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MOLECULAR CHARACTERIZATION OF FRESH WATER ACTINOBACTERIA USING 16S RDNA BASED TECHNIQUE

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ABSTRACT

Twenty four isolates of actinomycetes were collected from water and sediment samples of three major fresh water systems of Karimnagar, Andhra Pradesh, India at Lower Manair Dam, Manakondur Pond and Kothapally pond from July 2006 to June 2008. These isolates were screened for their diversity and biological significance. Among them, eight showed good antagonistic activity against ten test bacteria. Two of these eight isolates (LAM1 and LAM2) were found to produce wide spectrum of antibacterial agents. These most potent strains were selected and were subjected to molecular identification. 16S rDNA was successfully amplified from the genome of the selected isolates with 8F and 1492R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic analyzer. 16S rDNA amplicons of the strains were subjected to automated DNA sequencing. Partial 16S rDNA sequences obtained was compared directly with sequences in the NCBI database using BLAST as well as with the sequences available with Ribosomal Database Project (RDP). The sequence analysis revealed that LAM1 belongs to *Streptomyces sp* and LAM2 belongs to *Micromonospora sp*.

KEY WORDS: **Fresh water, Actinomycetes, Primers, NCBI, BLAST.**

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INTRODUCTION

Actinomycetes are widely distributed in nature and have long been a source of commercially valuable enzymes and therapeutically useful bioactive metabolites[1]. Since molecular structure determine molecular function, and molecular diversity underpins the diversity of life on earth, it follows that identifying biological diversity increases the chances of identifying novel molecules. In the case of bacteria, identifying new species and genera increases identification of novel bioactive molecules produced by such organisms which are unknown to science[2].

The traditional methods used for the identification of the actinomycetes are laborious, time consuming and often require a series of specialized tests[3-5]. Chemical criteria, such as the isomer of diaminopimelic acid (DAP) present in the cell wall and the diagnostic sugar present in the whole cell hydrolysate, have been used to separate the actinomycetes genera into broad chemotaxonomic groups. However, determination of these characteristics is time consuming and, in most cases, cannot identify an isolate to a single genus[6].

With the invention of polymerase chain reaction and DNA sequencing methods, elucidation of closely related taxon with better authenticity has been made successfully in comparison to other conventional methods[7-9]. PCR based methods have provided a rapid and accurate way to identify bacteria [3-4, 10-14]. In particular, amplified ribosomal DNA has proved to be very useful [5, 15]. The 16S rDNA is the most conserved gene in all the cells. Portions of this gene sequence from distantly related organisms are remarkably similar[16]. This means that sequence from distantly related organism can be precisely aligned, making the true difference easy to measure. For this reason genes that encode rRNA have been extensively used to determine taxonomy, phylogeny and estimate the rate of species divergence among the bacteria. Thus comparison of 16S rDNA sequence shows evolutionary relatedness among microorganism[16]. A large number of rDNA sequences from a variety of organisms were aligned and the secondary structure was deduced. Phylogenetic predictions were then made using the distance methods[17]. On the basis of rDNA sequence signatures, it is predicted that early life diverged into three main kingdoms namely Archea, Bacteria and Eukarya. Evidence for the presence of additional organisms in these groups has since been found by PCR amplification of environmental samples of DNA [18-21].

The present work was carried out to describe the isolation of antibacterial actinomycetes from water and sediment samples collected from three fresh water systems of Karimnagar, Andhra Pradesh. The identification of these antibacterial actinomycetes, based on cultural, morphology, and biochemical characteristics, as well as 16S rDNA methodology, is reported.

MATERIALS AND METHODS

A total of 144 water and sediment samples were collected from three freshwater systems of Karimnagar, Andhra Pradesh, India viz; Lower Manair Dam, Manakondur Pond and Kothapally Pond regularly every month during July 2006 to June 2008. Twenty four actinomycete strains with distinct characteristics were isolated by Double Agar Layer(DAL) method using actinomycetes isolation agar containing cycloheximide (50µg/ml) to minimize fungal contamination [22].

