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Characterization and antimicrobial potential of soil actinobacterium TFA1 isolated from Talakona forest, Andhra Pradesh

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ABSTRACT

The present study reports the characterization and antimicrobial potential of actinobacterial strain TFA1 isolated from Thalakona forest soil, Andhra Pradesh. Phenotypic, cell wall and molecular characteristics of strain TFA1 was studied by adopting standard procedures. Strain TFA1 produced powdery colonies on ISP2 agar medium with aerial and substrate mycelium. Cell wall analysis revealed the presence of LL- DAP and glycine but no sugar. PCR amplification of 16S rRNA gene of strain TFA1 yielded 1014bp sequence. During 16S rRNA sequence analysis, strain TFA1 (accession no KP893923) shows 99% similarity to *Streptomyces kurssanovii* NBRC 13192. In preliminary agar plug method, strain TFA1 showed activity against *S. aureus*, *B. cereus* and *E. coli*. Strain TFA1 produced bioactive metabolites in solid media on the 3rd day of fermentation whereas in liquid media the production of bioactive metabolite was observed on 5th day of fermentation. The ethyl acetate extract of strain TFA1 showed 14-18 mm inhibition against Gram positive and Gram negative bacterial pathogens.

INTRODUCTION

Emergence of drug resistance among common microbial pathogens is stern problem that accentuate the development of novel antimicrobials. Microbial resources from unexplored niches made inevitable contribution for antibiotic development process to combat the problem of antibiotic resistance (Harvey *et al.*, 2000). Among the microbial sources, actinobacteria are the most important source of bioactive metabolites and more novel molecules are still on the row to discover from this group of bacteria (Balagurunathan *et al.*, 2010). In particular, diverse actinobacteria from poorly studied unusual environments promises a raise in the prospect of discovering novel compounds (Jose *et al.*, 2013; Yuan *et al.*, 2014).Forest ecosystems are more complex and heterogeneous environments and they are characterized by a wealthy biological diversity (Lami *et al.*, 1993). Actinobacteria from forest ecosystems including from Western Ghats and Eastern Ghats of India are isolated and investigated for antimicrobial (Radhakrishnan *et al.*, 2014a) and enzymatic activities (Mohanapriya *et al.*, 2011). The present study reports the phenotypic and molecular characterization of actinobacterial strain TFA1 isolated from Talakona forest, Andhra Pradesh and its antimicrobial potential.

MATERIALS AND METHODS

Description of actinobacterial strain TFA1

Actinobacterial strain TFA1 (Figure 1) was isolated from the soil sample collected from Talakona forest (Estern Ghats), Andhra Pradesh **(**Lat. 13° 48′ 42″ N; Long. 79° 12′ 56″ E) using starch casein agar supplemented in the year 2013. Viability of strain TFA1 was maintained on ISP2 agar slants as well as in 30% glycerol broth at -20^{0}C.

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Fig. 1: Growth of actinobacterial strain TFA1 on ISP2 agar

Characterization of actinobacterial strain TFA1

Phenotypic characterization

The micro morphologicalstructures such as the aerial mycelium, substrate mycelium, mycelial fragmentation were observed under bright field microscope at 40X magnification (Balagurunathan *et al.*, 2010). The growth characteristics of strain TFA1 on different ISP (International Streptomyces Project) medium were studied ontryptone agar (ISP1), yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerolasparagine agar (ISP5), peptone yeast extract-iron agar (ISP6) and tyrosine agar (ISP7) (Shirling and Gottileb, 1966). Effect of different carbon sources (sugars such as glucose, fructose, sucrose, mannitol, inositol, xylose, rhamnose, raffinose, and cellulose) and nitrogen sources (amino acids such as tyrosine, asparagines and glutamine) were studied on the growth of strain TFA1 by adopting standard procedures (Mohanraj *et al.*, 2011). Effect of pH (5, 7, 9 & 11), temperature (20°C, 30°C, 40°C and 50°C) on the growth of strain TFA1 was studied using ISP2 agar medium (Radhakrishnan *et al.*, 2013). Isomers of diaminopimelic acid (DAP) and sugars in whole-cell hydrolysates of strain TFA1 were analyzed by adopting the method described by Hasegawa *et al.*(1983).

