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16S rRNA GENE SEQUENCE, SECONDARY STRUCTURE AND RESTRICTION SITES ANALYSIS OF ISOLATED ACTINOMYCETES FROM SOIL SAMPLES OF BOTANICAL GARDEN, KARNATAK UNIVERSITY, DHARWAD

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ABSTRACT

Current researchers are more emphasised towards microbes to meet the antibiotic requirement of mankind. And in this study we made an attempt for the search of antibiotics through isolation of actinomycetes and analysis of their antibiocity through 16s rRNA gene sequencing and identification of restriction site. An Actinomycete species (SN-4) was isolated from botanical garden soil samples of the Karnatak University, Dharwad, India. Morphological, physiological, biochemical and 16S ribosomal RNA studies suggested that the isolate belongs to *Streptomyces* sp. Preliminary screening of antimicrobial activity revealed that the SN-4 isolate was active against all the bacterial pathogens and can be used as source of antibiotics against various diseases causing pathogens. The rRNA secondary structure and the restriction sites of *Streptomyces* sp. SN-4 was predicted using Gene Bee and NEB Cutter online tools.

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INTRODUCTION

The Actinomycetes are called as a group of Gram-positive bacteria which form branched filamentous hyphae having resemblance with fungal characteristics. Actinobacteria are difficult to identify up to genus and species level. Usually, living organisms were classified, as stated to similarity and differences in their phenotypic characteristics into prokaryotes and eukaryotes. The actinomycetes classifications of these methods are difficult because of variations in phenotypic characteristics. Gupta and Woese (1983) started to study and sequence the 16S rDNA genes of different bacteria using DNA sequencing, a state that a good technology at the current time and used the sequences for phylogenetic studies.

In different instances, 16S rRNA gene sequencing has been mainly used in distinguishing between Actinomyces and non-Actinomyces anaerobic Gram-positive bacilli, which is frequently difficult in clinical microbiology laboratories (Woo *et al.,* 2002; Colmegna *et al.,* 2003). In the early workers, as a result, shows a wide spread using PCR and DNA sequencing, 16S rDNA sequencing plays essential role in the correct identification of bacterial and fungal isolates and also a discovery of novel strains in clinical microbiology fields. The use of 16S rDNA sequencing, there are many organisms are identified by novel bacterial species and belongs to novel genera have been discovered till now (Woo *et al*., 2008). Actinomycetes diversity and their biological activities are also a potential field of research in the Indian circumstance, there are many new species of actinomycetes are identified and

reported from part of the world (Arumugam *et al.,* 2011). Among the actinomycetes, the genus *Streptomyces* have produced useful secondary metabolites and the major source of a new bioactive molecule and good antibiotic producing organisms (Takahashi and Omura, 2003; Berdy, 2005). NEBcutter and GeneBee are powerful tools in DNA constructs and are currently fast sequenced tools to set restriction enzyme sites and secondary structures constructs are especially valuable. Each commercial software package is available to manipulate DNA sequences always includes one or more modules to detect restriction enzyme recognition sites and secondary structures of isolated organisms (Tamas *et al.,* 2003; Pachaiyappan *et al.,* 2012). The present study evaluates about morphological, cultural, physiological, biochemical and 16S rRNA, RNA secondary structure prediction of isolated actinomycet strain SN-4 from the soil samples.

MATERAIS AND METHODS

Isolation of microorganism

The soil samples were collected from different locations from Botanical garden of Karnatak University, Dharwad. The standard serial dilution culture method (El-Nakeeb and Lechevalier, 1963) was employed to isolate the Actinomycetes. Adequate serial dilution (10^{-1} to 10^{-5}) were prepared to form the enriched samples. 0.1 ml of the samples from the respective dilutions were placed on starch casein agar (Kuster and Williams, 1964). The inoculated plates were incubated at 30°C for one week. The growth of Actinomycetes was observed on the medium every 24h. All the isolated actinomycetes preserved on starch casein agar media at 4°C for further study (Williams and Cross, 1971).

