Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 6, 2014

Original Article

TAXONOMIC STUDIES AND PHYLOGENETIC CHARACTERIZATION OF POTENTIAL AND PIGMENTED ANTIBIOTIC PRODUCING ACTINOMYCETES ISOLATED FROM RHIZOSPHERE SOILS

[1]TELUGU VARALAKSHMI *, [2]KALVA MADHANA SEKHAR AND [1]PETLA BHASKARA BRAMHANANDHA CHARYULU

[1]Department of Microbiology, Sri Krishna devaraya University, Anantapur-515055, Andhra Pradesh, India. [2]Department of Plant Sciences, University of Hyderabad 500046, Andhra Pradesh, India
Email: iamtvaru@gmail.com

Received: 11 May 2014 Revised and Accepted: 12 Jun 2014

ABSTRACT

Objective: To investigate the production of antibiotics from pigment producing actinomycetes isolated from the rhizosphere of three different plant soils (Foxtail millet, Groundnut and Mulberry) from different localities of Anantapur region, Andhra Pradesh, India.

Methods: A total of 30 actinomycetes were isolated and tested against, pathogenic bacteria and fungi. Out of 30 isolates, 10 promising isolates [A2, A5, A8, A12, A14, A17, A21, A24, A27 and A29] showed activity against pathogenic bacteria and fungi by primary screening. Out of ten active isolates, potential isolate A2 was subjected to secondary screening, identification and fermentation methods. Identification is by morphological, biochemical and molecular methods. A phylogenetic tree was constructed by maximum parsimony method to identify up to the species level. The secondary structure of 16S rRNA and the restriction sites were also predicted using Genebee and NeBCutter online software's respectively. Fermentation is by submerged state to produce crude extracts. The fermented biomass was extracted by organic solvent extraction method and tested against the bacterial strains by agar well diffusion method.

Results: Molecular taxonomy and phylogeny revealed that the strain belongs to the genus *Streptomyces* and was designated as *Streptomyces rameus KCTC* 9767. RNA secondary structure predicted for 16S rRNA gene of *S. rameus* showed the free energy of -98.5kkal/mol and the restriction site analysis showed the GC and AT content to be 59% and 41%.The active metabolite was extracted using ethyl acetate and screened for antimicrobial activity. A maximum zone of inhibition were observed (29 mm) for Gram positive bacteria and (30 mm) for fungi. The MIC was 3.01 mg/ml against *Staphylococcus aureus* and 1.55 mg/ml against *Klebsiella pneumonia* and *Saccharomyces cerevisiae.*

Conclusion: The results of the present study revealed that rhizosphere actinomycetes have immense potential as a source of antibacterial compounds.

Keywords: Rhizosphere, *Streptomyces rameus*, Fermentation, Agar well diffusion method, MIC, Antibacterial activity.

INTRODUCTION

The usage of antimicrobial drugs for prophylactic or therapeutic purposes regularly in human, veterinary and agricultural purposes were favoring the survival and spread of resistant organisms. Due to this multidrug resistant pathogenic strains were causing substantial morbidity and mortality especially in elderly people and immune compromised patients. To overcome the present situation, there is an interest to discover novel antibiotics and new therapeutic agents by continuous screening of secondary microbial products produced from potential bacterial taxa [1].

The importance of soil sources for the discovery of novel natural products with a pharmaceutical potential has been proved during the last decade. Actinomycetes, belongs to the order Actinomycetales produce a wide range of secondary metabolites.

More than 70% of the naturally derived antibiotics which are in clinical use were derived from soil actinomycetes [2]. Among the 140 described actinomycete genera, only a few are responsible for the production of over 20,000 microbial natural products. Particularly, the genus *Streptomyces* accounts for about 80% of the actinomycete natural products reported to date [3]. Natural organic compounds produced by microorganisms are important for screening to produce a variety of bioactive substances [4]. Compound of actinomycete origin, in particular are valuable in the field of bioactive natural products [5].

Plant root produce organic compounds into the rhizosphere soils, contains high microbial biomass and activity when compare to non-rhizosphere soils. These organic compounds may inhibit the growth of some microbial population [6]. Plant rhizosphere soils represent a unique biological niche with a diverse microflora comprised of bacteria, fungi, actinomycetes, protozoa and algae. This community was supported nutritionally by a high input of organic materials derived from the plant roots and root exudates that are necessary for microbial growth [7]. However, the composition and quantity of root exudates varies depending on the plant species [8] and physical environment such as humidity and temperature [9].

The actinomycetes are well known important saprophytic bacteria in the rhizosphere, where they may influence the plant growth and protect plant roots against the invasion of root pathogenic fungi [10]. Filamentous soil bacteria belonging to the genus *Streptomyces* are rich sources of antibiotics, which are extensively using in pharmaceuticals and agrochemicals [11]. At present greater research has been taking place for novel antibiotics from different untouched habitats. Some of them they isolated from rhizosphere of *Azadirachta indica, Hibiscus rosasinensis*. No significant studies have been conducted so far to isolate and evaluate the pigment producing actinomycetes from rhizosphere of foxtail millet, groundnut and mulberry plants.

The current study was designed to describe the isolation of an pigment producing actinomycetes associated with the root system of the three different rhizosphere plant soil samples (*Setaria italica, Arachis hypogaea* and *Morus alba*) which generates an antimicrobial compounds. Isolation, characterization and identification of the isolate were reported. The primary bioactive substance was isolated by fermentation and extraction process, its biological activities were determined. The active metabolites extracted from the isolate were subjected to MIC against the Gram positive, Gram negative bacteria and Fungi. The active metabolites were partially purified by TLC.

MATERIALS AND METHODS

Sampling area

The soil samples were collected from the rhizosphere of all the three plants (means the soil present around the roots).

Sample collection

Soil samples were collected from the rhizosphere with the sterile spatula and transferred to ploythene bags which are labeled and transported to microbiology laboratory and stored at 4°C for further studies.

