Int. J. Chem. Sci.: 10(4), 2012, 2237-2247
ISSN 0972-768X
www.sadgurupublications.com

ANTIMICROBIAL ACTIVITY OF ACTINOMYCETES STRAIN ISOLATED FROM SOILS OF UNUSUAL ECOLOGICAL NICHES

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ABSTRACT

Microbial pathogens are developing resistance against existing antibiotics, stressing the urgency for discovery of new therapeutic compounds. Actinomycetes, alone produce 70-80% of the available antibiotics. The chances of isolating undiscovered strains from the terrestrial habitats have diminished so that the search for novel products has switched to rare genera of actinomycetes from normal habitats or to discovery of strains/species found in unusual habitats. Rare genera of actinomycetes can be selectively isolated using various physical and chemical pre-treatment methods. Primary and secondary screening of isolates was done to determine their antimicrobial potential both qualitatively and quantitatively. Chemical structures of active metabolites were ascertained using chromatographic and spectrophotometric techniques. Since production of novel and more efficient antibiotics needs detection of high yielding bacteria, in the current study, we evaluated 5 soil samples (collected randomly from different zones of Jaipur (Rajasthan) viz. rhizosphere of plants, preserved green areas and forest soils) towards their antibiotic production potential using FTIR spectroscopy and HPLC methods. Based upon FTIR and HPLC analyses, the isolate A5 displayed promising results, inhibiting some important pathogenic bacteria and fungi.

Key words: Rare actinomycetes, Antimicrobial compounds, Screening methods, Chromatographic analyses, Spectrophotometric analyses.

INTRODUCTION

The ever increasing knowledge in the area of pathogen`s drug resistance has evoked the discovery of new antibiotics by the screening of microbes. Last few decades has

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witnessed the production of novel antibiotics from different microorganisms. At present, aerobic Actinomycetes have attracted considerable attention of bacteriologist, geneticist and ecologist because of the production of novel antibiotics[1].

A large number of actinomycetes have been isolated and screened from soil in the past few decades, accounting for 70-80% of relevant secondary metabolites available commercially. Consequently the possibility of isolating novel actinomycetes strains from the terrestrial habitats have diminished so that the search for novel products has switched in emphasis to rarer genera of actinomycetes or to well characterized ones that are found in unusual environments. The logic behind these approaches is that such strains may be producers of novel bioactive compounds.

Microbial screening programs have started taking into account the ecological significance of antibiotic producing microorganisms. It is difficult to isolate rare actinomycetes genera by using conventional isolation methods. Novel genera can be isolated taking into account several factors during the isolation procedure, such as the selection of ecological habitats for sample collection, chemical and physical pretreatment of the samples, use of specific selective media, fine-tuning of culture conditions and genus-specific methodologies for screening of isolates[2].

Jaipur is well known for its huge and unexplored diversity. The diverse climatic and soil conditions support the existence of diverse species of actinomycetes which may prove to be a potential source for effective metabolites active against major pathogens. Such molecules, if found and identified, would be utilized to formulate new antibiotics. Hence, the present study made an attempt to estimate the actinomycetes populations in different soil types (the rhizosphere of plants, preserved areas and forest soils) of Jaipur, so as to screen for their antimicrobial properties. Further, the identified antagonistic actinomycetes were characterized based on morphological, biochemical, cultural and physiological characteristics. The antibacterial and antifungal compounds were characterized using HPLC and IR spectroscopy.

EXPERIMENTAL

Collection of soil samples

The soil samples were collected from various locations of Jaipur, which come under green forest cover. Several habitats in different areas were selected for the isolation of *Streptomyces* strains. These habitats included the rhizosphere of plants, preserved areas and forest soils. The samples were taken unto a depth of 11-15 cm from the soil surface. The soil

samples from sterile plastic bag were sieved asceptically to remove small pieces of stone and organic matter. The sample was homogenized using sterile mortar and pestle. The samples were placed in polyethylene bags to avoid external contamination and kept in 4°C until pretreatment.

Soil pretreatment

Soil pretreatment is required for inhibiting or eliminating unwanted microorganisms. In the present study one gram of dried soil was taken in 9 mL of distilled water, agitated vigorously and pre-heated at 50°C for half an hour[3]. Different aqueous dilution ranging from 10^{-3} to 10^{-7} of the suspension were applied onto Nutrient agar and Starch casein agar plates. Dry colonies of actinomycetes were selected and isolated (Fig. 1). Thus isolated colonies were preserved in Glycerol based media and stored at – 70°C[4].

