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Isolation, Identification and Characterization of Antimicrobial Activity Exhibiting Actinomycete *Streptomyces paradoxus* Strain KUASN-7 from Soil

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A B S T R A C T

Soil acts as a rich source of Actinomycetes, which are generally characterized as potent antibiotic producers. The research rationale of this study was to isolate and characterize potential Actinomycetes possessing antibiotic properties from the soils of Dharwad Karnataka, India. Further, isolates were characterized using microscopic, biochemical and molecular techniques. Initially, ten different Actinomycetes were isolated on starch casein agar media. Out of ten isolates, five of them were Gram-positive and were further screened for antimicrobial potency by cross streak method. Among 5 Actinomycetes isolates studied, isolate SN-7 was found to be most efficient against all the four pathogens tested, which confirmed its antimicrobial efficacy. The potent active isolate SN-7 was identified as *Streptomyces* strain by 16S rRNA gene sequence similarity analysis and given a strain no. SN-7. Genebee and NEB Cutter version 2.0 online tools were used to confirm 16S rRNA gene secondary structure and restriction sites on the DNA sequence respectively, which was deposited with NCBI with an accession number KX284898, *Streptomyces paradoxus* strain KUASN-7. In addition, *Streptomyces paradoxus* strain KUASN-7 was subjected for preliminary screening of bioactive metabolites using column chromatography and the eluted fractions were further characterized using UV and FTIR spectroscopy techniques.

Introduction

Actinomycetes are Gram-positive bacteria which exhibit fungi-like characteristic morphology and comprise a high G+C content. Studies on actinomycetes and their secondary metabolites are relatively important as they are prime sources of secondary metabolites-antibiotics (Al-Humiany *et al.*, 2010). Further, isolation of actinomycetes from different sources is quite essential to tackle those numerous pathogens that have acquired resistance to most prescribed antibiotics and to overcome them with the unique metabolite of these diverse actinomycetes. Hence, the quest for search of

novel metabolites antibiotics has gained significant importance in pharmaceutical industry. The soil, in particular, is an extensively exploited ecological niche and a prime source for diverse actinomycetes. Soil microorganisms produce numerous natural bioactive metabolites, including clinically important antibiotics and therapeutic molecules. However, extensive antibiotic usage has eventually lead to evolution of multi-drug resistant pathogens and thus entail discovering novel molecules, including antimicrobial that carry potential antibiotic properties. Searching for unknown microbial species is an effective approach for obtaining novel bioactive molecules (Sherman *et al.,* 2004; Bartlett *et al.,* 2006). Thus, there is dire necessity to find effective alternative drugs, especially antibiotics, to control and overcome the increasing spread of multi-drug resistant organisms and life-threatening diseases (Pompliano *et al.,* 2007).

However, as numerous studies have confirmed that actinomycetes are potent source of antibiotics, especially genus *Streptomyces* being most abundant and diverse group inhabiting soil (Reena *et al.,* 2012; Mukherjee *et al.,* 2017), they have been so isolated from unique extreme habitats such as cold/ hot springs and mangroves (Mukherjee *et al.,* 2017). Further, *Streptomyces* sp. have been source of several novel bioactive compounds which are antagonistic to pathogens (Bhattacharyya *et al.,* 2015). With the advent of polymerase chain reaction and gene sequencing tools, interpretation of a closely related taxon with superior authenticity has been convenient in comparison to other conventional techniques. The notable application of molecular techniques in analysis of bacterial genomes has contributed extensively to our knowledge of bacterial diversity and taxonomy. The identification of actinomycetes using conventional techniques such as microscopic and biochemical is often unreliable, as biochemical characters of few microorganisms are unsuitable for more effective identification of species and strain (Leung *et al.,* 2000). Hence, frequently rapid and reliable technique such as ribonuclease-resistant oligo-nucleotides of the 16S ribosomal RNA (rRNA) is extensively followed for suitable identification of microorganisms (Komagata *et al.,* 1993; Kizil *et al.,* 2008).

Further, phylogenetic analysis of the 16S rRNA gene sequence is one of the emerging key areas in the field of diversity and evolutionary studies (Sharon and Michael, 2007). The spectrometric analysis is always been the first choice for screening and identification of novel compounds irrespective of the source. UV and FTIR analysis are such spectrometric techniques which are of immense use for the detection and identification of novel bioactive compounds more specifically. Several studies have used this technique in screening and identification of compounds from microbial sources (Singh *et al.,* 2016).

