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Characterization of antimicrobial compounds from *Streptomyces* isolates

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ABSTRACT

In this research we evaluated the potential of antibiotic production and characterize the FTIR spectroscopy and HPLC (High performance liquid chromatography) analysis pattern of Streptomyces from various semi-arid locations of Jaipur, Rajasthan. Regarding this, five soil samples were collected randomly from three different green cover areas of Jaipur. Following the extraction of secondary metabolite, the HPLC and FTIR spectroscopy analysis was carried out for characterization of various extracts. Considering the coordinate analysis of HPLC and FTIR spectroscopy pattern, isolate A1 is producing Rifampin, isolate A2 might be potential producer of Capreomycin IA and Capreomycin IB while isolate A3 is producing Clavulanic acid. Besides A3 is also producing antifungal compounds. FTIR analysis showed peaks between 600-800 cm^{-1} showing Alkyl Halide, 1080-1360 cm^{-1} Amine group, 1400-1600 cm^{-1}Aromatic group, 1670-1820 cm^{-1}Carbonyl group and peaks between 1800-1830 cm^{-1} showing anhydride group. These results highlight the importance of Streptomyces isolates in antibiotic and antifungal production.

Keywords: Actinomycetes, *Streptomyces*, HPLC, FTIR, Antimicrobial compounds

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INTRODUCTION

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. A large number of actinomycetes have been isolated and screened from soil in the past several decades, accounting for 70-80% of relevant secondary metabolites available commercially [1]. 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 [2,3].

Actinomycetes have occupied a prominent position in the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites including antibiotics with diverse chemical structure and biological activities. The Actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA [4]. The majority of Actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population which may vary with the soil type [5].

Streptomyces is the largest antibiotic genus, producing both antibacterial and antifungal range of compounds. They produce over two thirds of the clinically useful antibiotics of natural origin. Nevertheless, a periodic replace of the existing antibiotics is necessary to prevent transmissible resistance among microorganisms to available antibiotics already marketed. 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.

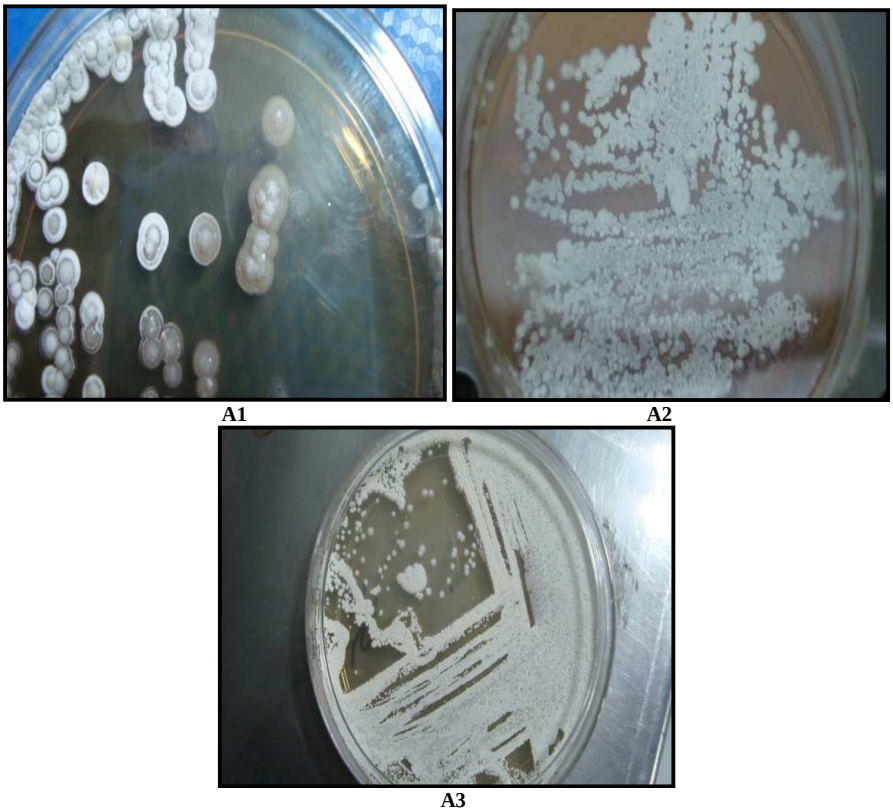
EXPERIMENTAL SECTION

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 desert region. Total five soil samples were collected from different areas of Jaipur.