Pretreatment of soil samples

Soil samples were subjected to three physical and chemical pretreatment methods in order to facilitate the better isolation of actinomycetes population [12]. These soil samples were air-dried and subjected to Heat treatment up to 40-45°C for 15 h to kill the Gram Negative bacteria, 1.4% of phenol solution was mixed with one gram of soil sample and incubated at room temperature for 10 min to kill the normal bacteria and to the equal amounts of soil sample and $CaCO_3$ were mixed with sufficient amount of water and incubated at room temperature for one week to enrich the soil for better isolation of actinomycetes.

Isolation and maintenance

Soil samples were serially diluted by diluting one gram of soil sample in 9 ml of sterile distilled water and shaken vigorously to make a stock solution. From this stock solution, 1ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} by serial dilution method. Finally, 0.1ml of soil sample suspension from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were used to spread on sterilized starch casein agar medium by using L-shaped glass rod and incubated at 37°C for 7 days. Triplicates were maintained for each dilution. After 7 days, the plates were observed for actinomycetes colonies and sub-cultured on starch casein agar slants and stored at 4°C for further studies. For every 30 days the cultures were sub-cultured freshly for better bioactive production.

Bacterial strains

In the present study pathogenic organisms used were *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Micrococcus luteus, Klebsiella pneumonia, Pseudomonas aeuroginosa* and filamentous fungi like *Saccharomyces cerviciae* and *Candida albicans*.

Primary screening of isolates

During the primary screening, isolates were screened against selected bacterial strains by using perpendicular streak method. Antagonistic activity of isolates against Gram positive and Gram negative bacteria was primarily screened by using perpendicular streak method. In this method, nutrient agar media was used and each plate was streaked with individual isolates at the center of the plate and incubated at 37°C for 7 days. Later, 24 h fresh sub-cultured test bacteria were prepared and streaked perpendicular to the isolates and incubated at 37°C for 24 h [13].

Production of crude extracts

The potential actinomycete was subjected to submerged state fermentation method to produce crude extracts. A loopful of the isolate actinomycete strain A2 from the 3rd day culture age was inoculated into 250 ml Erlenmeyer flasks containing 100 ml of liquid starch casein medium. The flasks were incubated on a rotary shaker (200 rpm) at 35°C for 7 days. The total volume was filtered by centrifugation at 5000 rpm at 10°C for 30 minutes. To the equal volume of the clear filtrate, ethyl acetate was added and subjected to the shaking on a rotary shaker at 250 rpm for 2-3 h. The organic phase was separated from aqueous phase by using separating funnel and concentrated to dryness under vacuum using rotary evaporator at a temperature not exceeding 50°C [14].

Secondary screening of the isolate

In the secondary screening the isolate is subjected to submerged state fermentation method to produce crude extracts and antimicrobial activity was checked against test bacteria at regular intervals (i.e., 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h) by agar well diffusion method.

Agar well diffusion method

Nutrient agar plates were prepared and spread with test bacteria. Then wells were prepared by using sterile cork borer (6mm in diameter). A volume of 50 µl of crude extracts was dispensed into each well and allowed to diffuse for 2 h and incubated at 37°C for 24 h. The sterilized phosphate buffer was filtered and used as control. After 24 h of incubation, zone of inhibition around each well was recorded.

Determination of antimicrobial activity

The (MIC) minimum inhibitory concentration has been determined by the two fold serial dilution method [15]. In this method pathogenic strains were used. A volume of 1ml of nutrient broth was dispensed into test tubes 1-10 and 2ml into test tube11 (broth control). 1ml of the crude extract solution was added into the test tubes 1 and 2 and 2 ml to test tube 12 (crude extract control). One millimeter of well mixed solution was transferred from test tube 2 to 3 and this process was continued serially up to the test tube10 by mixing and changing the micropipette tips at each dilution. Finally 1ml was discarded from test tube 10 and 0.1 ml of standardized inoculums (test organisms) was added into test tubes1-10 and incubated at 37°C for 18-24 h. After incubation, by observing the growth of bacteria in the test tubes MIC value was determined.

Taxonomic studies of actinomycete isolate

Morphological characteristics of the most potent produce isolate A2 grown on starch casein medium at 37°C for 7 days was examine under light microscopy. Biochemical characteristics such as Catalase test [16], Melanin pigment [17], Nitrate reduction [18], H_2S production, Citrate utilization, Coagulation of milk as well as the Utilization of Carbon sources [19] were monitored using standard methods. The cultural characteristics like color of aerial and substrate mycelium were studied on different ISP and Non-ISP media in accordance with the guide lines established by the ISP (International *Streptomyces* Project) [20]. Colors characteristics were assessed on the scale developed by Kenneth and Deane [21].

DNA isolation, Amplification and Sequencing of the 16S rRNA gene

The isolated actinomycete strain A2 was grown for 7 days on a starch casein agar slant at 35°C. Two ml of a spore suspension were inoculated into the starch casein broth and incubated for 3 days on a shaker incubator at 200 rpm and 35°C to form a pellet of vegetative cells (pre-sporulation). The preparation of total genomic DNA was conducted as described by [22].

PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF and Strep R [23]. The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 µM dNTPs and 2.5 units of Taq polymerase, in 50 µl of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C and 2 min of extension at 72°C.

The PCR reaction mixture was then analyzed via agarose gel electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents. The 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method [24]. The 16S rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit

Sequence similarities and Phylogenetic analysis

The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software [25]. The Phylogenetic tree was displayed using the TREEVIEW program.

Secondary structure prediction and Restriction site analysis

The RNA secondary structure of the isolate A2 was predicted using Gene bee online software (http:// www.genebee. msu.su/services/ rna2_reduced.html) by greedy method and the restriction sites of the DNA of the strain were analyzed by NEB cutter version 2.0 (http://tools.neb.com/NEBcutter2/).