Hence, the present study was undertaken to isolate and characterize potential actinomycetes strains from the soil of Botanical garden, Karnatak University, Dharwad. Further, the promising isolates were screened for their antimicrobial ability and preliminary screening of bioactive compounds using spectroscopy.

Materials and Methods

Isolation of actinomycetes from soil samples

Soil samples were collected from different locations of Botanical garden, Karnatak University, Dharwad, Karnataka, India. The soil sample (50g) was collected in sterile, autoclavable polythene bags. Soil samples (Red soil) were cleaned to remove the stones

and debris and air dried in shade for three to four days at room temperature at 32 to 35 °C.

The standard serial dilution plate culture technique was followed for the isolation of actinomycetes. Dilutions ranging from 10^{-1} to 10^{-5} were considered for isolation. 0.1 ml aliquot of sample from the respective dilutions was dispensed on two agar media viz. starch casein and glycerol asparagine agar (Williams and Kuster, 1964). The inoculated plates were kept for incubation at 30 °C for duration of 7-8 days. A growth characteristic of the isolates (actinomycetes) was recorded every 24 h. The colonies identical to actinomycetes were observed under microscope and sub-cultured subsequently.

Morphological and cultural characteristics

The morphological and cultural characterization of sub-cultured/selected actinomycetes strain was determined by growing the isolates on starch casein and glycerol asparagine agar medium at 30°C for 7 days (Amono *et al.,* 1987). The morphological characters viz. colony characteristics, aerial hyphae, substrate mycelium and spore were examined (Gottlieb and Shirling, 1966). 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 isolate colonies were subsequently observed by phase contrast microscope (Carl Zeis Axio A1) (Erko *et al.,* 2003).

Primary Screening of antimicrobial activities of pure isolates by cross streak method

Isolated strains were subjected for evaluation of antimicrobial potential. The pathogenic bacteria such as *Aspergillus fumigatus* (MTCC8877), *Enterobacter aerogenes* (ATTC2822), *Staphylococcus epidermidis* (MTCC435) *and Shigella flexneri* (MTCC1457) were procured from IMTECH, Chandigarh, India. Nutrient agar media was used for the antimicrobial activity and cross streak method was followed as described by Dezfully and Ramanayaka (2015). Briefly, each sterile plate was streaked with each specific isolate and incubated at 37 °C for seven to ten days. Later, 24 h fresh sub cultured test bacteria was prepared and streaked perpendicular to the actinomycete isolates. Then plates were incubated at 37 °C for 24 h. After incubation, plates were observed for inhibitory activity.

Scanning electron microscopy

After the preliminary examination of antimicrobial activity, a unique isolate which exhibited potential antimicrobial activity was further subjected for micro-morphology and external morphology studies using scanning electron microscopy (JEOL JSM-5610, Japan) and sample was prepared according to the protocol described by (Srinivasan *et al.,* 2014).

Physiological and Biochemical characteristics of the isolate

Physiological and biochemical characterization of the unique isolates were studied to understand the basic physiology of the soil actinomycetes.

Several physiological and biochemical tests such as different pH, temperature, Gram staining, spore staining, motility, NaCl tolerance, starch, casein, urea, gelatin, carbohydrate and nitrate reduction test were performed (Prakasham *et al.,* 2014).

Genomic DNA extraction and analysis of 16S rRNA and DNA sequence

Genomic DNA was isolated following the

protocol of Sambrook *et al.,* (1998) and the quality of the DNA was examined by agarose gel electrophoresis. 16S rRNA genes were amplified using locus specific primers 5'-GAAGCGCTCACGGCCTA-3' (forward primer) and 3'-CGGAGTGTCCATG TTCAGGGAACG-5' (reverse primer). The PCR reaction mixture contained 50 ng of DNA, 1 µl of 16S forward primer (400 ng) and 16S reverse primer (400 ng), 4 µl of dNTPs (2.5 mM each), 10 µl of 10X Taq DNA polymerase assay buffer, 1 µl of Taq DNA polymerase enzyme (3U/µl), and 7 µl Millipore water was added to make the total reaction volume to 100 µl, amplification was performed using automated thermal cycler (Model; ABI2720, Applied biosystems USA), with a following conditions; Initial denaturation at 96 °C for 5 min (25 cycles), denaturation at 96 °C for 30 sec, Extension at 50°C for 30 sec and final extension at 60 °C for 1.30 min. The PCR amplicons were examined on agarose gel electrophoresis (1%) with the aid of 500 bp DNA ladder. Purified PCR amplicon was sequenced (applied biosystems Sanger sequencing 3500 series genetic analyzer) and used to interrogate the NCBI database via BLAST web portal (Valan *et al.,* 2008).

