].

Pigmentation

The pure isolates were streaked on tryptone yeast extract agar (Himedia, Mumbai, India) and peptone yeast extract iron agar (Himedia, Mumbai, India) and incubated at room temperature for 7 to 14 days. After incubation, the color of mature sporulating aerial mycelium and reverse side pigment were observed and recorded [26]. The production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colours) was also noted [27].

Morphological characterization

Morphological observations were made by slide culture technique. One cm^2 starch casein agar blocks were cut from the petriplate and the actinomycetes isolates were inoculated individually on to the respective agar blocks. The inoculated agar blocks were placed on the center of the individual glass slides and covered with coverslips. The setup was placed inside a sterile petriplate containing moistened cotton and incubated at room temperature for 7 days. After incubation, the morphological observations were made with the light microscope. The slides were examined under microscope of 100X. Active purified isolates of actinomycetes were characterized by comparing their morphology of spore bearing hypae with the actinomycetes morphologies as described in Bergey's manual [28].

Biochemical characterization

The biochemical characterization of actinomycete colonies were analyzed following the directions given by Bergey's manual of systematic bacteriology [29].

Genotypic characterization

16s rDNA sequencing

Colonies were picked up with a sterilized toothpick from mother culture plate and suspended in 0.5 ml of sterilized saline in a 1.5 ml centrifuge tube. The samples were centrifuged at 10,000 rpm for 10 minutes. After removal of supernatant, the pellets were suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA) and incubated at 56ºC for 30 minutes and then heated at 100ºC for 10 minutes. After heating, supernatant was used for PCR amplification. PCR reactions were performed by adding 1µl of template DNA in 20 µl of PCR reaction solution. 27F/1492R primers was used, and then 35 amplification cycles were performed at 94ºC for 45 seconds, 55ºC for 60 seconds, and 72ºC for 60 seconds. Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore). Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

BLAST analysis: BLASTN (optimized for megablast) searches were manipulated with the sequences of isolated strains A1 and A2. The corresponding sequences of representative species were used for phylogenetic analyses.

Construction of phylogenetic tree

The Maximum parsimony (MP) tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm [30] with search level 0 in which the initial trees were obtained by the random addition of sequences (10 replicates). Evolutionary analyses were conducted in MEGA6 [31].

RESULT AND DISCUSSION

Screening of Actinomycetes from marine soil sample

Marine ecosystem covers almost 70% of the earth surface [32]. Soil is the most extensively studied ecological niche [33]. Since organisms present in these marine environments are extremely rich sources of bioactive compounds [34, 35, 36], attempts were made to isolate the native microbes present in the coastal area of Kanyakumari, Tamil Nadu [37]. The organisms which withstand the physicochemical variations throughout the year were screened and used for identification and other studies. The present study was focused on actinomycetes because as the physicochemical parameters of the marine site of Kanyakumari varies, the type and number of bacterial and fungal colonies were also varied but few actinomycetes colonies were found to persist throughout the year. About 52 actinomycete colonies were isolated by systematic serial dilution and plating technique from the marine soil samples collected from Kanyakumari coast. The isolates were small to larger in size, round, powdery with regular and irregular margins, grayish white to pure white in color. The reverse side pigmented colonies were also noted (plate 1). Based on the colony morphology and coloration which was observed on the master plate, two predominate colonies in the sampling site were selected for further studies.