Morphological characterization:

Isolation of actinomycetes of morphological characterization studied as substrate mycelium, aerial mycelium and sporulation status of the SN-4 strain (Williams and Wellington, 1980). The growth characters including aerial mycelium, substrate mycelium, pigmentation and growth activity of all selected isolates were studied on different media (Goodfellow, 1989).

Scanning electron microscope features

The ultra structure of mycelium and arrangement of spores were observed under scanning electron microscopy. Using the scanning electron microscope at the National Institute for Interdisciplinary Science and Technology (CSIR), Thiruvananthapuram. The sample preparation was determined as per the protocol (Srinivasan Vijay *et al.,* 2014).

Antimicrobial activity

Actinomycetes isolates were primarily screened for their antimicrobial activity. The test bacteria used for primary screening were *Pseudomonas aeruginosa* (*P. aeruginosa*) (MTCC424), *Staphyaloccous aureus* (*S. aureus*) (ATTC25923) *Escherichia coli (E.coli)* (MTCC40), *Klebsiella pneumonia (K. pneumonia)* (MTCC9238) and *Bacillus subtilis (B. substilis)* (MTCC121) as the test organism. Activities were assessed using nutrient agar media. Each plate was streaked with each isolate at the centre of a plate and incubated at 37°C for 7-10 days. Later, 24 h fresh sub cultured test bacteria prepared and streaked perpendicular to the actinomycete isolates. Then the plates were incubated at 37°C for 24 h. After incubation, the antagonism observed by inhibition of tested bacteria (Sohan *et al.,* 2015).

Physiological and biochemical characters

Many Physiological and biochemical characterization of the isolated strain SN-4 were performed such as pH, temperature, NaCl, gram staining, spore staining, motility, starch, casein, urea, carbohydrate and nitrate reduction test were conducted according to Gottlieb (1960).

Molecular identification of active strain SN-4

Isolation of DNA

The fresh culture (1.5 ml) was centrifuged (10,000 rpm for 3 min) to obtain the pellet and dissolved in 0.5 ml of lysis buffer (100 mM Tris pH 8.0, 50 mM EDTA, 10% SDS). 0.5 ml of saturated phenol and 0.2 ml chloroform were added and incubated at 550C for 10 min. Centrifuged at 10,000rpm for 10 min and to the supernatant equal volume of Chloroform: isoamyl alcohol (24.:1) and 1/20th volume of 3M sodium acetate were added. Centrifuged at 10,000rpm for 10 min and to the supernatant was added with 3 volumes of chilled absolute alcohol. Precipitated DNA was separated by centrifugation. Pellet was dried and dissolved in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) and store in cool. The quality of the DNA was examined by spectrophotometer and agarose gel electrophoresis (Sambrook *et al.,* 1998).

Analysis of 16S rRNA sequence

The 16S rRNA genes were amplified using primers forward primer 5'-GAAGCGCTCACGGCCTA-3' and reverse primer 5'-CGGAGTGTCCATGTTCAGGGAACG-3'. The PCR mixture contained DNA, 1 µl of 16s forward primer 400ng and 16s reverse primer 400ng, 4 µl dNTPs (2.5 mM each), 10X Taq DNA polymerase assay buffer 10 µl, Taq DNA polymerase enzyme (3U/µl) 1 µl, Water X µl and total reaction volume 100 µl and amplified in automated thermal cycler (ABI2720). The following conditions were used for the PCR amplification. Initial denaturation at 96°C for 5 min, followed by 25 cycles of denaturation at 96°C for 30 sec, Hybridization 50°C for 30 sec and final extension 60°C for 1.30 min. The PCR products were electrophoresed on 1% agarose gel with 500 bp DNA ladder as the size reference used. Purified PCR amplicon was sequenced (Applied Biosystems Sanger sequencing 3500 series genetic analyzer) and used to interrogate the NCBI database via the BLAST web portal (Fenical *et al.,* 1999; Valan Arasu *et al.,* 2008).