16S rRNA phylogenetic, secondary structure and restriction sites analysis

Sequenced DNA data were compiled and analyzed and matched with the GenBank database using Basic Local Alignment Search Tool (BLAST) algorithm from the NCBI website (www.ncbi.nlm.nih.gov). The DNA sequence was aligned and a phylogenetic tree was constructed by neighbor-joining method using the software MEGA4 (Tamura *et al.,* 2007). The SN-7 culture was deposited in gene bank and the accession number is KX284898. The RNA secondary structure of the isolate *Streptomyces paradoxus* strain KUASN-7 was predicted, using the GeneBee online software (http://www.genebee.msu.su/services/rna2_reduced.html) with the greedy method and restriction sites of the NRC-77 DNA were analyzed by NEB cutter V2.0 (http://tools.neb.com/NEBcutter2) (Wang *et al.,* 2007).

Preparation and preliminary characterization of crude methanolic extract

Streptomyces paradoxus strain KUASN-7 was grown in starch casein agar media. The pH of the medium was adjusted to 7.0. The culture was grown with continuous shaking on a rotary shaker (150 rpm) at 30°C for 14 days. After fermentation of the culture, biomass was harvested by centrifugation (5000 rpm) at 20 °C for 20 minutes and then the mycelia were washed thrice with sterile distilled water under aseptic conditions. The washed fraction was finely homogenised with the aid of mortar and pestle and subsequently methanol was added in the ratio of 1:1 (w/v) and mixture was allowed to mix in orbital shaker for overnight. Finally, the extract was filtered using Whatman filter paper (no.1). The filtrate was then dried using a rotary evaporator (maintained at 50 °C). The semi dried extract was then collected in clean glass vials and stored at 4 °C for further analysis (Augustine *et al.,* 2005). The crude methanol extract was chromatographed over a silica gel column chromatography (60x120 mesh Ramken Sigel) and eluted with mixtures of $CHCl_3$:MeOH (chloroform: methanol; 60:40). Finally, fractions were collected and subjected for further analysis (Saravana *et al.,* 2014). The methanolic extract was analysed using Perkin-Elmer Lambda 30 UV/VIS spectrophotometer (AH and Aysel 2003) in UV region (190 to 700 nm). FTIR analysis of active extract was performed using Shimadzu IR-470 plus with scanning mode from 400 to 4000 cm^{-1} range and plotted as intensity versus wave number (Al-Humiany *et al.,* 2010).

Results and Discussion

Screening and isolation of potential isolates

The unique and clearly distinguishable typical colonies of microbial isolates were seen after 7 days incubation. In the dilution plate pigmented, powdery, smooth and rough colonies of actinomycetes were observed. Ten actinomycetes were isolated from soil samples from botanical garden of Karnatak University, Dharwad. Out of 10 isolates, five isolates were found to be Gram-positive bacteria and inoculated in starch casein agar (SCA) media for further analysis. The morphological appearances of isolates are depicted in Figure 1. The growth characteristics of five strains in different media are summarized in Table 1. Actinomycetes considered as prolific producers of novel antimicrobial agents and thus huge numbers of these antimicrobial compounds are discovered by screening natural habitats such as soil, water and extreme environments (Duraipandiyan *et al.*, 2010; Zotchev, 2011). A vast taxonomic range of actinomycetes known to produce secondary metabolites with potential biological activities such as antibiotics, antifungal, anticancer, antiviral, immunosuppressant and other industrially important compounds (Newman and Cragg, 2007). The organisms showing moderate to excellent growth on SCA and GAA media showed varying pigment patterns.

Color of the substrate mycelium and aerial spore mass varied distinctly. The five isolates showed different colors of pigmentation SN-1, SN-4 and SN-7 showed white color, while SN-2 and SN-3 showed blue and grey color, respectively. The observations made in the present study were in accordance with the existing literature (Ndonde and Semu, 2000; Rizk *et al.*, 2007).