Cultural characterization

The selected actinomycetes were designated as A1 and A2. The size, shape and margin of the two selected actinomycete colonies were observed on starch casein agar after 7 to 14 days of incubation (Plate 1). The size of the isolate A1 was about 5mm, round shaped with irregular margin. The isolate A2 was about 4mm size and the shape was round with regular margin. For the grouping and identification of actinomyctes color of the aerial mycelium is one of the prominent identification characters of *Streptomyces* isolates at species level [38]. The colors of the mature sporulating aerial mycelium are white, grey, red, green, blue and violet [39]. The International *Streptomyces* Project (ISP) [27] has recommended recording of aerial mycelia color in different media for use as a taxonomic character, which was also followed in the present study. In the present study, starch casein agar, actinomyctes isolation agar and yeast extract agar were used to check the growth condition and aerial mass color of the selected isolates. Das *et al*. [26] also recorded the mature sporulating aerial mycelium color on starch casein agar and yeast extract agar. According to Shirling and Gottileb [27], when the aerial mass color falls between two color series then both the colors are recorded. If aerial mass color of a strain showed intermediate color tints, then in that case also both the color series should be noted. The growth condition and aerial mass color of the isolates in three different media were given in table 1 and plate 2. The actinomycetes isolate A1 showed excellent growth and abundant aerial mycelium formation on starch casein agar. The strain A1 showed good growth and aerial mycelium on actinomycete isolation agar whereas only moderate growth was seen on yeast extract agar. The aerial mass color of the isolate A1 was white in all the three media checked. The isolate A2 showed good growth and white aerial mycelium on both starch casein and actinomycete isolation agar. In the yeast extract agar, excellent growth and abundant aerial mycelium were found. The aerial mass color of the strain A2 was grayish pink on yeast extract agar. Vanajakumar *et al*. [40] have also reported that white color series of actinomycetes were the dominant forms.

Pigmentation

The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive and not distinctive when tested using peptone yeast extract iron agar. A color with low chroma such as pale yellow, yellowish brown or olive occurs; these are included in the not distinctive group [26]. In accordance with the aerial mycelium color series established in the Bergey's manual of determinatives bacteriology [41] and in the category 4 of the Bergeys manual of systemic bacteriology, the isolates could be grouped in the following series: grey and white. The pigmentation of strains A1 and A2 on tryptone yeast extract agar and peptone yeast extract iron agar after the incubation period of one to two weeks was shown in table 2 and plate 3. The grouping was also made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colors) on the medium. The strains are grouped as melanoid pigment producing (+) and non producing (-) [27]. As per the instructions of Shirling and Gottileb [27] the melanoid pigment production was tested on tryptone yeast extract agar and peptone yeast extract iron agar. On the media tested, the strain A1 produced black and yellowish brown pigments on tryptone yeast extract agar and yellowish orange pigment on peptone yeast extract iron agar. The strain A2 produces soluble yellowish orange pigment on tryptone yeast extract agar and a pale yellow pigment on peptone yeast extract iron agar. Labeda [42] also studied the presence of diffusible pigments on agar plates in order to characterize the actinomycetes. Formation of melanin by actinomycetes was given a prime importance in identification of species specially *Streptomyces* species [43,44]. The production of melanoid pigment by strain A suggested that the strain belongs to the genus *Streptomyces.* The color of the aerial mycelium of A2 was white to pale pink

which depends on the type of media. The vegetative mycelium was almost colorless. Similar observation was made by Ali *et al.* [45] who has isolated strains with red to deep orange soluble pigments which were formed on some media especially Sato medium A, yeast extract-malt extract and oatmeal agar media. In the present study, the color of the reverse substrate mycelium was yellowish orange on tryptone yeast extract agar and melanoid pigments are not produced. As per the findings of Ali *et al.* [45], the strain A2 was suggested as it belongs to the genus *Nocardiopsis.*

Morphological characterization

Characteristics of the spore bearing hyphae and spore chains can be determined by light microscopy using cover slip culture [46, 47] and slide culture techniques [48]. In this study, the spores of A1 are smooth, appeared as long chain and oblong in shape which resembles the genera *Streptomyces* as described by Songara and Kaur [49] and Waksman and Henrici [50]. So the isolate A1 was identified as a representative of genus *Streptomyces*. The isolate A2 shows similar morphology to that of a *Nocardiopsis* isolate with a distinct substrate and aerial mycelium with conidial chains observed on microscopic examination. So the isolate A2 was identified as a representative of genus *Nocardiopsis* as per the findings of Vimal *et al*. [51] (Plate 4)

Biochemical Characterization

The result of biochemical tests for the isolates A1 and A2 were given in table 3. The gram staining result shows that the isolates A1 and A2 were gram positive. All the two isolates were found to be non motile and showed positive results for methyl red test, catalase and urease. The isolates were negative to indole production and vogas proskauer test. The isolate A1 was acid fast negative, failed to produce H_2S and showed negative result to triple sugar iron agar test. It reduces nitrate and utilize citrate. Meanwhile, the isolate A2 was acid fast positive, produces H_2S, showed positive result to triple sugar iron agar test and it failed to reduce nitrate and does not utilize citrate.