Purification of antimicrobial compound

Purification by TLC

The crude extracts of the isolate A2 was partially purified by TLC by using silica gel slides. The solvents used include chloroform and methanol (24:1, v/v), methanol and acetic acid (9.2:0.8), n-butanol, acetic acid, water (4:1:0.5) as a solvent system. The solvent was allowed to run though silica gel layer until it reaches up to 1 cm of the top of the slide. Then they were removed from the bottles. The solvents were allowed to evaporate from the slides and transferred to screw cap bottles containing a few crystals of iodine and subjected to bioautography. Streptomycin is used as a reference antibiotic.

UV maximum absorption

The spot obtained from the TLC was collected, dissolved in methanol and subjected to UV maximum absorption spectrum. Streptomycin is used as a reference antibiotic.

Data analysis

The data collected from the above methods were subjected to one way ANOVA to compare the level of significance ($p \leq 0.05$) within the isolates by using statistical package Sigma Plot 11.0 and the results were interpreted.

RESULTS

Actinomycetes isolation and primary screening

30 isolates were isolated from the rhizosphere soils, only 10 isolates showed antimicrobial activities against at least one of the tested bacteria. The results in primary screening revealed that the isolate A2 exhibited broad spectrum activity against Gram positive bacteria like *B. subtilis* and *S. aureus*, fungi *S. cerevisiae* and *C. albicans* and Gram negative bacteria such as *P. aeruginosa, M. luteus, E.coli*, *K. pneumonia*. A12, A21 and A29 showed highest zone of inhibition against Gram positive bacteria. A5, A8, A14 and A24 showed potential activity against Gram positive bacteria when compared to Gram negative bacteria. A17 and A27 showed highest zone of inhibition against Gram negative bacteria. A8, A14, A17, A24 and A29 showed activity against fungi like *S. cerevisiae* and *C. albicans*. All the 10 isolates showed greater antimicrobial activity against Gram positive bacteria, fungi when compared to Gram negative bacteria. The results were tabulated in Table 1.

Table 1: Primary screening of Antimicrobial properties produced by actinomycetes isolated from rhizosphere soils

	*Mean values of inhibition zones (in mm) against							
	Bacteria					Fungi		
Isolates	***B. subtilis***	***S. aureus***	***P. aeruginosa***	***M. luteus***	***K. pneumonia***	***E. coli***	***S. cerevisiae***	***C. albicans***
A2	29.0±0.20	29.0±0.26	27.0±0.24	18.0±0.20	18.0±0.24	18.0±0.27	29.0±0.22	30.0±0.20
A5	22.0±0.40	20.0±0.60	18.0±0.37	16.0±0.22	15.0±0.37	17.0±0.22	-	15.0±0.28
A8	20.0±0.65	21.0±0.34	18.0±0.32	16.0±0.21	16.0±0.32	16.0±0.17	16.0±0.37	16.0±0.27
A12	25.0±0.30	23.0±0.18	-	-	-	-	-	-
A14	20.0±0.10	24.0±0.23	20.0±0.28	14.0±0.40	16.0±0.28	14.0±0.21	15.0±0.38	18.0±0.28
A17	-	-	18.0±0.33	-	14.0±0.33	14.0±0.32	18.0±0.22	18.0±0.15
A21	22.0±0.31	20.0±0.20	-	-	-	-	-	-
A24	20.0±0.33	22.0±0.15	19.0±0.28	12.0±0.22	12.0±0.28	12.0±0.40	15.0±0.31	15.0±0.17
A27	-	-	20.0±0.25	12.0±024	15.0±0.35	10.0±0.34	-	-
A29	24.0±0.22	22.0±0.31	-	-	-	-	15.0±0.32	14.0±0.40

Values are mean±SD of three replications

Table 2: Secondary screening of Antimicrobial properties produced by actinomycete isolate A2 at different intervals

	*Mean values of inhibition zones (in mm) against								
	Bacteria					Fungi			
A2	***B. subtilis***	***S. aureus***	***P. aeruginosa***	***P. mirabilus***	***E. Coli***	***M. luteus***	***K. pneumonia***	***S. cerevisiae***	***C. albicans***
24 h	15±0.45	15±0.38	8±0.41	8±0.24	5±0.35	6±0.31	8±0.44	10±0.42	15±0.36
48 h	20±0.32	19±0.30	10±0.51	8±0.43	8±0.32	8±0.40	12±0.21	12±0.41	18±0.21
72 h	22±0.21	23±0.20	20±0.48	10±0.48	10±0.28	12±0.28	12±0.25	17±0.38	20±0.18
96 h	25±0.22	25±0.18	23±0.32	16±0.33	14±0.24	14±0.27	14±0.30	22±0.32	25±0.20
120 h	24±0.18	23±0.24	20±0.28	14±0.20	12±0.30	12±0.18	12±0.34	19±0.31	20±0.17
144 h	22±0.23	20±0.26	12±0.33	12±0.18	12±0.34	12±0.20	12±0.37	17±0.44	18±0.31
168 h	12±0.42	13±0.43	10±0.50	8±0.24	8±0.40	8±0.30	8±0.21	12±0.46	16±0.43

Values are mean±SD of three replications

Fig. 1: Antimicrobial properties produced by the isolate A2 against *Bacillus*, *Staphylococcus*, *Psuedomonas* and *E.coli*

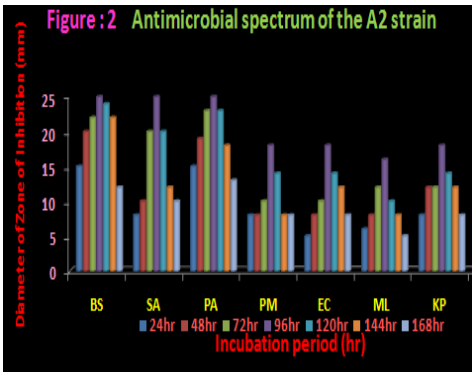


Fig. 2: Antimicrobial spectrum of the A2 strain

Secondary screening of the isolate A2

The crude extracts were extracted at regular intervals i.e., 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h from A2 potential isolate by using submerged state fermentation and solvent extraction method and subjected to secondary screening by agar well diffusion method. A2 isolate showed maximum zone of inhibition (25 mm) at 96 h i.e., fourth day of incubation. The isolate showed high activity against positive bacteria like *B. subtilis* and *S. aureus*, fungi *S. cerevisiae* and *C. albicans* and Gram negative bacteria such as *P. aeruginosa* and less activity against the *E.coli, M. luteus* and *K. pneumonia.* The results were statistically significant≤(P0.05) and the results were tabulated in Table 2. and (Fig. 1 and 2).