Antimicrobial activity

Five promising isolates were then evaluated for their inhibitory activity against three bacterial pathogens and one fungal pathogen by cross streak method. The antimicrobial activity is tabulated in Table 2. Out of 5 isolates, SN-1 and SN-3 showed no inhibition against tested pathogens, Whereas, SN-2 showed very minimal activity against *S. epidermidis* (MTCC435) and SN-4 exhibited antibacterial activity against *S. flexneri* (MTCC1457) strains. However, SN-7 inhibited the growth of all four pathogens [*A. fumigatus* (MTCC8877), *E. aerogenes* (ATTC2822), *S. epidermidis* (MTCC435) and *S. flexneri* (MTCC1457)] tested indicating potential antimicrobial activity against test microorganisms. Screening of antimicrobial activity of five isolates against four pathogenic organisms showed that SN-7 exhibited maximum zone of inhibition Figure 1a and 1b. Thakur *et al.*, (2007) reported the similar observation, wherein they isolated 110 actinomycetes strains from the forest soil of North East India and strains were characterized by conventional methods followed by assessment for their antagonistic activity against test microorganisms.

Scanning electron micrograph (SEM)

The scanning electron micrograph of promising strain SN-7 is depicted in Figure 1. The image shows aerial mycelium with cells of 1.25 µm (approx) in size arranged in pseudo hyphal form.

Table.1 Cultural characteristic of active isolates of actinomycetes

Isolates	Medium	Growth	Aerial mycelium	Substrate mycelium	Pigment
SN-1	SCA	Moderate to good	White	Colorless to white	White
	GAA	Poor			
SN-2	SCA	Good	Grey beige	Blue	Blue
	GAA	Moderate to Poor			
SN-3	SCA	Good	Olive yellow	Light yellow	Grey
	GAA	Poor			
SN-4	SCA	Moderate to good	Signal white	White	White
	GAA	Moderate to good			
SN-7	SCA	Good	Grey white	White	White
	GAA	Moderate to Poor			

Legend: SCA=Starch casein agar media; GAA=Glycerol aspergine agar media

Table.2 Primary screening of active isolates of actinomycetes using cross streak method against pathogenic organisms

Microbes	SN-1	SN-2	SN-3	SN-4	SN-7
***A. fumigatus* (MTCC8877)**	-	-	-	-	+++
***E. aerogenes* (ATTC2822)**	-	-	-	-	+++
***S. epidermidis* (MTCC435),**	-	+	-	-	+++
***S. flexneri* (MTCC1457)**	-	-	-	+	+++

Legend: +++ - Good activity, ++ - Moderate activity, + - Weak activity, - - No activity

Fig.1 Characterization of isolate of *Streptomyces* strain SN-7: Morphology of sporophores studied by scanning electron microscopy (A). Zone of inhibition against pathogenic bacteria by cross streak method (B and C)