Genotypic characterization

Genomic DNA was isolated, purified, amplified and sequenced with help of Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). The obtained sequences for strains A1 and A2 were given in table 4. There are 1482 nucleotide bases for strain A1 and 992 bases for strain A2. Nucleotide sequences were compared to those in the GenBank database with the Basic local alignment search tool (BLAST) algorithm to identify known closely related sequences (Table 6).

BLAST analysis

Isolation of *Streptomyces* sp. ANU 6277 was reported from the laterite soil collected at the Acharya Nagarjuna University, Guntur [34]. *Streptomyces* sp. isolated from saline farmlands of Punjab and Pakistan has been also reported [33]. Using the BLAST search engine the NCBI data bank, sequences homologous to our isolate A1 were collected and subsequently aligned using Clustal W (DDBJ). A phylogenetic tree was constructed based on maximum parsimony. The BLAST search analysis revealed 96% similarity of the strain A1 with *Streptomyces clavuligerus* strain MTCC 7037 (EU146061.1) and 95% similarity with the isolate *Streptomyces clavuligerus* strain LCB69 (FJ867927.1). Based on molecular phylogeny the strain A1 was designated as *Streptomyces* sp. N56. The genus *Nocardiopsis* was described by Meyer [52] and Nocardia belongs to the family Thermonosporaceae is a non-streptomycete group of actinomycete. *Nocardiopsis* genus is an aerobic actinomycete that includes several species [53]. From Indian marine sediment samples, few potential bioactive *Nocardiapsis* have been reported previously. A protease-producing, crude oil degrading marine *Nocardiopsis* sp.NCIM 5124 has been reported by Dixit and Pant [54]. A biosurfactant producing marine actinobacteria, *Nocardiopsis alba* MSA 10 have been reported by Gandhimathi *et al.* [55] and the sponge associated actinomycetes, *Nocardiopsis dassonvillei* MAD08 having 100% activity against multidrug resistant pathogens have been reported by Selvin *et al.* [56].

Table 1 Growth condition and aerial mass color of isolates A1 and A2

S.no	Growth medium	Growth Condition		Aerial mass color	
		A1	A2	A1	A2
1	Starch casein agar	Abundant	Good	White	White
2	Actinomycetes isolation agar	Good	Good	White	White
3	Yeast extract agar	Moderate	Abundant	White	Grayish pink

Table 2 Pigment productions by strains A1 and A2 on tryptone yeast extract agar and peptone yeast extract agar

S.no	Growth medium	Reverse side pigment		Malanoid pigment		Soluble pigment	
		A1	A 2	A1	A 2	A1	A 2
1	Tryptone yeast extract agar	Yellowish brown	Yellowish orange	Present	Absent	Present	Absent
2	Peptone yeast extract iron agar	Yellowish orange	Pale yellow	Absent	Absent	Absent	Absent

Table 3 Biochemical characterization of strains A1 and A2

S.no	Biochemical test	Isolates	
		A1	A2
1	Gram staining	+	+
2	Motility	-	-
3	Acid fast staining	-	+
4	Indole production	-	-
5	Methyl red	+	+
6	Vogas proskauer	-	-
7	Catalase	+	+
8	H_2S production	-	+
9	Urease	+	+
10	Nitrate reduction	+	-
11	Triple sugar iron agar	-	+
12	Citrate utilization	+	-

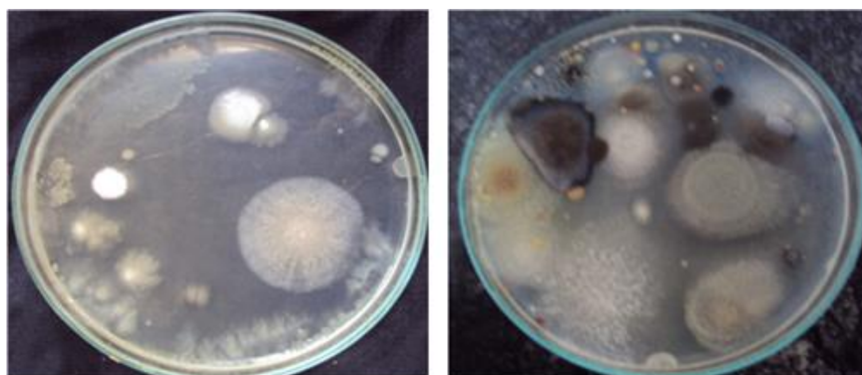


Plate 1 Isolation of actinomycetes from marine soil sample using actinomycetes isolation agar supplemented with 1% glycerol