Screening for antibacterial activity:

The isolated actinomycetes strains were tested for their antibacterial activity against ten test bacteria namely *Bacillus subtilis* (MTCC 441), *Proteus vulgaris* (MTCC 426), *Staphylococcus aureus* (MTCC 96), *Pseudomonas aeruginosa* (MTCC 424),

Enterobacter aerogenes (MTCC 111), *Salmonella typhi* (MTCC 733)*, Escherichia coli (*MTCC 40), *Sarcina lutea* (MTCC 1541), *Shigella flexneri* (MTCC 1457) *and Klebsiella pneumonia* (MTCC 7162). Among, eight isolates showed good antagonistic activity against test bacteria. Two of these eight isolates (LAM1 and LAM2) were showed very potent antagonistic activities which were selected and identified.

Taxonomic studies of actinomycete isolates:

Morphological characteristics of the most potent producer strains (LAM1 and LAM2) were examined under light microscope. Physiological and biochemical characteristics were monitored using standard methods. The cultural characteristics were studied in accordance with the guidelines established by the International *Streptomyces* project[23]. Colors were assessed on the scale adopted by Kornerup and Wanscher [24].

Molecular identification of LAM1 and LAM2:

DNA isolation:

The most potent antibacterial actinomycete strains (LAM1 and LAM2) were grown for 7 days on a starch nitrate agar slant at 30°C. Two ml of a spore suspension were inoculated into the starch nitrate broth and incubated for 3 days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (Pre-sporualtion). The preparation of total DNA was conducted as described by Sambrok *et al* [25]*.* The separated DNA quality is evaluated on 1.2% agarose gel.

Amplification and sequencing of the 16S r DNA gene:

Fragment of 16S rDNA gene of LAM1 and LAM2 was amplified by PCR from the above isolated DNA. The PCR amplicon of LAM1 and LAM2 were purified to remove contaminants.

Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

Sequence similarities and phylogenetic analysis:

The 16S rDNA gene sequence of LAM1 and LAM2 strains was used to carry out BLAST with nrdatabase of NCBI genbank data base. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

RESULTS

The isolated actinomycetes were screened with regard to their potential to generate bioactive compounds. The most potent producer strains LAM1 and LAM2 were selected and identified. The characterization of LAM1 and LAM2 was done by following the guide lines adopted by the International *Streptomyces* project[23]. Two of twenty four cultures were found to exhibit various degrees of activities against Gram-positive and Gram-negative bacteria (Table 1).

Table 1
Antibacterial activity of LAM1 and LAM2 strains.

Isolates	Test Organisms (inhibition zone in mm)									
	B.s.	S.a.	S.l	E.c.	K.p.	P.v.	P.a.	S.t.	S.f	E.a
LAM 1	20	18	17	12	13	15	15	17	9	14
LAM 2	15	12	13	12	11	7	13	5	8	7

B.s - Bacillus subtilis ***P.v-Proteus vulgaris***
S.a- Staphylococcus aureus ***P.a.-Pseudomonas aeruginosa***
S.l- Sarcina lutea ***S.t- Salmonella typhi***
E.c- Escherichia coli ***S.f- Shigella flexneri***
K.p- Klebsiella pneumonia ***E.a- Enterobacter aerogenes***

Cultural characteristics of actinomycete isolates

The most potent antagonistic actinomycetes strains were selected and characterized. The cultural characteristics of LAM1 showed that the aerial mycelium is white and substrate mycelium is also white and no diffusible pigments were observed. The cultural characteristics of LAM2 shows that the aerial mycelium is yellow and substrate mycelium is yellowish white and no diffusible pigments were observed. The morphological and biochemical characteristics of LAM1 and LAM2 were summarized in Table 2 which indicates that the LAM1 strain belongs to Streptomyces group and LAM2 belongs to Micromonospora group.

