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Isolation, Identification and Characterization of *Streptomyces* sp. SN-2

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The present investigation is carried out for the isolation and molecular characterization of actinomycetes from the soil samples from Karnatak University campus, Dharwad, Karnataka, India. Totally four actinomycetes isolated, out of four isolated only one actinomycetes strain (SN-2) showed positive results for antimicrobial activity. SN-2 isolated actinomycetes was further analysed for morphological, physiological, biochemical and 16S ribosomal RNA gene sequencing. The SN-2 strain gene sequence was predicted with secondary structure and restriction sites were identified with the help of Genebee and NEBcutter tools. This investigation clearly indicates that the isolated strain SN-2 as *Streptomyces* sp.

Keywords: Actinomycetes, *Streptomyces*, Antimicrobial, 16S rRNA, Cross streak method.

Actinomycetes are unicellular organisms and mode of reproduction is through formation of special types of spores. Actinomycetes are in close relationship with eubactera and they are highly filamentous. It is classified into actinomycetales order, genus actinomycetes it include *Streptomyces, Actinomyces, Arthrobacter*. Actinomycetes are belonged to gram-positive bacteria and 55% of high Guanine and cytosine (G+C) content present in DNA[1]. The rare actinomycetes diversity is dominant in aquatic and terrestrial ecosystem and isolated actinomycetes in all most all possible selective methods[2,3]. Nowadays researchers are more emphasized towards isolation of actinomycetes from different ecological niche. A number of rare actinomycetes were isolated from different regions of soil[4,5], marine sources[6,7] and hyper saline habitats[8]. A large number of *Streptomyces* sp. isolated and screened from soil samples[9,10].Therefore the possibilities a novel *Streptomyces* organisms are isolated from terrestrial habitats have diminished, more than 500 species of actinomycetes are used for the production of secondary metabolites[11].

Researchers are searching new genera from soil environments on a regular basis and discovering new metabolites produces never reported earlier. Actinomycete genera identified by cultural and molecular techniques from different marine ecological niches include *Streptomyces*, *Streptosporangium, Nocardiopsis* and *Microbacterium*[12]. A large number of *Streptomyces* sp. have been isolated and screened from different habitats in the past several decades[13,14]. Above 500

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species of *Streptomyces* it produces as 70 to 80% of important secondary metabolites. Actinomycetes are promised source of large bioactive compounds and these are clinical effects and important applications for pharmaceutical and agricultural purpose[15,16,17].

MATERIALS AND METHODS

Collection and Processing of Samples

The soil samples were collected at 2 to 6cm depth from different locations from Karnataka University campus, Dharwad. Totally 10 soil samples are collected using clean stainless steel scoop and a plastic spoon. About 500 g of soil samples were collected in sterile polythene bags. All the collected soil samples were cleaned to remove the stones and debris and air dried for three to four days at room temperature. Cleaned and air dried samples were refilled in the respective polythene bags were stored at 4 °C for further analysis[18].

Isolation of Actinomycetes

The soil samples were air dried for 24 h at 40 °C and crushed with mortar and pestle. The isolation of actinomycetes was done by the standard serial dilution method. The standard serial dilution plate culture method was employed to isolate the actinomycetes[19]. Adequate serial dilution (10^{-1} to 10^{-5}) were prepared from the enriched soil samples. 0.1 ml of the samples from the respective dilutions were inoculated on starch casein agar medium supplemented with amphotericin B-50 (25µg/ml) and tetracyclin (25µg/ml) and spread using L rod. Finally, incubated at 30 °C for one week, every 24 h we observed the growth of actinomycetes colonies. The actinomycetes isolated colony was observed based on morphological characters like sporulation and pigmentation they were purified as per the method[20].

Microscopic Characteristics of Isolated Actinomycetes

Microscopic characters were observed by isolated strains on starch casein agar and glycerol aspergine agar media. The selected isolates were streaked on the surface of the media by streak plate method and plates were incubated at 28 °C for 7 days. Morphological characters such as colony characters, aerial hyphae, substrate mycelium and pigmentation were observed[21]. The colony colors of the aerial and substrate mycelium were described according to the colors of the RALcode and arrangement of aerial hyphae and spore surface isolates colonies were subsequently observed by phase contrast microscope. For the micro-morphology and external morphology of the SN-2 strain using scanning electron microscopy analysis at national institute for interdisciplinary science and technology (CSIR) Thiruvanantapuram, a sample preparation was determined as per the protocol[22].