Bacterial strain and growth condition

In the present study a total of 37 actinomycetes were isolated from five soil samples collected from three 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 aseptically 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 is required for inhibiting or eliminating unwanted microorganisms. In the present study one gram of dried soil was taken in 9ml of distilled water, agitated vigorously and pre-heated at 50^0C for half an hour [6]. 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 [7].



A1 **A2**

A3

Fig. 1: Three actinomycetes isolate A1, A2 and A3 having antimicrobial activity

Test microorganisms

Antibacterial activities were tested for in vitro against bacteria and fungi including Gram positive Bacteria *viz. Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, Gram negative Bacteria *viz. Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 8739, *Proteus vulgarius* ATCC 13315, *Klebsiella pneumonia* ATCC 10031 and Fungal strains namely *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404. These strains 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: Zone of inhibition by active isolates against reference Bacterial and Fungal strains

Actinomycetes isolates	Activity against (in mm)							
	E.coli	***P.aur.***	***P. vul.***	***Kleb.***	***S.aur.***	***B.subtilis***	***A.nig.***	***C.albicans***
A1	20	18	13	5	-	17	-	-
A2	22	19	10	-	-	-	-	-
A3	25	16	-	-	18		12	10

Isolation of antibacterial metabolites

The selected isolates were cultured in Nutrient broth and incubated at 28°C for 7 days. The bacterial cultures were then filtrated using Whatman filter paper. Antibacterial compounds were recovered from the filtrate by solvent extraction with ethyl acetate in the ratio 1:1 (v/v) and were 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°C [8].

Table 2: Morphological, biochemical and physiological characteristic of actinomycetes isolates

Characteristic	Actinomycetes isolates		
	A1	**A2**	**A3**
Aerial mycelium	+	+	+
Gram staining	+	+	+
Spore chain morphology:			
Rectiflexibiles	-	-	-
Spirals	+	+	+
Verticillat	-	-	-
Degradation of:			
Starch	+	+	+
Casein	+	+	+
Gelatine	+	+	+
Tyrosine	+	+	+
Xanathine	+	+	+
Urease	-	-	-
Utilization of sugar	+	+	+
Growth on MacConkey agar	-	-	-
Resistance to:			
Neomycin	-	-	-
Rifampicin	-	-	-
Penicillin G	+	+	+
Growth with Nacl			
3%	+	+	+
5%	+	+	+
7%	+	+	+
10%	-	-	-
Growth at different Temp.			
10^0C	+	+	+
27^0C	+	+	+
40^0C	-	-	-

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 (2000)[9] 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 4^0C till further use.

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	Colum name	Size of Colum	UV (λ)nm
Capreomycin	Ammonium bi sulphate solution + Methanol	L10	(150×4.6mm)	268
Rifampin	Water+ACN+ Phosphate buffer+ Citric acid+ Sodium per chlorate	L7	(100×4.6mm) 5mm	254
Clauvlanic Acid	Sodium phosphate buffer+ Methanol	L1	(300×4mm) 10mm	220

Fourier transform 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

A large number of actinomycetes have been isolated and screened from soil in the past several decades, accounting for 70-80% of relevant secondary metabolites available commercially [1].Consequently the possibility of isolating novel actinomycetes strains from the usual 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 [3,10]. Thus in the present investigation an attempt was made to isolate actinomycetes from unusual environmental of desert region i.e. green cover areas like forest reserve park.