Table 2
Morphological and biochemical characteristics of LAM1 and LAM2 strains

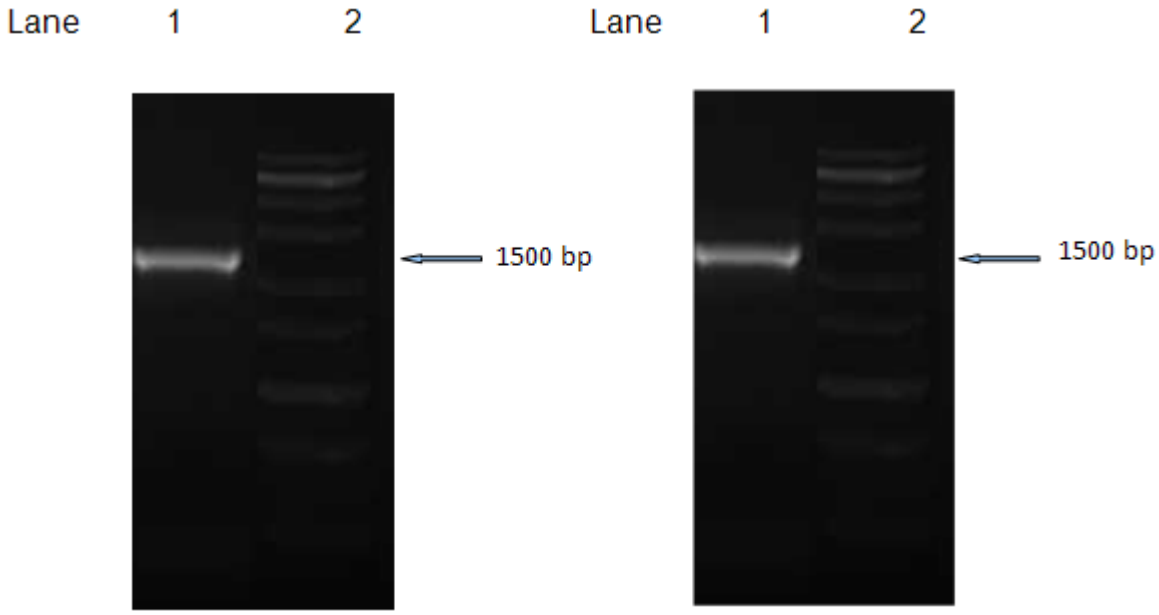
Characteristic	LAM1	LAM2
Morphological		
Aerial mycelium color	White	Yellowish white
Substrate mycelium color	White	Yellow
Colony diameter(mm)	3	4
Colony margin	Filamentous	Filamentous
Colony elevation	Flat	Convex
Spore chain	Spiral	Hook like
Spore surface	Warty	Smooth
Biochemical		
Indole production	-	-
Methyl red	-	-
Voges proskaur	-	-
Citrate utilization	+	+
H2S production	-	-
Nitrate reduction	-	-
Melanin production	+	+
Starch hydrolysis	+	+
Gelatin hydrolysis	+	+
Lipid hydrolysis	-	-
Casein hydrolysis	+	+
Carbon source utilization		
Starch	+	+

Dextrose	+	+
Fructose	+	+
Maltose	+	+
Nitrogen source Utilization		
D-Alanine	+	+
L-Arginine	+	+
L-Phenylalanine	+	+
L-Tyrosine	+	+

Molecular identification of LAM1 and LAM2

DNA was successfully isolated from LAM1 and LAM2 cultures. The quality of DNA was evaluated on 1.2 % Agarose gel, a single band of high-molecular weight DNA has been observed for both the cultures.

Fragment of 16S rDNA gene of LAM1 and LAM2 was amplified by using PCR and subjected to Agarose gel electrophoresis. Only single discrete PCR amplicon band of 1500 bp was observed for LAM1 and LAM2 DNA samples in Agarose gel (Fig. 1). The amplified DNA of LAM1 and LAM2 were subjected to purification to remove contaminants.



(Sample: LAM-1) **(Sample: LAM-2)**

Figure.1

Gel Image of 16SrDNA amplicons

Forward and reverse DNA sequencing reaction of PCR amplicon of LAM1 and LAM2 was successfully carried out with 8F and 1492R primers using BDT v3.1 cycle sequencing kit on ABI 3730xl Genetic analyzer. The consensus sequence of 1271bp 16S rDNA gene was generated for LAM1 and 1351bp 16S rDNA was generated for LAM2 from forward and reverse sequence data using aligner software (Fig. 2-7).