Table 4 Sequences of strains A1 and A2 obtained by 16s rDNA sequencing

S.no	Strains	Sequences
1	A1	CGACGCGTCTATACATGCAGTCGAGCGAATCTGAGGGAGCTTGCTCCCAAAGA TTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGAT AACTCCGGGAAACCGGGGCTAATACCGGATAATATCTATTTATACATATAATT AGATTGAAAGATGGTTCTGCTATCACTTACAGATGGGCCCGCGGCGCATTAGC TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAG GGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT GAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTA CCGGAGTAACTGCCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAAC TACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTAT TGGGCGTAAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCACGG CTCAACCGTGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAA AGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCA GTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT AAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTC CGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCC CGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC CAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGACTTTCCCCTTCGGGGGA CAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG TTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTTGGG CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA AATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTAC AAAGGGCTGCAAGACCGCGAGGTTTAGCCAATCCCATAAAACCATTCTCAGTT CGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCG GATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA CACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTGGAGCCAG CCGCCTAAGGTGGGACAGATGATTGGGGTGAAGTCGTAACAGGGTTAACCCGT AA
2	A2	GGGCCGACGTTGTCGGCTTATTGGGCGTAAGAGCTCGTAGGCGGCGTGTCGCG TCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCTGCAGTGGATACGGGCATG CTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCA GATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACG CTGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA TGCCGTAAACGTTGGGCGCTAGGTGTGGGGACTTTCCACGGTTTCCGCGCCGT AGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTTGCTTAATTCGAC GCAACGCGAAGAACCTTACCAAGGTTTGACATCACCCGTGGACTCGCAGAGAT GTGAGGTCATTTAGTTGGCGGGTGACAGGTGGTGCATGGCTGTCGTCAGCTCG TGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTT GCCAGCACGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAG GAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAAC ATGCTACAATGGCCGGTACAATGGGCGTGCGATACCGTAAGGTGGAGCGAATC CCTAAAAGCCGGTCTCAGTTCGGAATTGGGGTCTGCAACTCGACCCCATGAAG GTGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGGTGAATACGTTCCCG GGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGCAACACCCGAAACTT GCGGCCTAACCCCTTGTGGGAGGGAGTGAATTGAAAGGTGGGGGCTTGGCAAT TGGGACGAATCTACGGGGGGGGGCGCCACAGAAAAAAGGT

Table.5 Results of similarity searches between 16S rRNA genes of A1, A2 and GenBank accessions using BLASTN Algorithm (optimized for megablast)

S.no	Isolates	Highest identical species	Accession number	Sequence identity (%)	E- value
1	A1	*Streptomyces clavuligerus* strain MTCC 7037	EU146061.1	96	0
		Streptomyces clavuligerus strain LCB69	FJ867927.1	95	0
		Streptomyces sp. LD48	AY641538.2	94	0
		Streptomyces sp. A515 Ydz-FQ	EU384279.1	94	0
		Streptomyces sp. ess_amH1	KF996505.1	95	0
		Streptomyces sp. mixed culture J3-44	KR029211.1	94	0
		Streptomyces indiaensis strain IF 5	FJ951435.1	94	0
		Streptomyces sp. VEL17 gene	AB914463.2	94	0
		Streptomyces sp. VEL27 gene	AB909959.1	94	0
2	A2	*Nocardiopsis dassonvillei* strain ADVJ1	KT781122.1	99	0
		Nocardiopsis dassonvillei subsp. dassonvillei strain y47	KF306364.1	99	0
		Nocardiopsis dassonvillei subsp. dassonvillei strain y18	KF306354.1	99	0
		Nocardiopsis dassonvillei subsp. dassonvillei strain y4	KF306353.1	99	0
		Nocardiopsis dassonvillei strain L2	JN862844.1	99	0
		Nocardiopsis dassonvillei strain HR10-5	JN253591.1	99	0
		Nocardiopsis dassonvillei subsp. dassonvillei strain D21	HQ132775.1	99	0
		Nocardiopsis dassonvillei strain HBUM49460	GQ163475.1	99	0
		Nocardiopsis dassonvillei subsp. albirubida strain VTT E-062983	EU430536.1	99	0
		Nocardiopsis dassonvillei subsp. albirubida strain VTT E-062983	EU430536.1	99	0