Molecular characterization

The genomic DNA of actinobacterial strain TFA1 was isolated using the InstaGeneTM Matrix Genomic DNA isolation kit. Mycelial growth of actinobacterial strain was picked from ISP2 agar plates and suspended in 1ml of sterile water in a microfuge tube. The content was centrifuged for 1 minute at 10,000 rpm to remove the supernatant and the cells were pelleted. Then 200 µl of Insta Gene matrix was added to the pellet and incubate at 56 °C for 15 minutes and vortexed at high speed for 10s (Delalibera *et al.*, 2007). The mixture was heated in a boiling water bath for 10 minutes and then centrifuged at high speed to separate the matrix. Twenty microliters of the resulting DNA was used in a 50-µl PCR reaction to construct a 16S rRNAgene library. General primers for bacteria 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') were used to amplify 16S rRNA genes from DNA extracted from the actinobacterial strain TFA1. Final concentrations for 50-µl PCR reactions were as follows: 2 µl diluted DNA (10–100 ng) (or 20 µl for DNA extracted using InstaGeneTM Matrix), 0.2 µM of each primer, 0.2 mM dNTPs, 5 units of *Taq* polymerase and 1X *Taq* polymerase buffer. The reaction conditions were: 94 ^{o}C for 3 min, 35 cycles at 94 ^{o}C for 30 s, 55 ^{o}C for 1.5 min, and 72 ^{o}C for 2.5 min, and a final extension at 72^{o} C for 5 min. By using Montage PCR clean up kit (Millipore), the unincorporated PCR primers and dNTPs have been removed. Single-pass sequencing was performed on each template using 518F/800R primers (518F 5'-CCAGCAGCCGCGGTAATACG-3' and 800R 5'- TACCAGGGTATCTAATCC-3'). Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (Applied Biosystems).

The 16S rRNA sequence of actinobacterial strains were subjected to BLAST similarity search tool. By comparing the sequence similarity with non redundant database of nucleotide sequences deposited at NCBI (http://www.ncbi.nlm.nih.gov/) through BLAST program, closely related homologs were identified. The program MUSCLE 3.7 was used for to process the multiple alignments of sequences. The stability of relationship was admittance by performing bootstrap analysis for 1000 replicates. Divergence times for all branching points in the topology were calculated with the Real Time method using the branch lengths contained in the inferred tree. Bars around each node represent 95% confidence intervals were computed (Tamura *et al.*, 2011). All positions containing gaps and missing data were eliminated. The evolutionary history was inferred by using the Neighbor-Joining method in the MEGA 6 software. Identification of species through sequence similarity between query sequence and reference sequence based on criteria, for 99% or above similarity assigned to reference species, 99-95% similarity assigned to corresponding genus, less than 95%, assigned to corresponding family (Bosshard *et al.*, 2003). The 16S rRNA sequence of potential actinobacterial cultures were submitted to Genbank to get the accession number.

Antimicrobial activity of actinobacterial strain TFA1

Antimicrobial activity of strain TFA1 was studied by adopting agar plug method against *Staphylococcus aureus, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa* and *Candida albicans* (Radhakrishnan *et al.*, 2014b). Effect of solid and liquid medium on bioactive metabolite production from the strain TFA1 was studied using ISP2 medium. Antimicrobial activity was tested for every 24 hours against *S. aureus*. Crude bioactive metabolite from the agar medium was extracted using ethyl acetate and tested for antimicrobial activity by adopting disc diffusion method (Radhakrishnan *et al.*, 2014b).

RESULTS AND DISCUSSION

Phenotypic characteristics of strain TFA1

The characterization of a strain is a key element in systematics of prokaryotes including actinobacteria. Although

various new methodologies have been developed over the past 100 years, both the newer methodologies and those considered to be traditional remain key elements in determining whether the strain belong to known taxon or constitute a novel one (Tindall *et al.*, 2010). Actinobacterial classification was originally based largely on morphological observations and physiological characteristics (Shirling and Gottileb, 1966; Nonomura, 1974). The advent of chemotaxonomic criteria has provided reproducible and reliable tools to identify the genera at genus level (Labeda, 1990). In the present study, strain TFA1 shows good growth on all ISP medium except in ISP1 medium. Under bright field microscopic observation, strain TFA1 showed the presence of rectus flexible type aerial mycelium and non fragmented substrate mycelium (Figure 2).