Determination of MIC

MIC data of the crude extracts of the isolate A2 ranged from 1.05 to 3.01 mg/ml against Gram positive bacteria (*S.aureus*) and Gram negative bacteria ranged from 1.02 to 1.55 mg/ml and fungi showed MIC at 6.02 mg/ml. The results were represented in Table 3.

Morphological characteristics of selected isolate A2

Results of morphological characteristics of the selected isolate A2 revealed that the growth was excellent on ISP and Non-ISP media except in ISP4 and ISP9 and Czapeck's Dox medium. The color of aerial and substrate mycelium varied on ISP and Non-ISP media. The color of aerial mycelium of the isolate A2 on starch casein agar medium was grey and substrate mycelium was light yellowish brown or light grayish yellow and produces yellow color pigment. The results were tabulated in Table 4. and (Fig. 3).

Fig. 3: Morphology of *Streptomyces rameus* KCTC 9767

Biochemical characteristics of the isolate A2

Biochemical characteristics of the isolate A2 was positive for hydrolysis of Starch, Protein, Catalase, Melanin production, H_2S production, Nitrate reduction, Coagulation of milk and negative for IMViC, Urease test. All the biochemical results were tabulated in Table 5. Growth on thallous acetate with 0.001% and sodium azide with 0.01% was negative and growth on phenol with 0.1% was positive. The incubation period of the isolate is 37°C for 7 days and the isolate showed resistance to some antibiotics such as Amphicillin (10µg), Cephalexin (30µg), Erythromycin (15µg) and Rifampicin (5µg).

Table 3: Biological activities (MIC) of the crude extract

Test organisms	MIC (mg/ml) concentrations
Staphylococcus aureus	3.01
Bacillus subtilis	1.05
E.coli	1.52
Micrococcus luteus	1.52
Klebsiella pneumonia	1.55
Pseudomonas aeruginosa	1.02
Saccharomyces cerevisiae	1.55
Candida albicans	6.02

Table 4: Morphological characteristics of the isolate A2

S. No.	Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1.	Nutrient agar	Good	246-L.Gray Grayish white	76-1-y-br Light yellowish brown	Yellow
2.	Czapeck's Dox agar	Moderate	246-L.Gray Grayish white	92-y White Yellowish white	No
3.	Starch casein agar	Very good	92-y White Yellowish white	76-1-y-br Light yellowish brown	Yellow
4.	Potato dextrose agar	Very good	246-L.Gray Grayish white	92-y White Yellowish white	No
5.	Tryptone yeast extract agar (ISP-1)	Very good	246-L.Gray Grayish white	76-1-y-br Light yellowish brown	58m- br Brown
6.	Yeast extract malt extract agar (ISP-2)	Very good	92-y White Yellowish white	76-1-y-br Light yellowish brown	Yellow
7.	Oat meal agar (IPS-3)	Very good	92-y White Yellowish white	76-1-y-br Light yellowish brown	Yellow
8.	Inorganic salts starch agar (ISP-4)	Good	246-L.Gray Grayish white	92-y White Yellowish white	Yellow
9.	Glycerol asparagine agar (ISP-5)	Very good	246-L.Gray Grayish white	92-y White Yellowish white	58m- br Brown
10.	Peptone yeast extract iron agar (ISP-6)	Very good	246-L.Gray Grayish white	76-1-y-br Light yellowish brown	58m- br Brown
11.	Tyrosine agar (ISP-7)	Very good	92-y White Yellowish white	76-1-y-br Light yellowish brown	58m- br Brown
12.	Inorganic salts agar (ISP-9)	Moderate	246-L.Gray Grayish white	92-y White Yellowish white	Yellow

Table 5: Biochemical characteristics of isolate A2

Characteristic	Result
Spore mass	Gray
Spore surface	Smooth
Spore chain	Spiral
Color of substrate mycelium	Light yellowish brown
Diffusible pigment	Yellow pigment
Hydrolysis of Protein and Starch	+
Catalase test	+
Production of melanin pigment	+
H2S Production	+
Nitrate reduction	+
Citrate utilization	-
Urea test	-
Coagulation of milk	+
Utilization of	
D-Mannose	+
D-Glucose	+
D-Galactose	+
Rhamnose	+
Raffinose	+
Mannitol	+
L-Arabinose	+
Meso-Inositol	+
Lactose	+
Maltose	+
Trehalose	+
D-fructose	+
Sucrose	+
Starch	+
Growth with	
Thallous acetate (0.001)	-
Sodium azide (0.01)	-
Phenol (0.1)	+
Growth temperature	30°C (20-45°C)
Optimum pH	7.0 (5.5-7.5)
Resistance to antibiotics	
Amphicillin (10 µg); Cephalexin (30 µg); Erythromycin (15 µg); Rifampicin (5 µg)	+
Antimicrobial activity against	
Bacillus subtilis	+
Staphylococcus aureus,	+
Saccharomyces cerevisiae	+
Candida albicans	+

+= Positive, - = Negative

Identification of isolate A2

This was performed basically according to the recommended international key's and numerical taxonomy of *Streptomyces* species program. On the basis of the previously collected data and in view of the comparative study of the recorded properties of A2 in relation to the closest reference strain with a 99% similarity with *S. tricolor* strain AS.4.1867. It could be stated that actinomycetes isolate as *S. rameus* KCTC 9767 (Fig. 4).