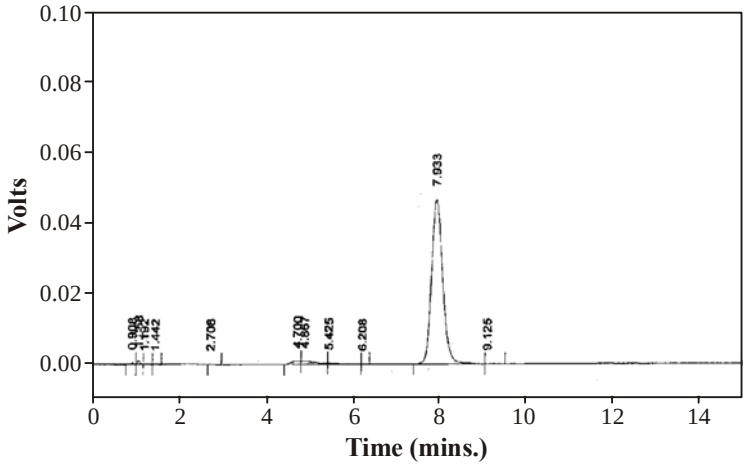

Fig. 1: HPLC chromatograms of extract of actinomycetes isolate A5

Detector A (237 nm) Pk #	Name	Retention Time	Area	Area percent
1		0.908	2259	0.25
2		1.058	4582	0.51
3		1.192	489	0.05
4		1.442	175	0.02
5		2.708	377	0.04
6		4.700	12673	1.42
7		4.867	20427	2.29
8		5.425	6346	0.71
9		6.208	168	0.02
10	Lincomycin	7.933	843553	94.56
11		9.125	1037	0.12

Screening of soil samples by crowded plate technique

A series of culture tubes containing 9 mL of sterile water was taken. From the stock culture, 1 mL suspension was transferred aseptically to the 1st tube (10^{-1}) and mixed well. Further serial dilutions were made to produce 10^{-5} suspensions were made. Suspension (0.1 mL) from each culture tube was spread on sterile Nutrient agar medium plates and starch-casein agar medium plates aseptically in a laminar-air flow cabinet. The plates were incubated at 27 ± 2°C for 72 h. The plates were observed intermittently during incubation. After 72 h, whitish pin-point colonies, characteristic of actinomycetes and with a clear zone of inhibition around them were seen. The pinpoint colonies with inhibitory or clear zone of inhibition were selected and purified into actinomycetes agar slants. The selected strains were further purified by multiple streaking method. The stock cultures of each selected strain was prepared and maintained in nutrient agar slants at 4°C. The actinomycetes colonies isolated from the crowded plate were selected for the further studies and labeled A1, A2… A5.

Test microorganisms

Antibacterial activities were tested for *in vitro* against bacteria and fungi that include:

- Gram positive Bacteria: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633
- Gram negative Bacteria: *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Proteus vulgarius* ATCC 13315, *Klebsiella pneumonia* ATCC 10031.
- Fungal strain: *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404.

They were used to determine the anti-microbial activity of isolated *Streptomyces* strains.

Primary screening of the antimicrobial activity

The primary antimicrobial activity was done by perpendicular streak method. In this method bacterial colonies were streaked on center of nutrient agar plates as a linear culture and incubated at 28°C for 7 days. After 7 days, the test microorganisms were inoculated perpendicularly to the linear cultures and incubated at 37°C for 48 h. Antagonism was measured by determination of size of inhibition zone (Table 1). The antimicrobial producer isolates inhibited the growth of test microorganisms and were selected for further experiments.

Table 1: Sensitivity of various microorganisms to the soil isolates

Isolate	Activity against							
	E.coli	***P.aur.***	***P. vul.***	***Kleb.***	***S.aur.***	***B.subtilis***	***A.nig.***	***C.albicans***
A1	+	+	+	+	-	+	-	-
A2	+	+	+	-	-	-	-	-
A3	+	+	-	-	+	-	+	+
A4	+	+	+	-	-	-	-	-
A5	+	+	-	-	+	-	+	+

Isolation of antibacterial metabolites

The selected isolates were cultured in nutrient broth and incubated at 28^{o}C for 7 days. After 7 days bacterial cultures were filtrated using Whatman filter paper. Antibacterial compounds were recovered from the filtrate by solvent extraction with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio 1 : 1 (v/v) and was shaken for 1 h for complete extraction. The ethyl acetate phase that contains antibiotic agent was separated from the aqueous phase. It was evaporated to dryness in a water bath at 80-90^{o}C[5].