Further, actinomycetes colonies that were showing point of zone of inhibition on nutrient agar media were selected for antibacterial screening. Total three isolates showed zone of inhibition on Nutrient agar plates. After sub-culturing, slants of isolates were stored at 4^0C in refrigerator and labeled A1, A2, and A3.Three 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 7th 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. In primary observation of Isolate A1, it showed well developed colony on Nutrient agar media; colony colour was white when

colonies were not fully developed (Fig.1) and later appeared grey on solid Nutrient Agar plate and produced red pigment on Starch Casein Agar. In microscopic characterization, presence of aerial mycelium with spiral arrangement of spores in spiral chain indicated that isolated actinomycetes belonged to *Streptomycetes* genus. Further it was found to be Gram positive in Gram staining reaction.

Isolate also showed antibacterial activity against both Gram negative and Gram positive bacteria. It showed maximum zone of inhibition against *E.coli* (20mm) followed by *P.auriginosa* (18mm), *B. Subtilis* (17mm), *Klebsiella* (5mm) and *P.vulgaris* (13mm) (Table 1). Isolate A1 showed maximum inhibition against *E.coli* and minimum for *P.vulgaris* in antibacterial screening by streak plate method. No activity was observed against fungal strains. Biochemical tests revealed that it showed positive reaction for starch hydrolysis with clear zone on media. Casein hydrolysis, Gelatin hydrolysis Tyrosin hydrolysis, and Xanathine hydrolysis also showed positive reaction but for Urease test negative reaction with no colour change of liquid media was observed. On Triple Sugar Iron Agar it showed the fermentation of lactose or sucrose but not for all kinds of sugars and no H_2S gas production was observed. No growth was observed on MacConkey agar due to its Gram positive property.

Optimum growth temperature was 27^0C for isolate A1. Good growth was observed at this temperature while it did not grow at 10^0C and at 40^0C thus indicating that it is mesophilic in nature (Table 2). The isolate was found to be resistant to inhibitory effect of wide range of NaCl concentrations. Growth was obtained at 3%, 5% and 7% but no growth was observed at 10% concentration of NaCl. It also showed resistance to Penicillin G and failed to grow in the presence of Neomycin and Rifampicin. All these result show that isolate A1 belonged to *Streptomyces* genera (Table 2).

Isolate A2 also showed antibacterial activity against Gram negative bacteria but lacked activity against *B. Subtilis*. However it was active against *E.coli* (22mm) followed by *P.auriginosa* (19) and *P.vulgaris* (10) as seen in Table 1. Isolate A2 showed maximum activity against *E.coli* and minimum against *P.vulgaris*. Isolate A2 did not form well developed colony on Nutrient Agar media with white colour of colony and it also produced yellow pigment on Starch casein Agar. In microscopic characterization it showed aerial mycelium with branching filaments; around 10 spores in long spiral chain and it again gave Gram positive reaction in Gram staining.

Besides, this isolate showed positive reaction for Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Tyrosine hydrolysis, Xanathine hydrolysis and negative reaction for Urease test. Further on triple sugar it showed the fermentation of lactose or sucrose by changing the colour of media from red to yellow. No growth was observed on MacConkey agar. Resistance to inhibitory effect of NaCl at various concentrations was also showed by isolate A2; growth was obtained at 3%, 5% and 7% but no growth was obtained at 10% concentration of NaCl. Isolate A2 failed to grow at high temperature such as 40^0C and at low temperature such as 10^0C but it grew well at 27^0C. Sensitivity was shown by isolate A2 against Neomycin and Rifampicin but it showed positive growth in the presence of Penicillin G (Table 2).