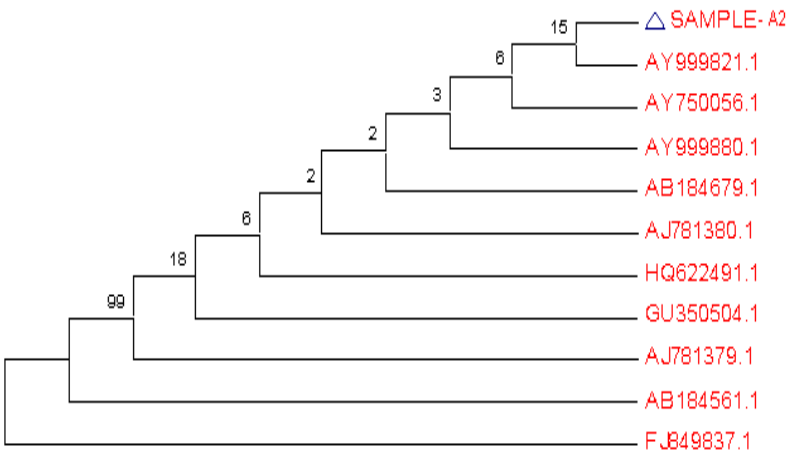


Fig. 4: Phylogenetic tree of *Streptomyces rameus* KCTC 9767

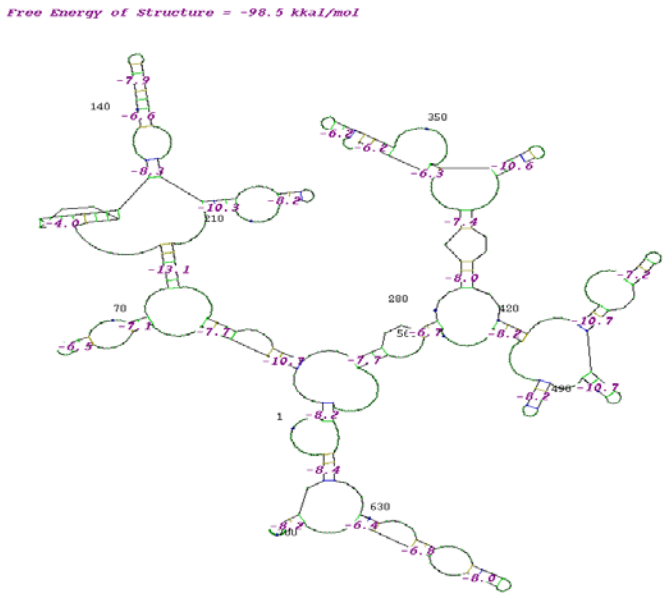


Fig. 5: Secondary structure prediction of 16S rRNA of the strain *Streptomyces rameus* KCTC 9767 was done using Genebee online software

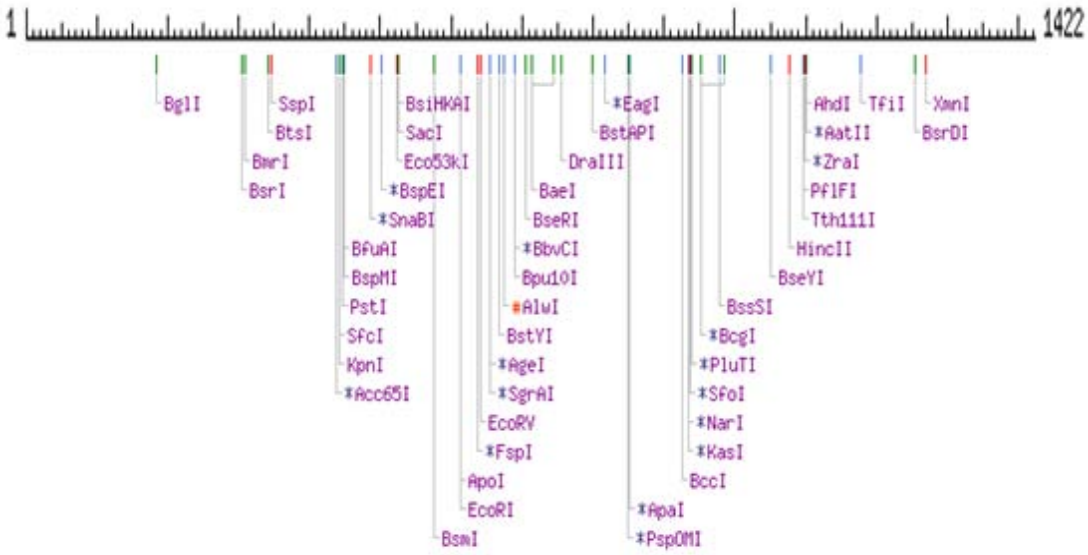


Fig. 6: Restriction sites of the strain *Streptomyces rameus* KCTC 9767 were predicted using NEB cutter

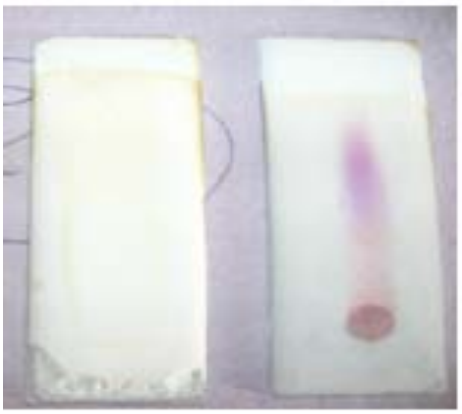
Secondary structure prediction and Restriction sites analysis

The RNA secondary structure predicted for 16SrRNA gene of *Streptomyces rameus* KCTC 9767 showed the free energy of -98.5 kkal/mol, threshold energy of -4.0 with Cluster factor 2, Conserved factor 2 and Compensated factor 4 (Fig. 5).

Similarly the restriction sites prediction of the 16SrRNA gene of *Streptomyces rameus* KCTC 9767 showed restriction sites for various commercial and NEB restriction enzymes such as EcoR1, EcoRV, BaeI etc. Also the restriction site analysis showed the GC and AT content to be 59% and 41% (Fig. 6).