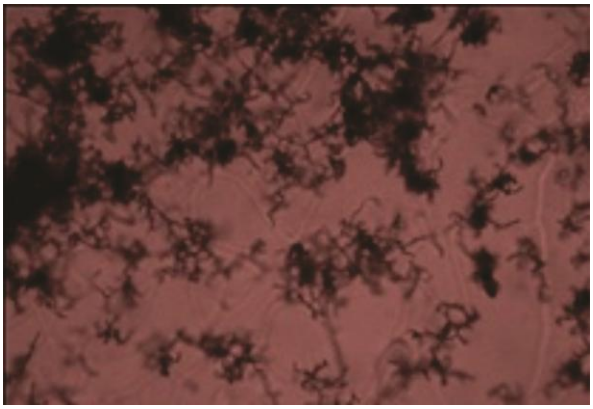

Fig. 2: Micromorphology of actinobacterial strain TFA1 undeerbright field microscope.

Strain TFA1 showed good growth on all the ISP media tested except ISP1 and ISP3 and it also utilized wide range of sugars, pH 7 & 9 and temperature 30˚C (Table 1). The whole cellhydrolysate of isolate TFA1 were rich diaminopimelic acid (DAP), with no characteristic sugar indicates chemotype I cell wall.The results of phenotypic characterisation revealed that the actinobacterial strain TFA1 belongs to a species of the genus Streptomyces. But it is not adequate in itself to differentiate between genera.

Table 1: Phenotypic characteristics of actinobacterial strain TFA1.

Characteristics	Strain TFA1
Micromorphology	
Aerial mycelium	Present
Substrate mycelium	Present
Fragmentation	Non-fragmented
Number of spores	30-40
Cultural characteristics	
Aerial mycelialcolour	Brown
Reverseside pigment	Absent
Diffusible pigment	Absent
ISP1 (Tryptone agar)	Moderate
ISP2 (Yeast extract malt extract agar)	Good
ISP3 (Oatmeal agar)	Good
ISP4 (Inorganic salts- starch agar)	Good
ISP5 (Glycerol aspargine agar)	Good
ISP6 (Peptone Yeast extract ironagar)	Good
ISP7 (Tyrosine agar)	Good
Utilization of	
Glucose	Good
Arabinose	Good
Sucrose	Good
Xylose	Moderate
Inositol	Good
Mannitol	Good
Fructose	Moderate
Rhamnose	Moderate
Raffinose	Moderate
Cellulose	Good
Growth at	
pH 5	Moderate
pH 7	Good
pH 9	Good
pH 11	Poor
20˚C	Poor
30˚C	Good
40˚C	Moderate
50˚C	No growth

Molecular characterization of strain TFA1

The analysis of 16S rRNA gene sequence has also revealed that the strain TFA1 belongs to a species of the genus *Streptomyces*. The PCR amplification the 16S rRNA gene of actinobacterial strain TFA1 yielded 1014 base pairs. During BLAST analysis the 16S rRNA gene sequence of strain TFA1 showed 99% similarity to the gene sequence of *Streptomyces kurssanovii* NBRC 13192 published in GenBank. The 16S rRNA gene sequence of strain TFA1 was published in GenBank with the accession no KP893923.

The phylogenetic relatedness of strain TFA1 with its closely related species was given in figure 3.