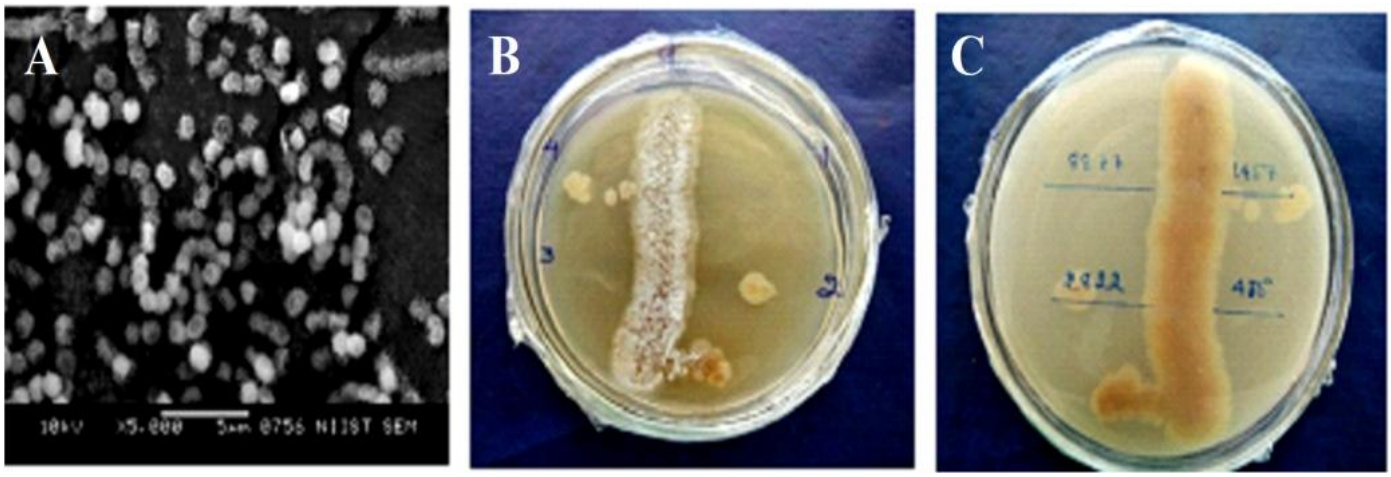


Table.3 Physiological and Biochemical characteristics of the strain SN-7

Test	SN-7
Growth at pH	
pH6.0	-
pH6.5	-
pH7.0	++
pH7.5	+++
pH8.0	+
Growth at Temperature	
20 ℃	-
25 ℃	
30 ℃	+
35 ℃	+++
40 ℃	-
Gram staining	+
Spore staining	+
Motility	-
NaCl tolerance	+
Substrate Hydrolysis	
Starch	+++
Casein	+++
Urea	+++
Gelatin	+++
Carbohydrate	++
Nitrate reduction	++

Legend: - - No growth, + - Normal growth, ++ - Moderate growth, +++ - Optimum growth

Table.4 FTIR absorption peaks and their associated functional groups detected in the *Streptomyces* strain SN-7 extract

Sl. No.	Absorption peak (cm^{-1})	Functional groups
1	618	C-Br stretching alkyl halides
2	868	C-Cl stretching alkyl halides
3	1048	C-N stretching aliphatic amines
4	1079	C-N stretching aliphatic amines
5	1112	C-N stretching aliphatic amines
6	1241	C-H wag alkyl halides
7	1386	CH_2 waging mode alkanes
8	1404	C-C stretching aromatics
9	1465	C-H bend alkanes
10	1580	1,3 Diketones of enol
11	1638	N-H bending 1º amines
12	2852	C-H stretching alkanes
13	2923	H-C=O:C-H stretching aldehyde
14	2957	C-H stretching alkanes
15	3420	O-H stretch alcohol

Fig.2 (a) Gel image showing the amplified product of SN-7, (b) Phylogenetic analysis of 16S rRNA gene sequence of SN-7.

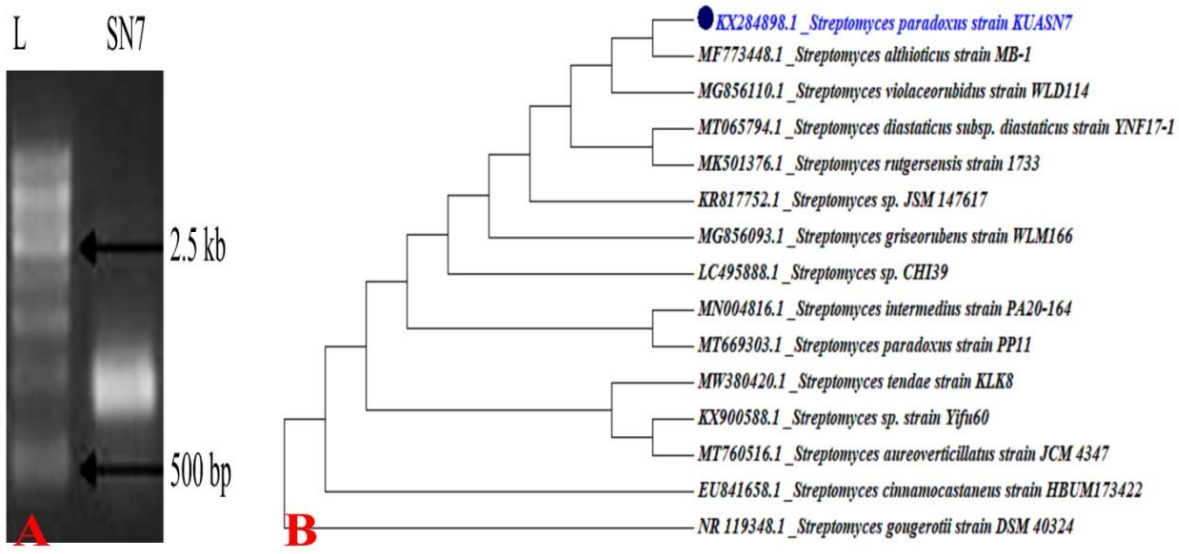


Fig.3 Secondary structure of 16S rRNA sequence of *Streptomyces paradoxus* strain KUASN7