Figure.2

LAM-1_8F_4093_015_A01.ab1: Data obtained with Forward primer LAM-1_8F_4093_015_A01 (906 bp)

CAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCACCGGCCGCATGGTCTGGTGGTGGAAAGCTCCGGCGG
TGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACA
CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGG
GATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCGAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCCTGTCGCGTCGGATGTGAAAGCCCGGGGCTTA
ACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAGTTCGGCAGGGGAGATTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC
ACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGTTGGGCACTAGGTGTGGGCGGCATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCT
AAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACAT
CGGAAAACTCTGGAGACAGGGTCCCCCTTTGGGTCGGTGTACAGGTGG

Figure.3

LAM-1_1492R_4093_013_B01.ab1: Data obtained with Reverse primer LAM-1_1492R_4093_013_B01 (918 bp)

GACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCAATGCTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAG
GCCCCAATCCGAACTGAGACCGGCTTTTTGAGATTCGCTCCACCTCACGGCATCGCAGCTCATTGTACCGGCCATTGTAGCACGTGTGCAGCCCAA
GACATAAGGGGCATGATGACTTGACGTCGTCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCGTGAGTCCCCATCACCCCGAAAGGCATGCT
GGCAACACAGGATAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCACCTGTACACCGACC
CAAAGGGGGACCCTGTCTCCAGAGTTTTCCGATGTATGTCAAGCCTTGGTAAGGTTCTTCGCGTTGCGTCGAATTAAGCCACATGCTCCGCCGCTTG
TGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTGCGGCACGGACGACGTGGA
ATGCCGCCCACACCTAGTGCCCAACGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTAT
CGGCCCAGAGATCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAATTCCAATCTCCCCTGCCGAACTCTAG
CCTGCCCGTATCGAATGCAGACCCGGGGTTAAGCCCCGGGCTTTCACATCCGACGCGACAGGCCGCCTACGAGCTCTTTACGCCCAATAATTCCG
GACAACGCTCGCACCCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCT

Figure.4

Consensus Sequence LAM-1 (1271 bp)

CAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATATGACCACCGGCCGCATGGTCTGGTGGTGGAA
AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCGGC
CTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCGAGTGA
CGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTATTGG
GCGTAAAGAGCTCGTAGGCGGCCTGTCGCGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCT
AGAGTTCGGCAGGGGAGATTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATC
TCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGG
GCACTAGGTGTGGGCGGCATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGC
TAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGG
CTTGACATACATCGGAAAACTCTGGAGACAGGGTCCCCCTTTGGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTGTGTTGCCAGCATGCCTTTCGGGGTGATGGGGACTCACGG
GAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACA
ATGGCCGGTACAATGAGCTGCGATGCCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGCCTGCAACTCG
ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC

Figure.5

LAM-2_8F_4093_011_C01.ab1: Data obtained with Forward primer LAM-2_8F_4093_011_C01 (913 bp)

AGGCCCTTCGGGGTACTCGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCTAGGCTTTGGGATAACCCCGGGAAACCGGGGCTAATAC
CGAATAGGACCACCGGCTGCATGGCTGGTGGTGGAAAGTTTTTCGGCCTGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTAC
CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGAGAG
TGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAAGACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAG
AGCTCGTAGGCGGCTTGTCGCGTCGACTGTGAAAACCCGCGGCTCAACCGCGGGCCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGA
CTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGATACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGGGGGACCTCTCCGGTTTCCTGTGCCGCA
GCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGCGGAGCATGCG
GATTAATTCGATGCAACGCGAAGAACCTTACCTGAGTTTGACATGGCCGCAAAACCG

Figure.6
LAM-2_1492R_4093_009_D01.ab1: Data obtained with Reverse primer
LAM-2_1492R_4093_009_D01 (929 bp)