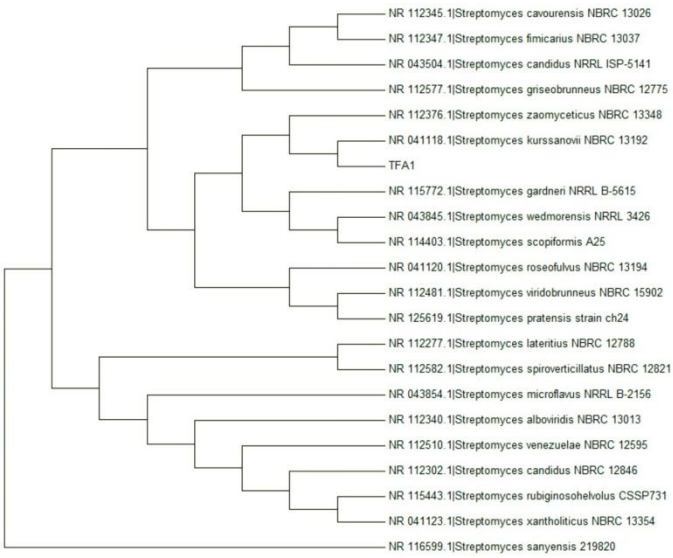

Fig. 3: Phylogenetic tree showing relationship of the *Streptomyces species TFA1* with closely related species of the genus *Streptomyces* inferred from aligned sequence of 16S rRNA gene. The optimal tree with sum of branch length = 0.95is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches**.** The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. There were a total of 1014 positions in the final dataset.

Antimicrobial potential of strain TFA1

In agar plug method, strain TFA1 inhibited *S. aureus* (15 mm), *B. cereus* (14 mm) and *E. coli* (10 mm). During submerged and agar surface fermentation process, strain TFA1 produced bioactive metabolites in submerged culture on 5th day of incubation whereas in solid culture bioactive metabolite production was observed even on 3rd of incubation. Effect of incubation period and medium consistency on bioactive metabolite production from the strain TFA1 was given in figure 4.

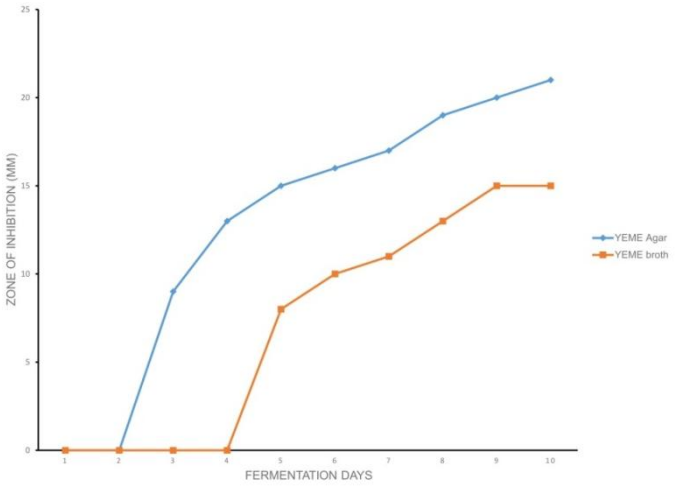

Fig. 4: Effect of incubation period and medium consistency on bioactive metabolite production by the strain TFA1

The morphology of actinobacterial mycelium is well suited for invasive growth on solid medium (Barrios-Gonzlez and Mejia, 1996). The hyphal mode of growth gives the filamentous organism the power to penetrate into the solid substrates. This also gives them a major advantage over unicellular microorganisms for the colonisation of the substrate and the utilisation of the available nutrients.

Therefore, SSF technology can be exploited as an alternative, allowing better oxygen circulation, especially when large quantities of secondary metabolites are required to be produced in short fermentation periods with minimal expenditure of media and downstream processing (El-Naggar *et al.,* 2009). In the present study, the bioactive metabolite from strain TFA1 was produced by agar surface fermentation, a variant of solid state fermentation. There are many reports on antibiotic production from actinobacteria through SSF using agricultural substrates (El-Naggar *et al.,* 2009). Further, studies on solid state fermentation using agricultural substrates may results in the economic production of bioactive compound from the potential actinobacterial strain TFA1.

Majority of the actinobacterial metabolites are extracellular in nature and they are extracted using the medium polar solvent ethyl acetate (Selvameenal *et al.,* 2009). The ethyl acetate extract from the strain TFA1 showed 18 mm inhibition against *S. aureus and B. cereus* and 14 mm inhibition against *E. coli* in disc diffusion method. This result indicates the medium polar nature of the active compound produced by the actinobacterial strain TFA1.Further production and isolation of active metabolites from the actinobacterial strain TFA1 is in progress.

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