Purification of antimicrobial compound

Separation of antimicrobial agent into individual components was carried out by thin layer chromatography using a different solvent system composed of chloroform and methanol (24:1, v/v), methanol and acetic acid (9.2:0.8), n-butanol, acetic acid, water (4:1:0.5). Among the different solvent system, chloroform and methanol (24:1) showed only one band with Rf value 0.75 showed only one zone of inhibition by bioautography (Fig. 7).



S A2

Fig. 7: TLC slides with solvent system chloroform, methanol, for extracts of A2 with streptomycin

The active fraction obtained from TLC was collected mixed in methanol and subjected to UV maximum absorption spectrum. The active fraction showed the maximum absorption spectrum between 200-210 nm.

DISCUSSION

Rhizosphere is one of the most important factors which determine the population structure of a specific soil area and produce certain compounds (such as cellulose, sugar, aromatic acid and amino acids) can attract some species and repel others, leading to the structure of different communities. The presence of large populations of Streptomycetes in the rhizosphere of samples is consistent with previous reports which showed that actinomycetes are very common in plant root systems [26, 27 and 28]. At present, the incidence of multidrug resistant organisms is increasing and compromising the treatment of a growing number of infectious diseases. Due to this reason, there is a need to develop new drugs which are effective against current antibiotic resistant pathogens. Actinomycetes have been proven as a potential source of bioactive compounds and rich source of secondary metabolites [29]. Isolation of antimicrobial compounds from the rhizosphere was an advance interest in present situation to isolate novel bioactive actinomycetes. Saadoun and Al-Momani; Saadoun; Ndonde and Semu reported that 83% of soil actinomycetes were active against one or more of the test organisms [30, 31 and 32]

In the present study 30 actinomycetes were isolated from rhizosphere and only 10 isolates showed wide range of zone of inhibition against Gram positive bacteria and fungi than Gram negative bacteria in the primary screening. Hamdi, Hussen and Saadoun reported that streptomycete isolates appear to be highly active against Gram positive bacteria when compare to Gram negative bacteria [33, 34 and 31] Mohamed and Crawford showed the percentage of actinomycetes isolate active against fungi during screening studies [35 and 36].

Among the 10 isolates only one i.e., A2 isolate has broad spectrum activity and subjected to secondary screening. In the secondary screening the isolate A2 was subjected to submerged state fermentation to produce crude extracts. Submerged state fermentation process produced higher yields because presence of sufficient water, but immiscible in organic solvents like ethyl acetate. A2 isolate produced higher yields when compared to Gebreselema [1]. The antimicrobial activity of the crude extracts was checked at every 24 h regular intervals up to 7 days, and highest zone of inhibition against test organism was observed at 96 h i.e., fourth day of incubation because the isolate produces two types of phases, trophophase and idiophase. In trophophase, the isolate utilizes the media to increase the biomass and in the idiophase it produces secondary metabolites. Prit and Righelato and Lurie reported that two phases were observed during antibiotic production. The first phase (trophophase) was characterized by a rapid growth (biomass production) and the second phase (idiophase) was characterized by a slow growth and maximal productivity of antibiotics [37 and 38]. Sejiny observed the growth pattern of five *Streptomyces* isolates for 12 days of incubation. Biomass and bioactive metabolite reached maximum at stationary phase and then decreased. The bioactive metabolites extracted from these five isolates showed high antimicrobial activity against a Gram-positive bacterium, *Staphylococcus aureus* [39]. Porter reported that all streptomycetes possessed antimicrobial properties, if proper culture conditions were taken for antibiotic production [40]. The crude extracts of the isolate A2 showed wide range of inhibition zone against tested bacterial strains when compared to reports of Narayana and Vijayalaksmi [41]. Ilic, gave results clearly that the Gram positive bacteria were highly susceptible to the tested crude extracts when compare to Gram negative bacteria. Gram negative bacteria have lipopolysaccaharide as an outer membrane which is impermeable to lipophilic extracts. But a Gram positive bacteria were more susceptible because lack of lipopolysaccaharide as an outer membrane [42].

The MIC results varied among the isolate A2 against *S. aureus, E. coli* and *C. albicans*. Sibanda and Lilia Fourati reported the similar results of MIC with *S. aureus* [43 and 44]. Therefore, the crude extracts obtained from the isolate has potent source for antibiotic production, which leads to the production of novel compounds, used for the treatment of infectious diseases. The report of antibacterial activities of actinomycetes isolated from rhizosphere soils of foxtail millet, ground nut and mulberry was first hand information as per our knowledge.

Different colors of aerial mycelium, substrate mycelium and pigment was observed on the different culture media in which the isolate was grown. Among the ISP and Non-ISP media used, growth of the isolate A2 was excellent in ISP media, except in ISP4 and ISP9 and Non-ISP media except Czapeck's Dox agar medium. Saadoun and Arai reported that most active species of *Streptomyces* were found in the grey and yellow series and no antibiotic producing species were described in the white and green series [31 and 45]. The A2 isolate in this study has an ability to hydrolyse starch and protein, utilizes all the carbon sources, produce melanin and hydrogen sulphide, reduces nitrate and coagulate the milk and has resistant to some antibiotics like amphicillin, cephalexin, erythromycin and rifampicin. The morphological characteristics and microscopic examination emphasized that the spore chain is spiral, spore mass is light grey, spore surface is smooth; substrate mycelium is light yellowish brown. All these results emphasized that the actinomycete isolate A2 belongs to the group Streptomycetes [46].

In the past years the identification of actinomycetes was difficult, slow and very expensive, but now a days by the molecular approach the identification process was easy and can obtain results in less time. Katoaka observed that the phylogenetic analysis based on the 120 bp sequence bearing the variable α region is useful for *Streptomyces* species identification [47]. They conclude that this type of the phylogenetic tree will serve as a useful tool for rapid identification of the phylogenetic localization of newly isolated *Streptomyces* strains and it is more effective than the conventional methods. The isolate A2 subjected to 16SrDNA sequence analysis and identified as *Streptomyces rameus* KCTC 9767. By the secondary structure prediction and restriction site analysis, one can calculate the free energy and percentage of GC and AT contents by using restriction site enzymes.