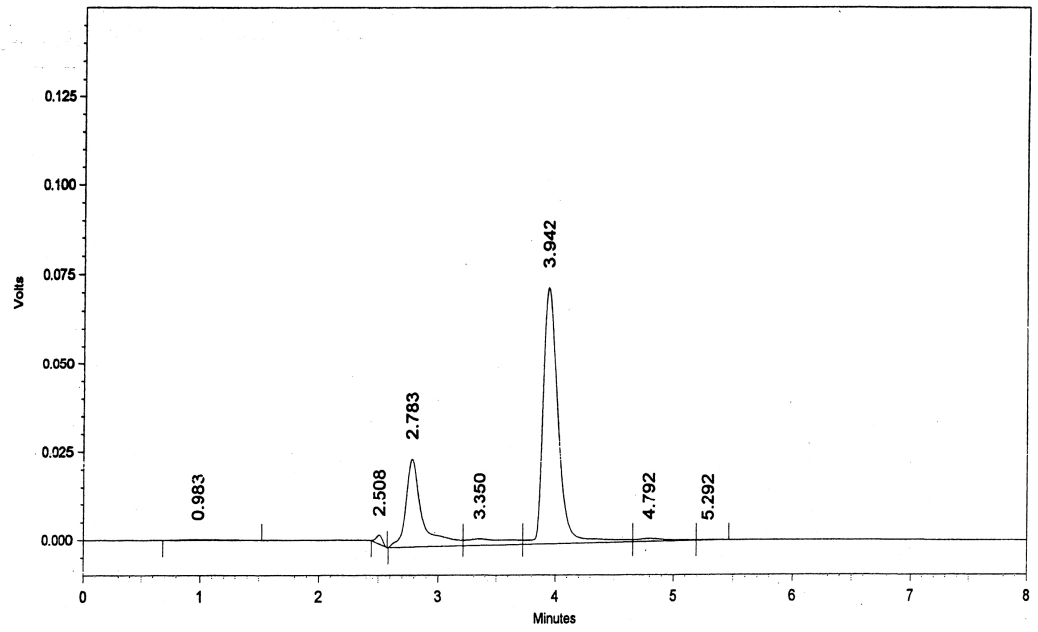
Isolate A3 showed broad spectrum of antimicrobial activity against both Gram positive, Gram negative and fungal strains. It showed antibacterial activity against two Gram negative bacteria *viz*. *E.coli* and *P.auriginosa* with zone of inhibition was 25 mm and 16 mm to respectively, and zone of inhibition was 18 mm against Gram positive bacteria such as *S.aureus*, It was also noted that it was also active against two fungal strains i.e. *A.niger* and *C.albicans* with 12mm and 10mm zone of inhibition (Table 1).

Isolate A3 appeared as a white powdery colony on solid agar and produced yellow pigmentation on Starch Casein Agar. Isolate A3 showed Gram positive reaction in Gram staining; in microscopic characterization aerial mycelium was found filamentous with long highly branched and non fragmenting, long chain containing 30 spores in spiral chain. Degradation of starch, casein, tyrosine, xanthine and gelatin was positive but negative reaction was observed for urease test. On Triple sugar iron agar it showed the fermentation of lactose, sucrose and H_2S production. No growth was observed on MacConkey Agar due to its selectivity for Gram negative bacteria. Isolate A3 also failed to grow at 10% concentration of NaCl but good growth was observed at 3%, 5% and 7% of NaCl. Moderate growth was observed in the presence of Penicillin G but it was sensitive to Neomycin and Rifampicin so no growth was observed in the presence of these antibiotics (Table2).

On the basis of macroscopic & microscopic characteristics, Gram reaction, biochemical and physiological characterization, all selected actinomycetes isolates were found to belong to *Streptomyces* genus (Table 2).