16S rRNA secondary structure and restriction sites analysis

The RNA secondary structure of the isolate SN-4 was predicted, using the GeneBee online software (http://www.genebee.msu.su/services/rna2_reduced.html) with the greedy method and the restriction sites of the SN-4 DNA were analysed by NEBcutter V2.0 (http://tools.neb.com/NEBcutter2) (Saltau *et al.,* 1987; Wang *et al*., 2007; Vincze *et al.,* 2003).

RESULTS

Collection and processing of samples

Fifteen soil samples were collected from Botanical garden of Karnataka University Dharwad and they are used for the isolation and enumeration of actinomycetes. All the samples collected were cleaned, dried and preserved at 4°C. The preserved samples were heat treated with an aim to achieve better qualitative and quantitative isolates of actinomycetes.

Isolation of actinomycetes

The unique and clearly distinguishable typical colonies of actinomycetes isolated from soils. A total of 14 actinomycetes were isolated from soil samples from Botanical garden of Karnatak University, Dharwad. Out of 14 isolates of only 4 isolated were found to be gram positive bacteria and these isolates used for further analysis.

Morphological and scanning electron microscope

Growth characteristics of 4 strains in different media were summarized in (Table-1). SN1, SN2, SN3 and SN4 isolates showed excellent growth in starch casein agar (SCA) and moderate growth was seen in glycerole aspergine agar (GAA).

All the 4 Actinomycetes isolates were tested for their inhibitory activity against five bacterial pathogens, such as *Pseudomonas aeruginosa, Corynebacterium, Escherichia coli, Klebsiella pneumonia and Bacillus subtilis* by streak culture technique. Out of 4 isolates, SN-1 and SN-3 showed no inhibited activity, SN-2 strain showed inhibition activity against *E. coli*. The SN-4 showed good inhibition activity against *Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumonia and Bacillus subtilis* of four pathogenic bacteria (Table-2). The isolate SN-4 exhibited good antimicrobial activity, hence it was chosen and used for further studies.

Physiological and biochemical characterization

The physiological and biochemical characteristics of actinomycetes isolate SN-4 are summarized in (Table-3). The pH ranges that chosen growth of the organism and pigment production was established to be 6.0 to 8.0. The optimum growth was observed at pH 7.5. Effect on temperature isolated strain SN-4 culture showed a good growth and pigment production was observed at 35°C. Gram staining activity of SN-4 showed a positive result. The biochemical test was performed and the isolate SN-4 showed positive results for NaCl starch, casine, urea and gelatine, carbohydrate and nitrogen reduction tests.

Table 1. Isolates showing growth on different culture media, aerial mycelium, substrates mycelium and pigmentation

Isolates	Media	Growth	Aerial mycelium	Substrate mycelium	Pigment color
SN-1	SCA	Moderate to good	White	Colourless to white	White
	GAA	Poor			
SN-2	SCA	Good	Grey beige	Blue	Blue
	GAA	Poor to moderate			
SN-3	SCA	Good	Olive yellow	Light yellow	Grey
	GAA	Poor			
SN-4	SCA	Moderate to good	Signal white	white	Cement
	GAA	Moderate to good			

SCA- Starch casein agar, GAA: Glycerol aspergine agar

The aerial and substrate mycelium color varied among the isolates such as SN-1 and SN-4 was observed with white pigment, SN-2 blue pigment, and SN-3 observed with gray pigment. Observations analyzed according to the colors of the RALcode. Scanning electron microscope (SEM) is helpful for the study of spore morphology of isolated SN-4. The spore morphology of isolated strain SN-4 as whorls attached to long, straight branches (Figure-1).