GTTGGGCCACCGGCTTCGGGTGTTGCCGACTTTCGTGACGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCTGATCT
GCGATTACTAGCGACTCCGACTTCACGGGGTCGAGTTGCAGACCCCGATCCGAACTGAGACCGGCTTTTTGGGATTCGCTCCACCTCGCGGTATCG
CAGCCCATTGTACCGGCCACTGTAGCATGCGTGAAGCCCTGGACATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCG
GCAGTCTTCGATGAGTCCCCGCCATAACGCGCTGGCAACATCGAACGAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCT
GACGACAGCCATGCACCACCTGTGACCGCCCCCGAAGGACCCGACATCTCTGCCGGTTTTGCGGCCATGTCAAACTCAGGTAAGGTTCTTCGCGTT
GCATCGAATTAATCCGCATGCTCCGCCGCTTGTGCGGGCCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGCGGCCGTACTCCCCAGGCGGGGCGCTT
AATGCGTTAGCTGCGGCACAGGAAACCGGAGAGGTCCCCCACACCTAGCGCCCAACGTTTACAGCGTGGACTACCAGGGTATCTAATCCTGTTCGCT
CCCCACGCTTTCGCTCCTCAGCGTCAGTATCGGCCCAGAGACCTGCCTTCGCCATCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAG
GAATTCCAGTCTCCCCTACCGAACTCTAGCCTGCCCGTATCGACTGCAGGCCCGCGGTTGAGCCGCGGGTTTTCACAGTCGACGCGACAAGCCGCC
TACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTCGCACCCTACGTCTTACCGCGGCT

Figure.7
Consensus Sequence LAM-2 (1351 bp)

AGGCCCTTCGGGGTACTCGAGCGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCTAGGCTTTGGGATAACCCCGGGAAACCGGGGCTAATAC
CGAATAGGACCACCGGCTGCATGGCTGGTGGTGGAAAGTTTTTCGGCCTGGGATGGGCTCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTAC
CAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA
TATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGAGAG
TGACGGTACCTGCAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAAGACGTAGGGTGCGAGCGTTGTCCGGAATTATTGGGCGTAAAG
AGCTCGTAGGCGGCTTGTCGCGTCGACTGTGAAAACCCGCGGCTCAACCGCGGGCCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGA
CTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGATACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGGGGGACCTCTCCGGTTTCCTGTGCCGCA
GCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGCGGAGCATGCG
GATTAATTCGATGCAACGCGAAGAACCTTACCTGAGTTTGACATGGCCGCAAAACCGGCAGAGATGTCGGGTCCTTCGGGGGCGGTCACAGGTGGT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCGATGTTGCCAGCGCGTTATGGCGGGGAC
TCATCGAAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACGCATGCTACAGTGGC
CGGTACAATGGGCTGCGATACCGCGAGGTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGG
AGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGCAACACCCGA
AGCCGGTGGCCCAACC

16S rDNA gene sequence of LAM1 and LAM2 obtained were matched with previously published bacterial 16S rDNA sequences available in NCBI using BLAST and the Ribosomal Database Project (RDP).

Comparison of the sequence of strain LAM1 with the corresponding sequences of representative strains showed that this organism formed a distinct phyletic line with a clade encompassed by *Streptomyces sp. 172606* (GenBank Accession Number: HQ992711.1) (Figure 8). Information about the other close homologs for LAM1 is indicated in alignment view table (Table.3)

Table 3
Sequence Producing Significant Alignments (LAM1)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ992711.1	*Streptomyces sp. 172606*	2348	2348	100%	0.0	100%
JN182145.1	*Streptomyces sp. FXJ6.306*	2337	2337	100%	0.0	99%
JF346529.1	*Streptomyces sp. FXJ7.385*	2337	2337	100%	0.0	99%
GQ163473.1	*Streptomyces violaceoruber strain HBUM49432*	2337	2337	100%	0.0	99%
EU119189.1	*Streptomyces sp. HBUM79010*	2337	2337	100%	0.0	99%
AB184183.1	*Streptomyces cacaoi subsp. cacaoi*	2337	2337	100%	0.0	99%
NR_041061.1	*Streptomyces cacaoi subsp. cacaoi strain NBRC 12748*	2337	2337	100%	0.0	99%
JN182149.1	*Streptomyces sp. FXJ6.323*	2331	2331	100%	0.0	99%

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
HQ610449.1	*Streptomyces cacaoi strain GY525*	2331	2331	100%	0.0	99%
GU144523.1	*Streptomyces cacaoi subsp. cacaoi strain W9609*	2331	2331	100%	0.0	99%

After comparison of LAM2 sequence with the corresponding sequences of representative strains showed that this organism formed a distinct phyletic line with *Micromonospora sp. MBRL 34* (GenBank Accession Number: JN560152.1) (Figure 9). Information about other close homologs for LAM2 is indicated in alignment view table (Table.4).