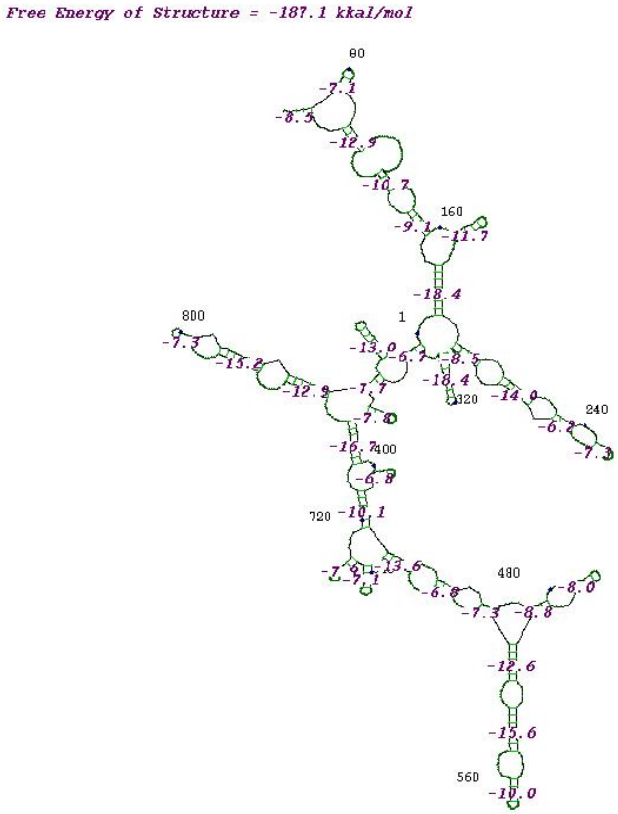


Fig.4 Restriction sites on the 16S rRNA gene sequence of *Streptomyces* strain SN-7