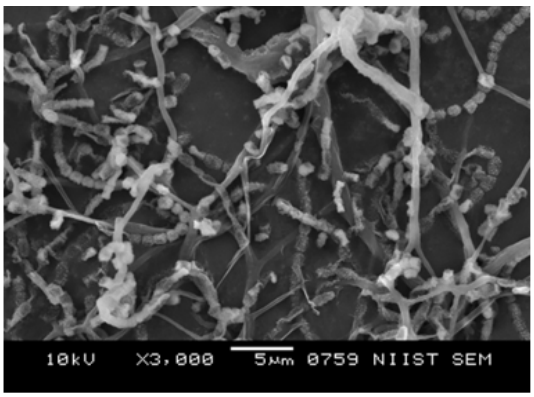

Figure 1. Morphology of sporophores of SN-4 isolated Actinomycetes studied by scanning electron microscopy

Primary screening of antibacterial activity

Molecular identification of isolate SN-4

The active strain SN-4 isolated genomic DNA according to the genomic DNA isolation kit. The isolated DNA was confirmed with 1% agarose gel. PCR product yielded a single amplicon of approximately size 1.5kb. The 16S rRNA gene sequence of strain SN-4 nucleotide sequence obtained was 829bp in length and was submitted to NCBI database (Accession No KX284897).

The phylogenetic tree constructed, results are showed that SN-4 isolated strain (lcl 153675) showed 98% similarity with *Streptomyces* sp. Therefore, SN-4 active strain indicated that *Streptomyces* sp. (Figure-2). The secondary RNA structure of active strain *Streptomyces* sp.SN-4 was predicted (Figure-3). This prediction showed that the free energy of the structure is -177.5 kcal/mol; the threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4; and the conservatively is 0.8. The prediction of restriction sites in the strain *Streptomyces* sp. SN-4 showed the restriction sites for various enzymes, such as *BsrI, SmlI, BmrI, MlyI, PleI, HinfI, BpuE I, Ssp I, BtsI, SfaNI, BsaHI* and possess 58% GC and 42% AT content (Figure-4).

Table 2. Zone of inhibition (<mm) of the isolates against tested bacteria using perpendicular streak method.

Bacterial pathogens	SN-1	SN-2	SN-3	SN-4
P. aeruginosa (MTCC 424)	-	-	-	+
S. aureus (ATTC25923)	-	-	-	+
E. coli (MTCC40)	-	+	-	+
K. pneumonia (MTCC9238)	-	-	-	+
B. subtilis (MTCC121)	-	-	-	+

+: Positive inhibition –: No inhibition

Figure 2: **Phylogenetic analysis of 16S rRNA gene sequence of *Streptomyces* Sp. SN-4**