Table 4
Sequence Producing Significant Alignments (LAM2)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JN560152.1	*Micromonospora sp. MBRL 34*	2495	2495	100%	0.0	100%
JN989296.1	*Micromonospora sp. S2909*	2410	2410	100%	0.0	99%
FR692085.1	*Micromonospora sp. BK65*	2399	2399	100%	0.0	99%
EU214959.1	*Micromonsopora sp. CNS-628_SD06*	2399	2399	100%	0.0	99%
FR692083.1	*Micromonospora sp. BK61*	2394	2394	100%	0.0	99%
GQ163463.1	*Micromonospora sagamiensis strain HBUM49500*	2394	2394	100%	0.0	99%
GQ163462.1	*Micromonospora sagamiensis strain HBUM49490*	2394	2394	100%	0.0	99%
FJ481620.1	*Micromonospora echinaurantiaca strain HBUM49493*	2394	2394	100%	0.0	99%
FJ481617.1	*Micromonospora echinaurantiaca strain HBUM49505*	2394	2394	100%	0.0	99%
FJ481614.1	*Micromonospora echinaurantiaca strain HBUM49497*	2394	2394	100%	0.0	99%

Phylogenetic Tree: (LAM1)

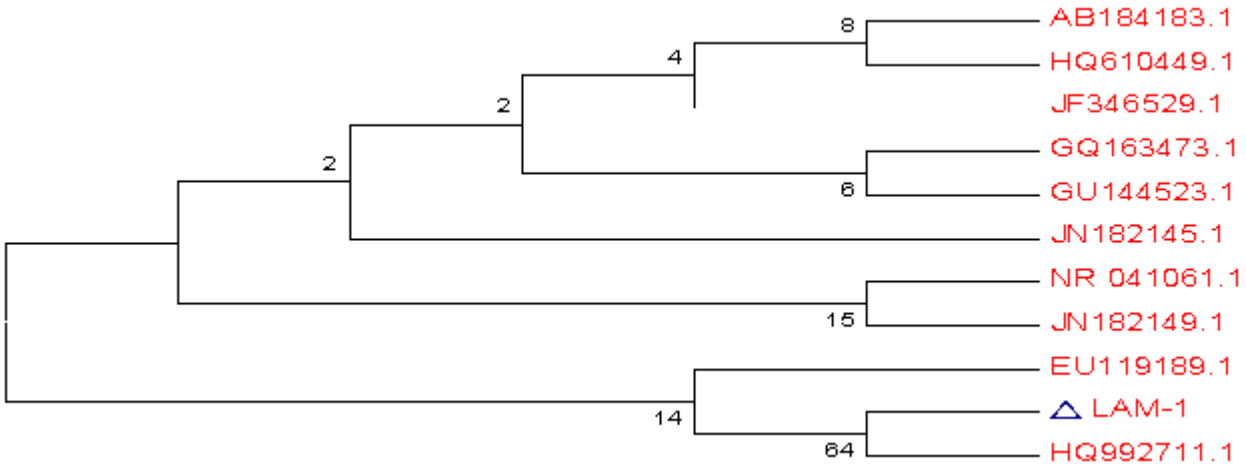


Figure.8
Phylogenetic tree showing evolutionary relationship of LAM1 with other members of Streptomyces along with their evolutionary distance.

Phylogenetic Tree (LAM2)