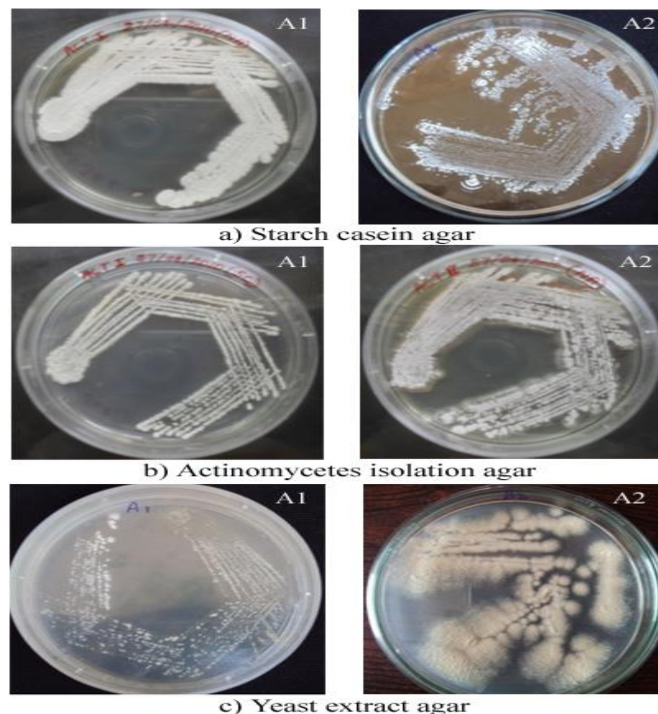
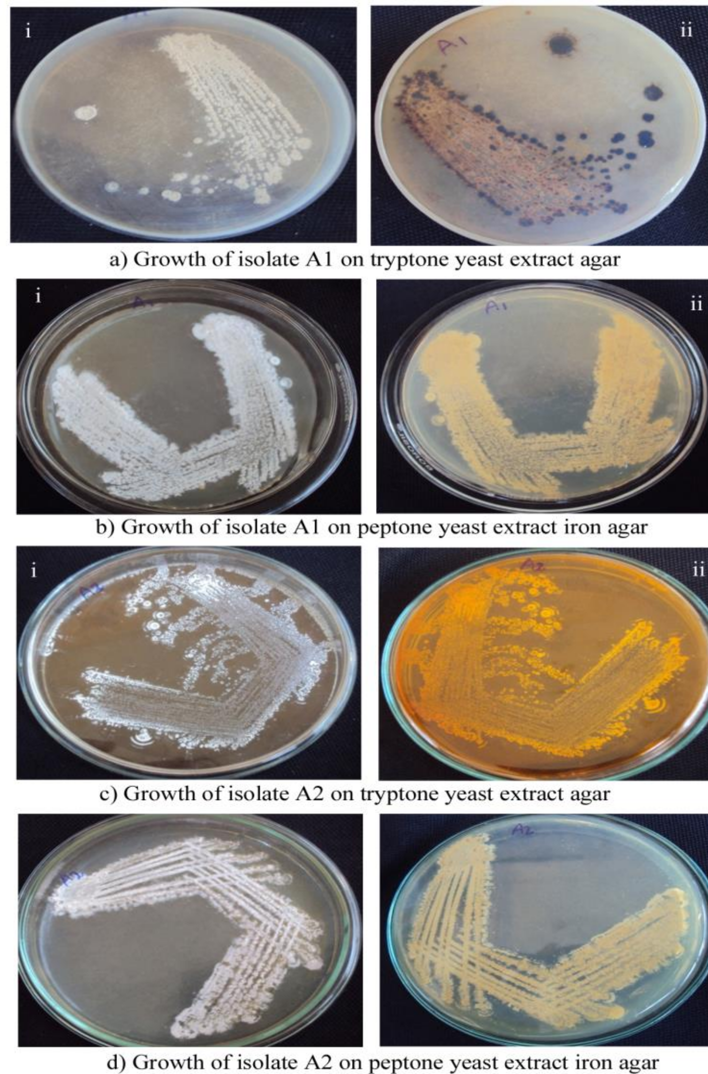