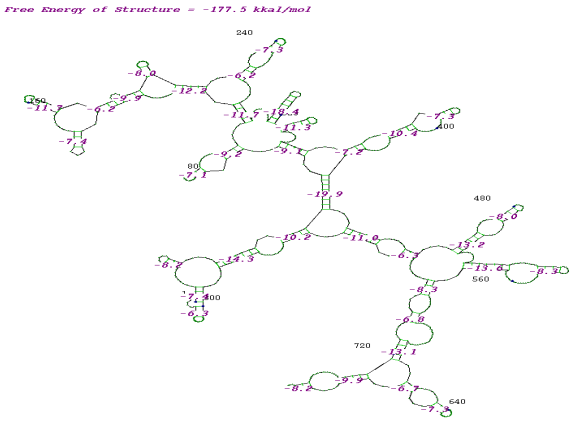

Figure 3. Restriction sites on the 16S rRNA gene sequence of SN-4

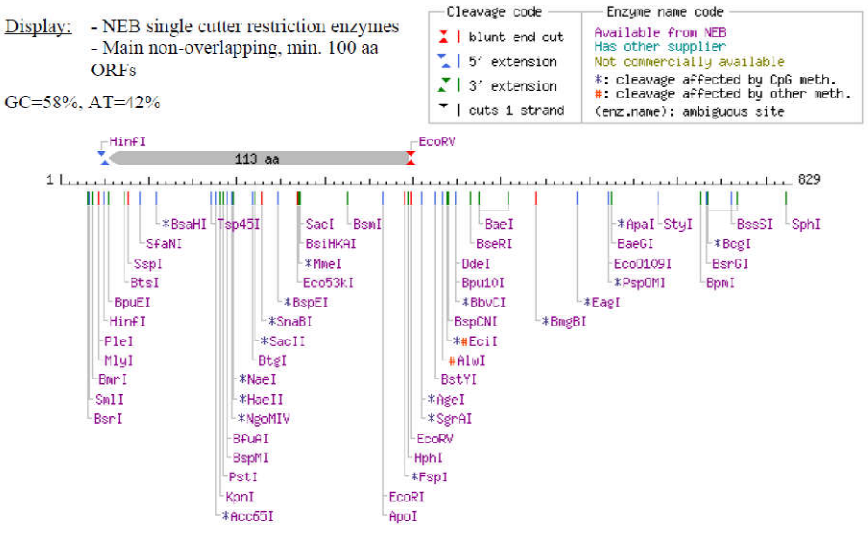

Figure: 4. The secondary structure of 16S rRNA sequence of SN-4

Table 3. physiological and Biochemical characteristics of the isolate SN-4

Types of Test	Strain SN-4
Growth at pH	
pH6.0	-
pH6.5	-
pH7.0	-
pH7.5	+
pH8.0	-
Growth at Temperature	
20^0C	-
25^0C	-
30^0C	-
35^0C	+
40^0C	-
Gram staining	+
NaCl	+
Starch	+
Casein	+
Urea	+
Gelatin	+
Carbohydrate	+
Nitrate reduction	+

-: Negative; +: Positive

DISCUSSION

In our experiments, fourteen different types of actinomycetes were isolated from the soils of a Botanical garden, Karnatank University, Dharwad. It is very difficult to isolated actinomycetes strains; it is always faced with difficulties in comparison to bacteria and fungi. Similarly, Sarayza and Hemashenpagam (2011) reported that this may be due to their long incubation period and different selective media incorporated with antibiotics like cycloheximide (50µg/ ml) and nystatin (50µg/ ml) was crucial inhibiting contaminating microorganisms. The morphological characterization of the actinomycetes isolates was studied by using different types of media. In our study, the growth of actinomycetes is seen in starch casein agar medium. The earlier described, isolation of actinomycetes producing a selection of different media for the different ecosystems and different methods used for the isolation of actinomycetes (Kitouni *et al.,* 2005). In the present investigation out of 14 isolates of only 4 isolates were found to be gram positive bacteria and these isolates used for further analysis. The morphological, cultural characteristics four isolates shows different colors. Similar findings we observed in earlier reports are (Pridham and Tresner, 1974; Kim *et al.,* 1999; Rizk *et al.,* (2007); Ndonde and Semu, 2000) chalky white, yellow, red, violet and green and gray colored series are dominant from different soil samples.

The aerial and substrate mycelium is colors consider as a most important characteristic of identification of actinomycetes. Preliminary screening of antimicrobial activity done with all actinomycetes isolates, only SN-4 isolate showed good inhibition zone. Similarly noted (Sreenivasa Nayaka and Gireesh Babu, 2014; Saadoun *et al.,* 1999 and Ouhdouch *et al.,* 2001) the range of antimicrobial activities among *Streptomyces* species has been previously observed in other soil isolates. The proper identification of actinomycetes is very difficult. The molecular technique is used for proper identification of actinomycetes species for 16S rRNA gene sequencing. Isolate SN-4 shows the highest similarity (99%) was obtained with the 16S rRNA gene of *Streptomyces* sp. Similar findings (Isik *et al.,* 2014 and Sandeep and Menaka, 2014) for identification of species and genus level for easier by the molecular method.

The primers which target particularly the 16S rRNA sequence of the actinomycetes are used for a particular identification of actinomycetes. The restriction site analysis showed GC 58% and AT 42% content. Similar findings we observed earlier report Pachaiyappan *et al.,* (2012) studied the secondary structure and restriction sites using a bioinformatics tool.

Conclusion

Actinomycetes are gram positive bacteria. It is difficult to identify up to species and genus level. There are many scientists who took the help of molecular studies for the identification of actinomycetes. With all the above analysis we can conclude that the actinomycetes strain *Streptomyces* sp. SN-4 which showed maximum inhibition activity against all the tested pathogens and which had 58% GC and 42% AT can be used as antibiotics for various diseases caused by the bacteria.

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