Purification of crude extracts has been carried out by TLC using a solvent system chloroform and methanol (24:1). The Rf value was 0.75 which reported only one spot. The result of bioautography showed only one inhibition zone around the spot. The bioautography of the purified antibiotic was conducted by Weinstein and Wagman, it is used to detect only one inhibition zone that reveals the purity of the antibiotic [48]. Atta, showed similar results with Rf value of 0.75 using same solvent system [49]. Augustine reported the results with Rf value 0.78 using a solvent system ethanol, water, chloroform (40:40:20) [50]. Ahmed reported the purification of the antibiotics by TLC by using a solvent system, benzene-ether (1:1). The Rf value was 1.37 which reported only one spot [51].

The UV spectral data of the ethyl acetate extract showed maximum absorbance peaks ranges between 200-210 nm and the characteristic of absorption peaks indicates presence of amino glycosides. The A2 isolate produce a broad spectrum of antimicrobial compound with different activities. Further investigation is needed in order to study the structure of active compound.

CONCLUSION

From this study we have concluded that, the antimicrobial substances produced by *S. rameus* KCTC 9767 isolated from rhizosphere soils was effective. By morphological and biochemical characteristics it is concluded that the isolate belongs to Streptomyces and the isolate produces yellow color pigment. In phylogenetic relation, it shows 99% similarity with S. tricolor with Bootstrap value 100. This strain produced maximum inhibition zone for Gram positive bacteria and fungi when compared to Gram negative bacteria. TLC and UV maximum absorption spectrum results revealed that the *S. rameus* KCTC 9767 produces an aminoglycoside antibiotic. All the purification data concluded that the *S. rameus* KCTC 9767 produce an aminoglycoside antibiotic, may be equal to that of Streptomycin. Purification and characterization of the antimicrobial compound was done for further studies.

ACKNOWLEDGEMENTS

I am grateful to UGC, University Grants Commission, Bahadurshah Zafar Marg, New Delhi for sponsoring Meritorious Fellowship for the progress of the work.