Cross Streak Method for Antimicrobial Activity

Totally four isolated actinomycetes were tested against bacterial pathogens by using cross streak method[23]. The pathogenic bacteria used as a test organism for primary screening such as *Pseudomonas aeruginosa (P. aeruginosa)* (MTCC424), *Staphylococcus aureus (S. aureus)* (ATTC25923) *Escherichia coli (E.coli)* (MTCC40), *Klebsiella pneumonia (K. pneumonia)* (MTCC9238) and *Bacillus subtilis (B. substilis)* (MTCC121) were obtained from IMTECH, Chandigarh. Activities were assessed using nutrient agar media. Each plate was streaked with each actinomycete isolate at the center of a plate and incubated at 37° for 10 days. These plates were then inoculated with microbial pathogens by streaking at 90° angle to the streak of actinomycetes culture and incubated over night at 37° C. The results were observed based on the inhibition level of the pathogen.

Morphological Characterization of Strain SN-2

The potent active actinomycetes were characterized based on the morphological according to the Bergey's systematic bacteriology manual[24,25]. The morphological characteristics were observed in aerial and substrate mycelium and pigmentation in different media such has starch casein agar and glycerol asparagine agar media.

Physiological and Biochemical characteristics

The physiological and biochemical characterization of the active isolated strain SN-7 many tests were performed such as pH, temperature and NaCl tolerance. Specific tests like gram staining, motility, starch, casein, urea, carbohydrate and nitrate reduction test[26].

Molecular Characterization of Active Strain SN-2

Amplification of 16s rRNA gene sequence from genomic DNA

The genomic DNA extracted from

strain SN-2[27]. The 16S rRNA genes were amplified using primers forward primer GAAGCGCTCACGGCCTA and reverse primer CGGAGTGTCCATGTTCAGGGAACG. The following conditions were used for the PCR amplification. Initial denaturation at 25 cycles of 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 machine (Sequence machine applied biosystems sanger sequencing 3500 series genetic analyzer) and used to interrogate the NCBI database via the BLAST web portal[28].

Species Identification and 16S rRNA Secondary Structure

The highest similarity of actinomycetes isolated strain SN-2 with the reference species was confirmed using the NCBI BLAST tool (http://www.ncbi.nlm.nih.gov/BLAST). The secondary structure and restriction site was identified by using bioinformatics tools of Genebee and NEBcutter obtained in online software's (http://WWW.genebee.msu.su/services/rna2_reduced.html, http://tools.neb.com/NEBcutter2)[29].

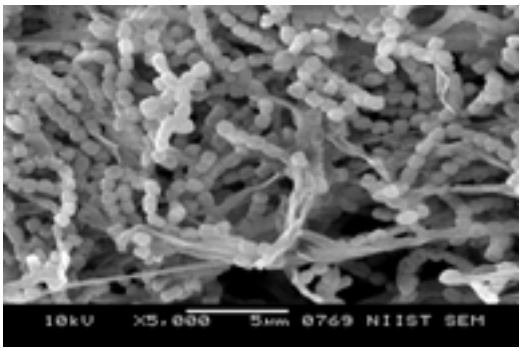

Fig. 1. SEM image showing isolated strain SN-2

RESULTS

Isolation of Actinomycetes

Actinomycetes are isolated using different media in starch casein and glycerol aspergine agar media. Isolated actinomycetes are identified according to the Bergey's manual of systematic bacteriology based on their morphology, sporulation and pigment production in the serially diluted plate 10^{-1} to 10^{-5}. Totally seven actinomycetes were identified and named as SN-1, SN-2, SN-3, SN-4, SN-5 SN-6 and SN-9, theses strains are tested for gram positive and gram negative bacteria. Out of seven only four isolates shows gram positive bacteria and these four isolates stored on starch casein agar media for further analysis.

Morphological Characterization

Morphological features of isolated colonies of four actinomycetes are described according to the colors of RALcode. The morphological characteristics of four isolates SN-1 as white, SN-2 blue, SN-3 gray and SN-4 as brown color pigmentation was observed.