Characterization of the isolates

The selected *Actinomycetes* via antibacterial tests were characterized through morphological and biochemical tests. Morphological methods consisted of macroscopic and microscopic methods. The mycelium structure, color and arrangement of spores on the mycelium, and other properties such as the color of colonies, soil pH and etc. were observed. The observed structures were compared with Bergey`s Manual of Determinative Bacteriology, Ninth edition[6] and the organisms were identified. Moreover several biochemical tests such as Casein hydrolysis, starch hydrolysis and urea hydrolysis, acid production from various sugars, NaCl resistance and temperature tolerance were done (Table 2).

Isolation of antibacterial metabolites

In the present study antibacterial compound was recovered from the filtrate by solvent extraction method using ethyl acetate as a solvent and used for the compound purification for HPLC and IR. Residues were collected in standard vials and stored in refrigerator at 4^{o}C till further use.

Table 2: IR spectroscopical data and their functional group identification of actinomycetes isolate A5

S. No.	Wave Number (cm^{-1})	Functional Groups
1	500-600	Alkyl Halide (C-Br)
2	600-800	Alkyl Halide (C-Cl)
3	1000-1400	Alkyl Halide (C-F)
4	1400-1600	Aromatic(C=C)
5	1640-1690	Amide (C=O)
6	1705-1725	Ketone
7	1720-1740	Aldehyde

High performance liquid chromatography (HPLC)

High performance liquid chromatography is a chromatographic technique used to separate a mixture of compounds in analytical chemistry and biochemistry with the purpose of identifying, quantifying and purifying the individual components of the mixture. In current study we used Shimadzu LC 500 for the analysis of unknown compound against the reference antibiotics mentioned in USP 34. Analysis method is also according to the USP 34 (United state pharmacopeia). Three different Columns (L1, L7 and L10) were used for analysis of sample and different mobile phase were prepared according to samples (Table 3).

Table 3: List of standard parameters used in HLPC method for sample identification

Standard	Mobile phase	Column name	Size of Column	UV (λ) nm
Monensin	Methanol + water + Glacial acetic acid	L1	(250 × 4.6 mm) 5 mm	520
Rifampin	Water + CAN + Phosphate buffer + Citric acid + Sodium per chlorate	L7	(100 × 4.6 mm) 5 mm	254
Clauvlanic Acid	Sodium phosphate buffer + Methanol	L1	(300 × 4 mm) 10 mm	220

Cont…

Standard	Mobile phase	Column name	Size of Column	UV (λ) nm
Lincomycin	Phosphate buffer + ACN + Methanol	L7	(250 × 4.6 mm) 5 mm	210
Capreomycin	Ammonium bi sulphate solution + Methanol	L10	(150 × 4.6 mm)	268

Fourier transforms infrared (FTIR) spectral analysis

With the use of IR we can identify chemical bond functional groups by the absorption of infrared radiation which excites vibrational modes in the bond, especially chemical bond of organic materials. Detection limits vary greatly, but are sometimes $<10^{13}$ bonds/cm^3 or sometimes sub monolayer. It is useful in the identification of solids, liquids, or gases. In present study we used Model Shimadzu FTIR 8700. Extracted amorphous part was used for FTIR, 1 mg of sample and 300 mg of KBr (IR grade) was mixed properly and a thin KBr disk was prepared for analysis, this disk was applied on FTIR instrument for analysis and the peaks obtained were observed and interpreted.

RESULTS AND DISCUSSION

Streptomyces, as the most important genus of *Actinomycetes,* are the most abundant soil microorganisms under a wide variety of conditions. *Actinomycetes* strains are characterized by the production of important extracellular bioactive compounds and majority of those strains belong to species within the genus *Streptomyces* which produce two–thirds of the clinically important antibiotics. This genus was confirmed to be promising bacteria against several pathogens and is well known for their potential to produce a large number of inhibitory metabolites used in industry and pharmacy[7,8].