REFERENCES

1. Gebreselema G, Feleke M, Samuel S, Nagappan R, J. Isolation of potential antibiotic producing actinomycetes from water and sediments of lake Tana, Ethiopia. Asian Pac. J. of Trop. Biomed. 2013;3(6):426-35.
2. Atta HM, Bayoumi R, Sehrawi M, Galal GF, Governorate KSA. EI-Taxonomic studies and phylogenetic Characterization of Streptomyces rimosus-KH- isolated from Al-Khurmah Governorate KSA. Researcher 2011;3(9):1223-55.
3. Bull AT, Stach JEM. Marine actinobacteria: new opportunities for natural product search and discovery. Trends in microbiology 2007;15(11):491-99.
4. Chhiaki I, Naoko K, Masazumi K, Naoko H. Takeshi k, Isolation and characterization of antibacterial substances produced by marine actinomycetes in the presence of seawater. Actinomycetol. 2007;21:27-31.
5. Arai T. Actinomycetes, the boundary Microorganisms. Toppan, Tokyo. 1976;123.
6. Li J, Zhao G-Z, Huang H-Y, Zhu W-Y, Lee J-C, Kim C-J, et al. Pseudonocardia rhizophila sp. nov., a novel actinomycete isolated from a rhizosphere soil. Antonie van Leeuwenhoek 2010;98(1):77-83.
7. Lynch JM. Introduction: some consequences of microbial rhizosphere competence for plant and soil1990. 1-10.
8. Smith WH. Character and significance of forest tree root exudates. Ecol. 1976;57:324-31.
9. Martin JK, Kemp JR. Carbon loss from roots of wheat cultivars. Soil Biol and Biochem 1980;12:551-54.
10. Yilmaz EI, Yavuz M, Kizil M, J. Molecular characterization of rhizosphere soil Streptomycetes isolated from indigenous Turkish plants and their antimicrobial activity. World j. of Biotechnol. 2008;24:1461-70.
11. Manteca A, Alvarez R, Salazar N, Yagüe P, Sanchez J. Mycelium differentiation and antibiotic production in submerged cultures of Streptomyces coelicolor. Appl. and Environmental Microbiology. 2008;74(12):3877-86.
12. Hong K, Gao A-H, Xie Q-Y, Gao H, Zhuang L, Lin H-P, et al. Actinomycetes for marine drug discovery isolated from mangrove soils and plants in China. Marine drugs 2009;7(1):24-44.
13. Williams ST, Goodfellow M, Wellington EM, Vickers JC, Alderson G, Sneath PH, et al. A probability matrix for identification of some Streptomycetes. J. of Gen. Microbiol. 1983;129(6):1815-30.
14. Westley JW, Evans RH, Sello LH, Troupe N, Liu CM, Blount JF. Isolation and characterization of antibiotic X-14547A, a novel monocarboxylic acid ionophore produced by Streptomyces antibioticus NRRL 8167. The Journal of antibiot. 1979; 32(2): 100-7.
15. Andrews JM. Determination of minimum inhibitory concentrations. The Journal of antimicrobial chemotherapy 2001;48 Suppl 1:5-16.
16. Jones kl. fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. Journal of bacteriology 1949;57(2):141-5.
17. Pridham TG, Anderson P, Foley C, Lindenfelser LA, Hesselting CW, Benedict RG, et al. A selection of media for maintenance and taxonomic study of Streptomycetes. Antibiot. Ann. J. 1957:947-53.
18. Gordon RE. Some criteria for the recognition of Nocardia madurae (Vincent) Blanchard. J. of Gen. Microbiology 1966;45(2):355-64.
19. Pridham TG, Gottlieb D. The Utilization of Carbon Compounds by Some Actinomycetales as an Aid for Species Determination. J. of bacteriol. 1948;56(1):107-14.
20. Shrilling EB, Gottlieb D, J. Methods for characterization of Streptomyces species. Int. J. of Syst. Bacteriol. 1966;16:313-40.
21. Kenneth LK, Deane BJ. Color universal language and dictionary of names. United States Department of Commerce National Bureau of standards Washington DC 4 2023.
22. Sambrook J, Fritsch EF, Maniaties T, Spring USA, Harbor S, New H. Molecular cloning. A laboratory Manual 2 edition Cold Spring, Harbor Laboratory press, Cold Spring Harbor, New York, USA.
23. Edwards U, Rogall T, Blöcker H, Emde M, Böttger EC. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic acids research 1989;17(19):7843-53.
24. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Sciences of the United States of America 1977;74(12):5463-7.
25. Hall TA. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nuc Acid Symp Series1999. 95-8 p.
26. Atalan E, Manfio GP, Ward AC, Kroppenstedt RM, Goodfellow M. Biosystematic studies on novel streptomycetes from soil. Antonie van Leeuwenhoek 2000;77(4):337-53.
27. Sembiring L, Ward AC, Goodfellow M. Selective isolation and characterisation of members of the Streptomyces violaceusniger clade associated with the roots of Paraserianthes falcataria. Antonie van Leeuwenhoek 2000;78(3-4):353-66.
28. Ouhdouch Y, Barakate M, J. Actinomycetes of Moroccan habitats; isolation and screening for antifungal activities. Eur. J. of Soil Biol 2001;37:69-74.
29. Suthindhiran K, Kannabiran K, J. Cytotoxic and antimicrobial potential of actinomycete species Saccharopolyspora salina VITSDK4 isolated from the Bay of Bengal Coast of India. Amer. J. of Infec. Dis 2009;5:90-98.
30. Saadoun I, Al-Momani F. Studies on soil Streptomycetes from Jordan. Actinomycetes 1997; 8 : 42-48.
31. Saadoun I, Mohammadi MJ, Al-Momani F, Meqdam M. Diversity of soil Streptomycetes in northern Jordan. Actinomycetes 1998;9:53-58.
32. Ndonde MJM, Semu E, J. Preliminary characterization of some Streptomyces species from four Tanzanian soils and their antimicrobial potential against selected plant and animal pathogenic bacteria. World Biotechnol 2000;16:595-99.
33. Hamdi YA, Ahmed D, Al-Tai AM, J. Genera and species of actinomycetes isolated from Iraq soils. Egy. J. of Microbiol. 1980;15:7-22.
34. Hussein AM, Rajab AM, Elgammal AA, Mansour FA, Sami E, Helmy M, et al. Taxonomy of gray pigmented Streptomyces spp. isolated from Egyptian soil Egy. J. of Bot 1980;23:9-16.
35. Mohamed ZK. Physiological and antagonistic activities of Streptomycetes in rhizosphere of some plants. Egy. J Phytopathal. 1982; 14 : 121-128.
36. Crawford DL, Lynch JM, Whipps JM, Ousley MA. Isolation and characterization of actinomycete antagonists of a fungal root pathogen. Appl. and Environ. Microbiol. 1993;59(11):3899-905.
37. Pirt SJ, Righelato RC. Effect of growth rate on the synthesis of penicillin by P. chrysogenum in batch and chemostat culture Appl. Microbiol. 1967;5:1284-90.
38. Lurie LM, Verkhotseva T, Levitov MM. Penicillin biosynthesis and two phase pattern of Pencillium chrysogenum development. Antibiot. 1975;20:291-95.
39. Sejiny MJ. Growth phase of some antibiotics producing Streptomyces and their identification. JKAU Sci 1991;3:21-9.
40. Porter JN, Nithelm JJ, Tresner HD. Method for preferential isolation of actinomycetes from soils. Appl Microbiol 1960;8:174-8.
41. Narayana KJP, Vijalakshmi M, J. Optimization of Antimicrobial methods production by Streptomyces albidoflavus. Res Res. J. Pharm. 2008;2:4-7.
42. Ilic SB, Konstantinovic SS, Todorovic ZB, Lazic ML, Veljkovic VB, Jokovic N. Characterization and antimicrobial activity of the bioactive metabolites in Streptomycetes isolates. Microbiol. 2007;76:421-8.
43. Sibanda T, Mabinya LV, Mazomba N, Akinpelu DA, Bernard K, Olaniran AO, et al. Antibiotic producing potentials of three freshwater actinomycetes isolated from the Eastern Cape Province of South Africa. Int. J. of Mol.Sci. 2010; 11(7):2612-23.

44. Fourati B, Serge F, Mehdi R, Lotfi M, Hartmut L. Lilia Purification and Structure elucidation of antifungal and antibacterial activities of newly isolated Streptomyces sp. strain US80 Res in Microbiol. 2005;156:341-47.
45. Arai T, Kuroda Y, S. Classification of actinomycetes with reference to antibiotic production. 261-76.
46. Williams, S.T., *et al*. Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, vol 4,1989;USA.
47. Katoaka M, Ueda K, Kudo T, Yoshida T, Fems. Application of the variable region in 16S rDNA to create an index for rapid species identification in the genus Streptomyces. FEMS Microbiol.Lett 1997;15:249-55.
48. Weinstein MJ, Wagman CH. Isolation, separation and purification of antibiotics, Elsevier Scientific Publishing New York 1978.
49. Atta HM. Application of biotechnology in search for antibiotic from environmental pollutants under solid state fermentation conditions;;PhD thesis, Faculty of sciences, Al-Azhar University, Cairo, Egypt 1999.
50. Augustine SK, Bhavsar SP, Kapadnis BP. A non-polyene antifungal antibiotic from Streptomyces albidoflavus PU 23. J. of Biosci. 2005;30(2):201-11.
51. Ahmed AA. Production of antimicrobial agent by *Streptomyces violachromogenes*. Saudi J. of Biosci. 2007;14(1):7-16.