Antimicrobial Activity

The primary screening of all the four cultures were tested for their inhibitory activity against five bacterial pathogens such as *P. aeruginosa, C. bacterium, E. coli, K. pneumonia* and *B. substalis* by streak culture technique. Out of four isolates SN-1, SN-3 and SN-9 showed no inhibition growth from all the tested pathogenic bacteria. Among them, SN-2 showed maximum inhibition growth activity of all the pathogenic bacteria and scanning electron micrograph (SEM)

Table 1. Preliminary screening of antimicrobial activity by cross streak method

Actinomycetes strains	Tested microbes				
	Pseudomonas aeruginosa.	*Coryne bacterium*	*E.coli*	*Klebsilla pneumonia.*	*Bacillus substalis.*
SN-1	+	-	-	-	-
SN-2	+	+	+	+	+
SN-3	-	+	-	-	-
SN-9	+	-	+	-	-

+: Positive inhibition -: No inhibition

picture shows aerial mycelium of SN-2 strain in Fig 1 and Table 1.

Physiological and Biochemical Characterization

The physiological and biochemical test results are summarized Table 2. The Physiological characterization of pH ranges studied from 6.0 to 8.0. The SN-2 showed the optimum growth at pH 7.5. The different temperatures were analysis at 20°C to 40°C. The optimum growth temperature at 35°C. The different biochemical tests were performed, such as NaCl, starch, casein, urea, gelatine, carbohydrate it shows the positive results and nitrate reduction it shows the negative results of isolated strain SN-2.

Table 2. Physiological and biochemical characterization of potent strain SN-2 isolate

Test	SN2
Growth of pH	
pH6.0	-
pH6.5	-
pH7.0	+
pH7.5	++
pH8.0	+
Growth at Temperature	
20^{0}C	-
25^{0}C	-
30^{0}C	+
35^{0}C	++
40^{0}C	+
NaCl tolerance	+
Hydrolysis of:	
Starch	++
Casein	++
Urea	++
Gelatin	++
Carbohydrate	++
Nitrate reduction	+

-: No growth; +: Normal growth; ++: Optimum growth

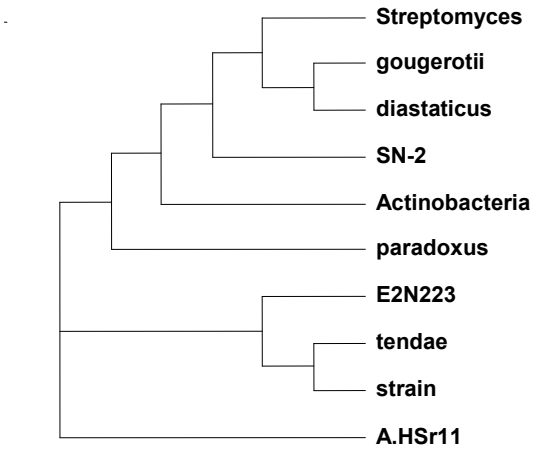

Fig. 2. Dendogram indication the phylogenetic relation of the *Streptomyces* SN-2

Molecular Identification of Potential Isolates by 16S rRNA gene Sequencing

The strain SN-2 isolated genomic DNA according to the bacterial genomic DNA isolation kit. The DNA was confirmed with 1% agarose gel and purified. PCR reaction mixture was set up to amplify the 16S rRNA gene. The amplified product was sequenced using ABI Sequencing machine (ABI 3500 XL Genetic Analyzer). The sequence obtained was 853 bp in length and was submitted to NCBI database and Genbank accession numbers are given (Accession No KX284895).

Sequencing and Phylogenetic Tree

The sequence initially characterized by BLAST analysis, which hit homologous 16s rRNA gene from various species. Most homologies of the sequence were found with 16S rRNA genes from *Streptomyces* sp. SN-2. The DNA sequence was