In the present study, systematic screening approach was applied on Jaipur (Rajasthan) soil for actinomycetes isolation. Although Jaipur belongs to Thar Desert area, the actinomycetes were isolated from the rare and unusual green cover areas of the so called desert region. Total five soil samples were collected from different climatic areas of Jaipur. All soil samples were collected from 11-15 cm depth due to oxygen requirement of actinomycetes but not from the surface due to high temperature of surface soil in Jaipur region.

After taking the pH of all soil samples it was found that all soil samples were neutral to alkaline in nature. Nature of soil samples were mainly black-humus and sandy. To kill spores of fungus heat treatment was applied on all soil samples. After heat treatment serial

dilution was done on the soil samples to reduce the colony count on agar plate and to reduce other bacterial colony to final countable range. Rough, chalky, powdery and single white, yellow, pink colonies were observed on Nutrient agar plates. Some colonies were very hard to pick from agar surface, which is also a characteristic of actinomycetes. These kinds of colonies were picked with help of hot nichrome loop. A total of 47 actinomycetes isolates were obtained in all.

Further, actinomycetes colonies that were showing point of zone of inhibition on nutrient agar media were selected for antibacterial screening. Total five isolates showed zone of inhibition on Nutrient agar plates. After sub-culturing, slants of isolates were stored at 4^{o}C in refrigerator and labeled A1, A2, A3, A4 and A5. Five isolates that were showing zone of inhibition were further tested for antibacterial and antifungal activity against two Gram positive, four Gram negative and two fungal strains. After 7^{th} day of streaking of active actinomycetes isolates, related test organisms were streaked on nutrient agar plates. Observation was done on different times and reference pathogens and observations were recorded.

Isolate A1 showed activity against both Gram positive and Gram negative bacteria but no activity against fungal strains. Isolate A2 showed activity against only Gram negative bacteria i.e. *E. coli* and *P. aeruginosa*. There was no activity against Gram positive and fungal strain. Isolate A3 showed broad spectrum of activity against both Gram positive, Gram negative and fungal strains. Isolate A4 showed activity against Gram negative bacteria only. There was no activity observed against Gram positive and fungal strains.

On the basis of macroscopic and microscopic characteristics, Gram reaction, biochemical and physiological characterization, all selected actinomycetes isolates were found to belong to *Streptomyces* genus. Isolate A5 showed broad spectrum against both Gram positive, Gram negative and fungal strains and was therefore selected for further analyses (Table 1). The morphology of isolate A5 showed a well defined colony on Nutrient Agar plate; colony colour was white; aerial mycelium was observed with long chain of spore containing more than 50 spores in rectiflexibiles chains in macroscopic characterization and Gram positive reaction was observed in Gram staining.

In biochemical tests, it showed positive reaction in Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Tyrosine hydrolysis, Xanthine hydrolysis and urease test. For utilization of sugar on Triple sugar iron agar it showed fermentation of lactose, sucrose with H_2S production. No growth has been observed on MacConkey agar.

Isolate did not show any growth at high concentration of NaCl i.e. 10% but it showed resistance to 3%, 5% and 7% NaCl concentration and moderate growth was observed at these concentrations. Isolate A5 also failed to grow at high temperature such as 40^{o}C and at low temperature such as 10^{o}C but 27^{o}C was optimum temperature for growth of isolate A5. There was no resistance observed against Neomycin and Rifampicin but growth was observed in the presence of Penicillin G.

For identification of antimicrobial compound produced by isolates, HPLC (High Performance Liquid Chromatography) was performed at test house. Extract compounds were analyzed at D.D. Pharmaceuticals, Jaipur. For the identification of compound by High performance Liquid Chromatography samples were prepared as mentioned in United State Pharmacopeia (USP), reference standard were also prepared by following USP protocol. For identification of active compound of three actinomycetes isolates, HPLC Shimadzu LC 500 was used for analysis; mobile phase, Column, length of UV detector, flow rate and injection volume used as described by USP.

In the present study, active actinomycetes isolate A5 showed 11 peaks in HPLC graph, with retention times of 0.9 min, 1.0 min, 1.1 min, 1.4 min, 2.7 min, 4.7 min, 4.8 min, 5.4 min, 6.2 min, 7.9 min and 9.1 min. Results indicate that 10th peak showed resemblance with standard of Lincomycin and thus isolate A5 may be producing Lincomycin with many other compounds.