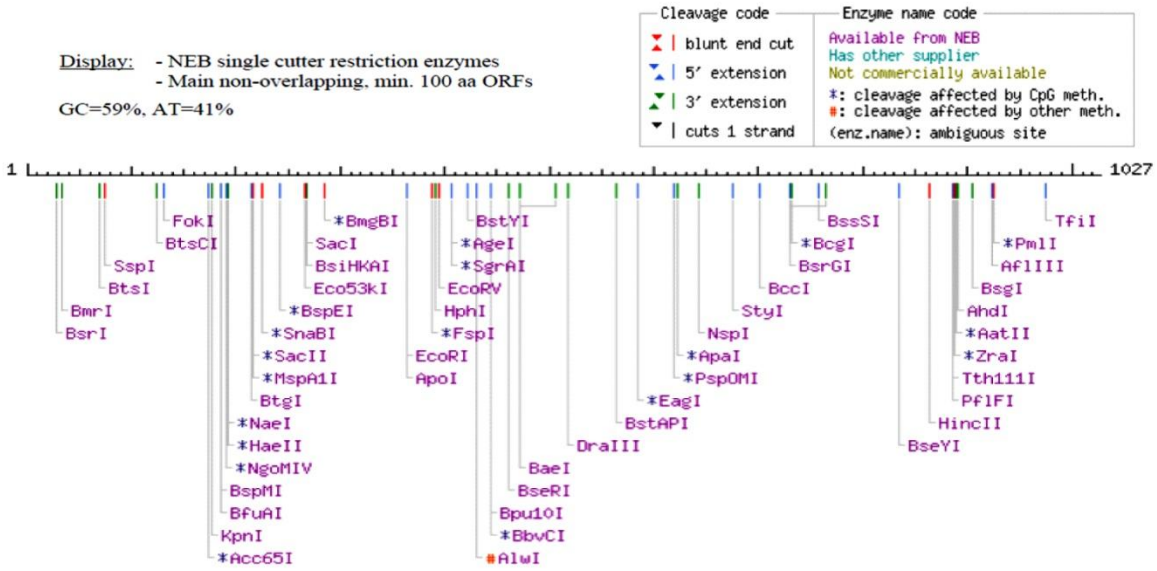


Fig.5 UV-Spectrum of *Streptomyces* strain SN-7

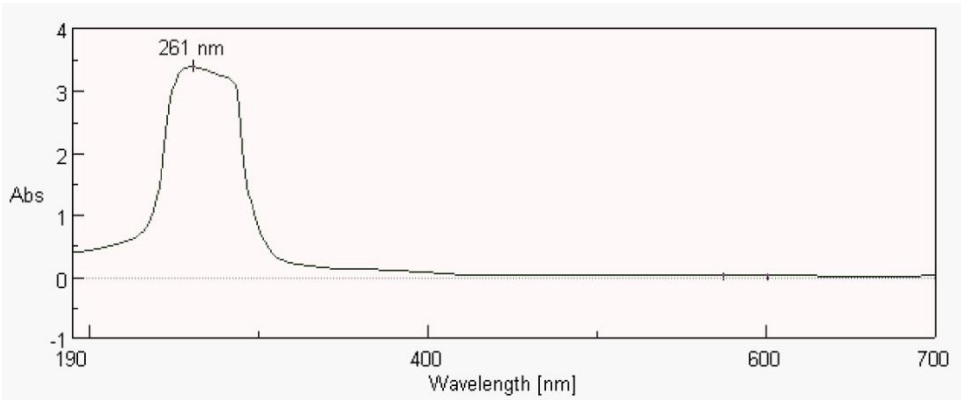
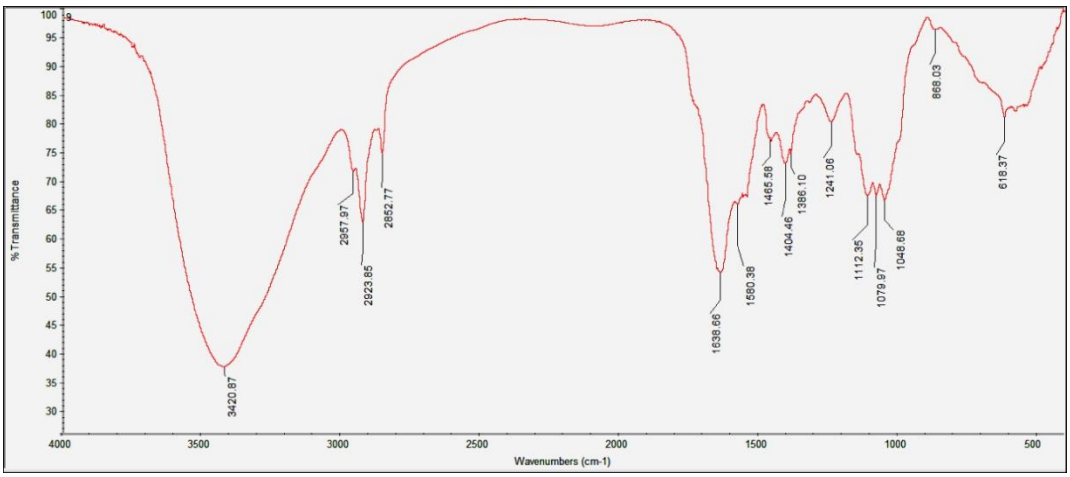


Fig.6 FTIR spectrum of *Streptomyces* strain SN-7



Physiological and biochemical characteristics of isolate SN-7

The physiological and biochemical characteristics of isolate SN-7 is summarized in Table 3. The SN-7 isolate at various pH levels for the growth and pigment production was found to be 5.0 to 8.0. The culture showed optimum growth and pigmentation at pH 7.5. However, pH below 7.5 and above 7.5 did not show any significant effect on growth and pigment production. Further, Growth characteristics of SN-7 at different temperature range (20 °C to 40 °C) showed optimum growth and pigmentation at 35 °C. SN-7 showed positive results against gram staining and spore staining. The biochemical characteristics of isolate SN-7 showed positive results against gram staining, spore staining, NaCl tolerance, hydrolysis of starch, casein, urea, and gelatin and motility test is negative, The carbohydrate fermentation and nitrogen reduction tests also showed positive results. The biochemical and physiological characteristics of the isolates differ from isolate to isolate and mainly depends on the growth conditions. In the present study physiological characteristics of SN-7 varied depending on the available nutrients in the medium and the physical conditions. Thus, it is concluded that on the basis of the present and previous studies that the nutrient compositions of the medium greatly influence the growth and morphology of organisms (Kamil *et al.,* 2014).