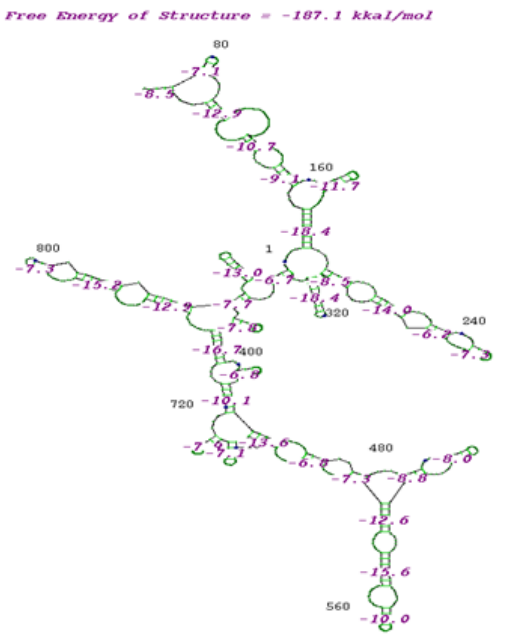

Fig. 3. Restriction sties on the 16S rRNA gene sequencing of the *Streptomyces* SN-2

Fig. 4. Secondary structure of 16S rRNA sequence of *Streptomyces* sp. SN-2

aligned and a phylogenetic tree was constructed by using MEGA4 software (Fig 2).

Secondary Structure and Restriction Site of *Streptomyces* sp. SN-2

The RNA secondary structure was predicted for the 16S rRNA gene of *Streptomyces* sp. SN-2 (Fig 3). This prediction showed that the free energy of the structure is -238.8 kcal/mol, the threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4 and the conservative is 0.8. The prediction of restriction sites in the strain SN-2 showed the restriction sites for various enzymes, such as *BsaB l, SnaBl, EcoR l, Agel and Bsal* (Fig 4).

DISCUSSION

In the present study, we are isolated actinomycetes from the soil samples of the Botanical garden of Karnatak, University, Dharwad. Totally four actinomycetes were isolated and screened for primary screening of antimicrobial activity by cross streak method against different pathogens. The results of the primary screening of antimicrobial activity clearly it indicates that only SN-2 actinomycetes were showed a high activity of inhibition growth for tested pathogenic organisms. These results are compared with the others totally thirty one actinomycetes are isolated from different niche habitats of Sheopur district, Madhya Pradesh and tested their antimicrobial activity of twelve pathogenic microorganisms. These actinomycetes isolates showed antibacterial activity against *S. aureus*, and less activity against *S. dysenteriae*[30]. Similarly actinomycetes are isolated from soil samples of Vengodu (village) in Kanchipuram district, Tamil Nadu, India. Totally thirty five actinomycetes isolated and screened for antibacterial activity by cross streak method and Loyola PBT VAS 10 showed good antibacterial activity against the tested bacteria[31].

All the four isolates were grown in starch casein agar and glycerol aspergine agar media, we observed different colors of growth characteristics like aerial, substrate and the pigmentations. Similar findings we observed from actinomycetes are isolated from soil samples in Belgaum, their morphological and cultural characteristics studied of the A-4 mutant showed cellular and aerial growth as well as soluble pigment formation in various ISP media[32,33]. Actinobacteria have different cultural characteristics in various kinds of culture media, which are important in the classification identification, general with spores, aerial hyphae, with or without color and the soluble pigment, different growth condition on various media as the main characteristics[34].

It is always facing to the difficult for identification of actinobacteria. The conventional culture methods are not satisfactory for analysis of actinomycetes. Therefore, 16S rRNA gene sequencing studies are one of the best methods for identification of actinomycetes genera. In the present investigation 16S rRNA amplified and sequenced for isolated strain, SN-2 belongs to the members of *Streptomyces* sp. Similarly in other

studies actinomycetes isolated different niche and identified from 16S rRNA gene sequencing[35,36]. The RNA secondary structure and restriction sites were predicted by using Genebee and NEBcutter online tools for the 16S rRNA gene of isolated *Streptomyces* sp. SN-2. Many researchers have been reported using these tools for prediction of secondary structure and restriction sites[37,38].

CONCLUSION

Actinobacteria group of gram positive bacteria and highly G-C content. Actinobacteria are produce variety of antibiotics. Soil has most important habitat for isolation of *actinomycetes* sp. Actinomycetes are difficult to identification up to species and genus level. The present investigation analysis for 16S rRNA gene sequencing, secondary structure and restriction site analysis. It is concluded that the identified strain SN-2 has *Streptomyces* sp. and also a good antibiotic producing organism.

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