Plate 2 Growth condition and aerial mass color of isolates A1 and A2



a) Growth of isolate A1 on tryptone yeast extract agar

b) Growth of isolate A1 on peptone yeast extract iron agar

c) Growth of isolate A2 on tryptone yeast extract agar

d) Growth of isolate A2 on peptone yeast extract iron agar

Plate 3 i) Aerial mass color and ii) Reverse side pigmentation of strains A1 and A2 on tryptone yeast extract agar and peptone yeast extract iron agar

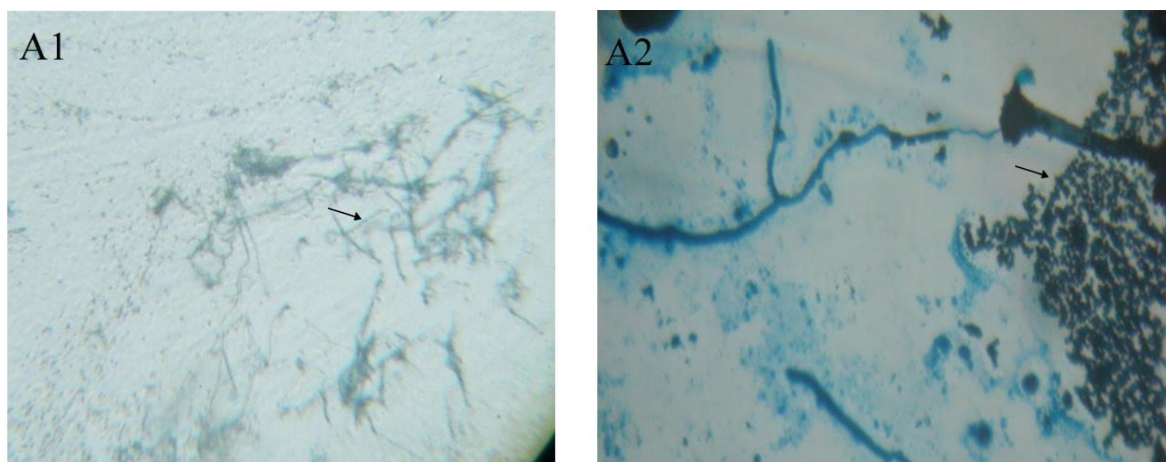


Plate 4 Spore chain morphology of strains A1 and A2

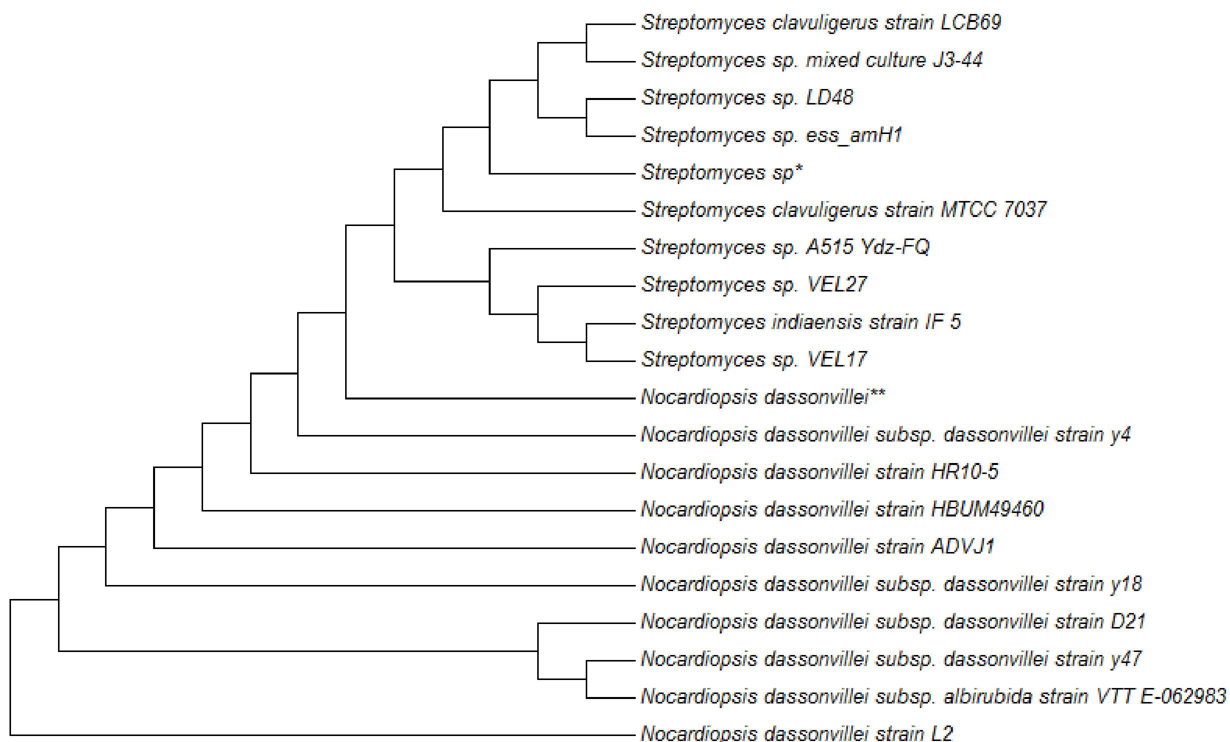


Figure. 1 Maximum Parsimony analysis of taxa (*isolate A1 and **isolate A2)

Isolation of *Nocardiopsis* sp have been reported in shore marine environment and mangrove ecosystem at 8 different locations of Kerala, West Coast of India by Remya and Vijayakumar [3]. In the present study, *Nocardiopsis dassonvillei* was one of the predominant organism isolated from the marine coast of Kanyakumari, Tamil Nadu and was confirmed by 16s rRNA sequencing. The 16S rRNA gene sequence of *Nocardiopsis dassonvillei* showed high similarity (99%) with 16S rRNA of *Nocardiopsis dassonvillei* strain L2 (KF306353.1). Therefore based on experimental studies and genetic DNA sequence analysis, it was concluded that present strains A1 and A2 were identified as *Streptomyces* sp. and *Nocardiopsis dassonvillei* respectively and the 16s rRNA sequences of A1 and A2 were submitted to GenBank under accession numbers KU174216 and AB896798. The strains were submitted as *Streptomyces* sp. N56 and *Nocardiopsis dassonvillei* and the data are simultaneously made available to EMBL in Europe and the DNA Data Bank of Japan. The results of similarity searches between 16S rRNA genes of A1, A2 and GenBank accessions using BLASTN Algorithm were given in table 5.