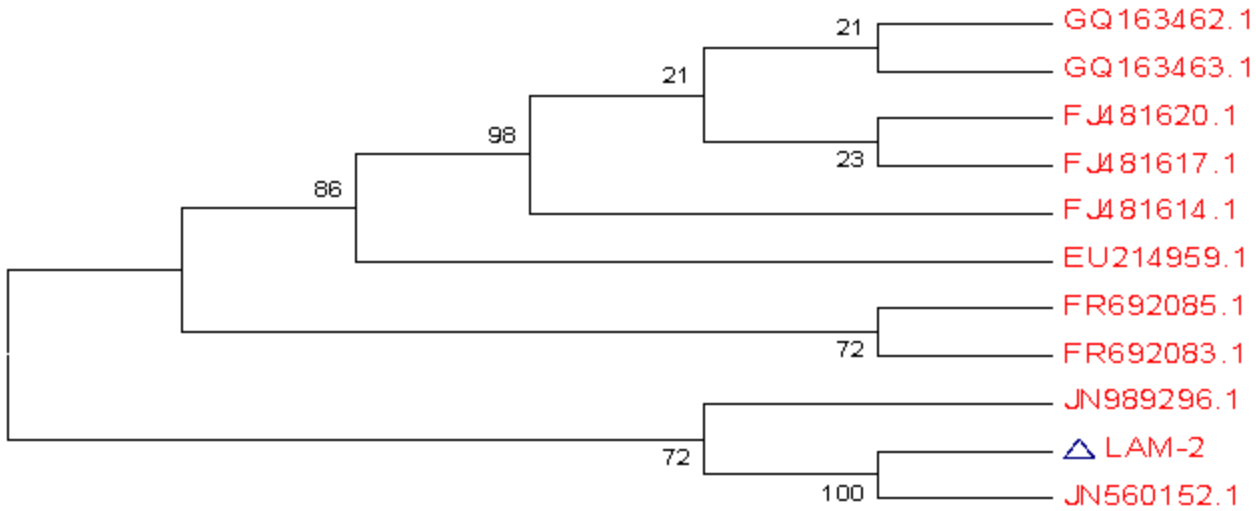


Figure.9
Phylogenetic tree showing evolutionary relationship of LAM2 with other members of Micromonospora along with their evolutionary distance

DISCUSSION

The natural habitat of most of actinomycetes is the soil but can also be found in aquatic environments[26-28]. The diversity of fresh water associated actinomycetes has been extraordinarily significant in several areas of science and medicine. The fresh water systems represent an underexplored environment for actinomycetes discovery. The sequence of 16S rDNA has been widely used to identify an unknown bacterium up to genus or species level[29]. Advances have been made in automating and minimizing the detection times using biochemical methods, however, biochemical identification is not accurate for determining the genotypic differences of microorganisms. A more accurate method for

genotype determination is that of molecular biological approach of ribotyping by comparing the similarities in 16S rDNA sequences. Molecular identification provides two primary advantages to phenotypic identification, a more rapid turn around time and improved accuracy in identification[30-31]. A whole array of taxonomic tools has been used to identify genera and supra generic groups of actinomycetes, but partial sequence analysis of 16S ribosomal DNA is the most significant [32-33]. Information obtained using molecular techniques is very useful in that it provides researchers with a powerful and independent data set in which hypothesis generated from other data, such as morphology and physiology, can be tested [34].

In a routine antibiotic screening strategy, the strains showing interesting activity or producing interesting metabolites in preparative screening are usually identified by 16S rDNA gene sequencing. The morphological, biochemical and physiological characterization of LAM1 and LAM2 strongly suggested that they are the members of *Streptomyces* and *Micromonospora* respectively.

In ribotyping studies, LAM1 exhibited genetic similarity with different sps of the genus *Streptomyces* and LAM2 exhibited similarity with different sps of *Micromonospora.* In most of the cases in this ribotyping study, the 16S rDNA up to 1271bp was obtained for LAM1 and 1377bp was obtained for LAM2. The isolates LAM1 and LAM2 exhibited 100% similarity and homology with *Streptomyces* sp172606 and *Micromonospora* sp. MBRL 34 respectively.

CONCLUSION

Sequence comparisons of specific genes were applied successfully to investigate the relationship among actinomycetes members on the basis of their nature of product and other characteristics such as antibiotic producers and pathogenic actinomycetes. It was shown that molecular weight and activity profile study of antibacterial compounds produced by actinomycetes is not sufficient for their identification.. Thus gene sequence level comparison was employed in this study. BLAST results revealed higher sequence similarity of LAM1 with 16S rDNA partial gene sequence of *Streptomyces* sps172606 and LAM2 with *Micromonospora* sps.MBRL*34*. Results of multiple sequence alignment analysis using CLUSTALW showed higher level of nucleotide conservation of LAM1 with *Streptomyces sp.172606* and LAM2 with *Micromonospora sp. MBRL.* This is further confirmed through evolutionary tree study using neighbor joining distance method of phylogenetic analysis. The present findings indicate that the fresh water systems harbor a diverse population of potent actinomycetes. The isolation, characterization and the study on fresh water actinomycetes can be useful in discovery of antibiotics and also confirmed by molecular characterization.

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