16S rRNA gene sequencing of isolate SN-7

In this result the active isolate SN-7 was identified as *Streptomyces* sp. based on the 16S rRNA gene sequence of the isolate and its phylogeny. Recent studies indicate that phylogenetic analysis has been key technique in the identification of *Streptomyces* sp. Several new species have been validated such as *Streptomyces thermoalcaltolerans*, *Streptomyces thermocarboxydovorans* and *Streptomyces viridis* isolated from different habitats (Song *et al.,* 2001). Further, 16S rRNA secondary structure and restriction site analysis of *Streptomyces* strain. SN-7 showed GC and AT content of 58% and 42%, respectively. The genomic DNA of SN-7 strain was isolated, analyzed by PCR amplification. The amplified product obtained and gel purified was further sequenced. A 1027 bp long, 16S rRNA partial gene sequence of isolate SN-7 was subjected to BLAST analysis, which confirmed that the SN-7 isolate belonged to the *Streptomyces* sp. (Figure 2a). The sequence of 1027 bp shared 98% sequence similarity with *Streptomyces* strain. The DNA sequences were aligned and a phylogenetic tree was derived by using MEGA4 software (Figure 2b).

The secondary RNA structure of SN-7 isolate 16S rRNA sequence was predicted (Figure 3). This prediction showed free energy of the structure as -238.8 kcal/mol, the threshold energy as -4.0 with cluster factor, conserved factor 2 and compensated factor 4 and the conservative factor of 0.8. The prediction of restriction sites in the strain SN-7 showed the restriction sites for various enzymes, such as *Bsr l, Bmr l, Bts l, Ssp l, Btsc I and Fok I* and possess GC and AT content of 58% and 42%, respectively (Figure 4).

UV-Visible Spectrophotometer and Fourier-transform infrared spectroscopy analysis of *Streptomyces* strain SN-7

The analysis indicated that methanolic extract comprised multiple functional groups. The UV spectral data for methanol extract of *Streptomyces* strain SN-7 fractions of column chromatography, the bioactive fraction exhibited a maximum UV absorption at 261 nm (Figure 5) and revealed the presence of

single major peak at 261nm. A similar observation has been reported previously (Cai *et al.,* 2008; Saravana *et al.,* 2014). FTIR study was carried out for identification of biomolecules present in the extract SN-7, the spectrum reveals that IR bands at 3420, 2957, 2852, 1638, 1580, 1465, 1404, 1386, 1241, 1112, 1079, 1048, 868 and 618 cm^{-1}(Figure 6 and Table 4). The band at 3420 cm^{-1} can be attributed to free OH stretching vibrations of alcohol. The sharp bands at 2923 cm^{-1} arise from stretching modes of aldehydic groups, whereas sharp bands at 1638 cm^{-1} correspond to N-H bending amines. The three medium peaks at 1048, 1079 and 1112 cm^{-1} may be from aliphatic amines of C-N stretching and band at 1386 cm^{-1} is due to CH_2 waging mode of alkanes. The peak at fingerprint region 618 and 868 cm^{-1} is assigned to carbonyl stretching of alkyl halides, these biomolecules are present in the KUASN-7 extract. Some important functional groups detected were alkyl halides, aliphatic amines, alkanes, aldehyde and alcohol group.

Currently, microbial infections have become a major clinical threat and are generally associated with a very high morbidity and mortality mainly due to the development of resistance by pathogens against existing antimicrobial agents. Therefore, antimicrobial susceptibility testing and discovery of novel antimicrobial agents have been extensively explored and continue to be developed. Findings from present study reveal that *Streptomyces paradoxus* strain KUASN-7 isolate shows promising antimicrobial properties. Further analysis is needed for purification and characterization of the purified secondary metabolite from SN-7 potent isolate.

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