Construction of phylogenetic tree

In this study, 16S rRNA gene of different *Streptomyces* species and *Nocardiopsis dassonvillei* strains (different strains of a species) were obtained by BLASTN search, however 9 strains for each isolates were selected on the basis of high identity (%) with good E value for phylogenetic analysis. The evolutionary history was inferred using the Maximum Parsimony method. Tree #1 out of 2 most parsimonious trees (length = 184) was shown on Figure 1. The consistency index is 0.820652 (0.808140), the retention index is 0.964630 (0.964630), and the composite index is 0.791626 (0.779556) for all sites and parsimony-informative sites (in parentheses). The analysis involved 20 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 562 positions in the final dataset. As shown in Figure 1 the strains belonging to *Streptomyces* species were closely related to strain A1 (*Streptomyces* sp. N56) and all strains belonging to *Nocardiopsis*

dassonvillei were relatively closely related to strain A2 (*Nocardiopsis dassonvillei*). Microorganisms inhabiting the marine ecosystems are more diverse and unique with the ability to produce unique chemical entities. These ecosystems need to be extensively studied to gain a complete knowledge and unravel its unexhausted reserve of secondary metabolites. Our searches for actinomycetes lead to the identification of *Streptomyces* sp. N56 and *Nocardiopsis dassonvillei*. These two isolates can be used for further studies.

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