Lincomycin is an antibiotic used to treat a wide variety of bacterial infections. In therapeutic doses the medication possesses bacteriostatic action. In high doses lincomycin may exert bactericidal action. Action mechanism of this antibiotic is associated with inhibiting protein synthesis of microbial cells. It is active against many gram-positive microorganisms for example, aerobic cocci like *Staphylococcus* sp., *Streptococcus pneumoniae*, *Corynebacterium diphtheriae* and anaerobic spore-forming bacteria like *Clostridium* sp. The medication is active against gram-negative anaerobic microorganism for example *Bacteroides* sp., *Mycoplasma* sp., etc. Besides it is also known to be effective against a number of fungi, viruses and protozoa (Australian hand book, 2010). Further Lincomycin, which belongs to a group of antibiotics called lincosamides is also effective against some species of *Plasmodium*. Lincomycin is specially used to treat severe bacterial infections in people who cannot receive penicillin antibiotics[9].

For identification of the antifungal compounds, extract were send to the test house

for FTIR. Fourier transform infrared spectroscopy (FTIR) graph of active actinomycetes isolate A5 showed many peaks. Related groups were defined on the basis of peak (Fig. 2). Peaks between 500-1400 cm^{-1} representing the Alkyl Halide group, 1400-1600 cm^{-1} showing Aromatic group, 1640-1690 cm^{-1} showing Amide group, 1705-1725 cm^{-1} showing Ketone group and peak between 1720-1740 cm^{-1} showings Aldehyde group (Table 2).

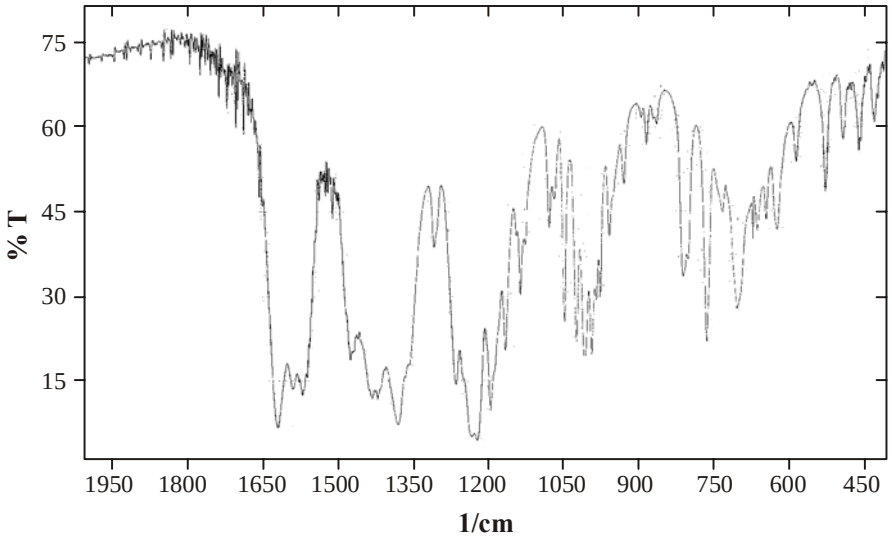

Fig. 2: IR spectrum of secondary metabolites from active actinomycetes isolates A5

The gram positive, filamentous *Streptomycetes* are the most studied and well known group of *Actinomycetes* which have a great ability to produce most important secondary metabolites such as antibiotics, anti tumors, anti viral, anti fungal, etc. since some of the most important antibiotics that are used in medicine are obtained from *Streptomyces* resources, the investigation on the production of this kind of secondary metabolites from *Streptomyces* species is of significance. In the present study, the actinomycetes isolated from soil samples of unusual ecological niches of Jaipur, Rajasthan. This study demonstrates the potential of *streptomycetes* in production of antibiotics and further evaluates the antimicrobial activity of the various isolates through the HPLC and FTIR spectroscopy. The isolate A5 displayed promising results and Lincomycin, specially used to treat severe bacterial infections in people who cannot receive penicillin antibiotics, was obtained and characterized using HPLC. Further, this strain is also producing important antifungal compounds as seen from FTIR spectrum. This analysis would be valuable to tackle drug resistant infections in the future.

ACKNOWLEDGEMENT

Authors are thankful for the financial support provided by UGC Major Project (File No. 40-113/2011) and CSIR JRF (F. No. 09/149(0581)/2010-EMR-I and F. No. 09/149(0581)/2011-EMR-I). Authors also acknowledge help and support of D. D. Pharma, Jaipur.

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Revised : 27.07.2012 *Accepted : 30.07.2012*