Shimadzu CLASS-VP V6.14 SP1
Method Name: C:/CLASS-VP/METHODS/RIFAMPIN
Data Name: C:/CLASS-VP/DATA/2011/NOV/RIFAMPIN/V006
User: System
Sample ID: RIFAMPIN SAMPLE
Column Name: LICROSPHERE ZK9 L7

Acquired: 11/19/2010 02:17:00 PM
Run Time : 8 min
Vial No.: 10006
Injection Volume:50µL
Flow Rate: 1.5 ml/min
Column No: L12



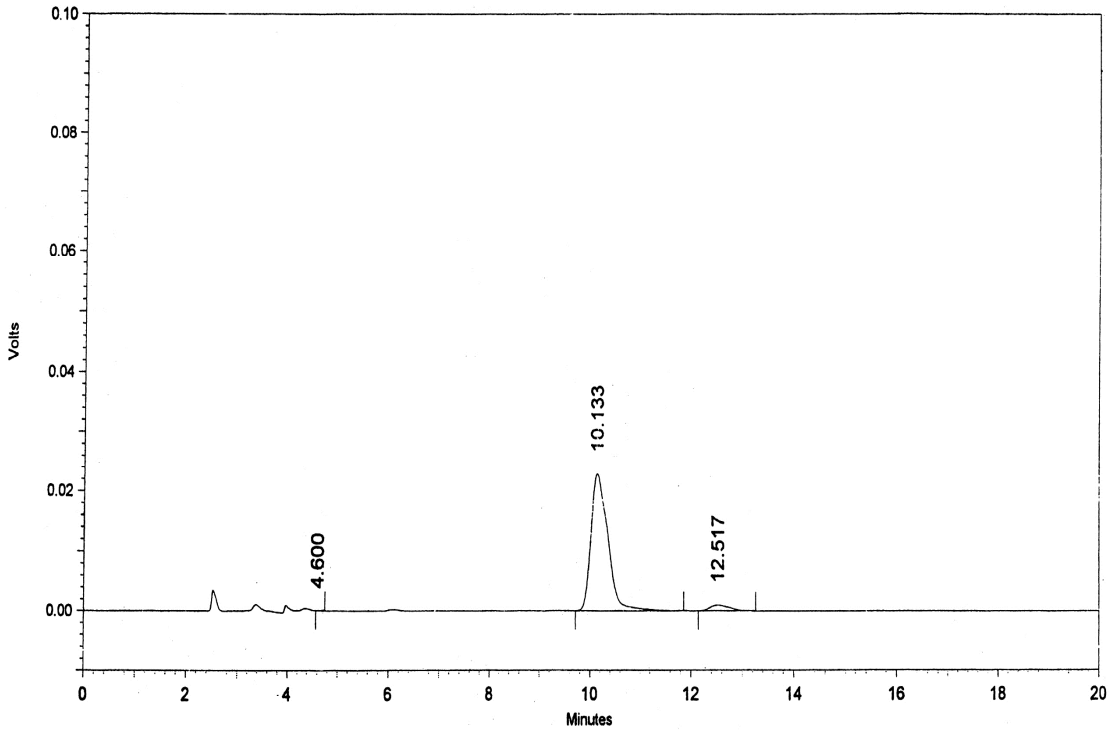
Detector A (210nm)

Pk #	Name	Retention Time	Area	Area Percent
1		0.983	4514	0.46
2		2.508	9191	0.94
3		2.783	239862	24.56
4		3.350	49628	5.08
5	RIFAMPIN	3.942	654785	67.03
6		4.792	16886	1.73
7		5.292	1965	0.20
Totals			976831	100.00

Fig. 2: HPLC chromatogram of extract of actinomycetes isolates A1

Shimadzu CLASS-VP V6.14 SP1
Method Name: C:/CLASS-VP/METHODS/CAPREOMYCIN
Data Name: C:/CLASS-VP/DATA/2011/NOV/CAPREOMYCIN/V002
User: System
Sample ID: CAPREOMYCIN SAMPLE
Column Name: Agilent(XBD)L1

Acquired: 11/03/2010 09:02:00 AM
Run Time : 20 min
Vial No.: 10002
Injection Volume:20µL
Flow Rate: 1.5 ml/min
Column No: L12

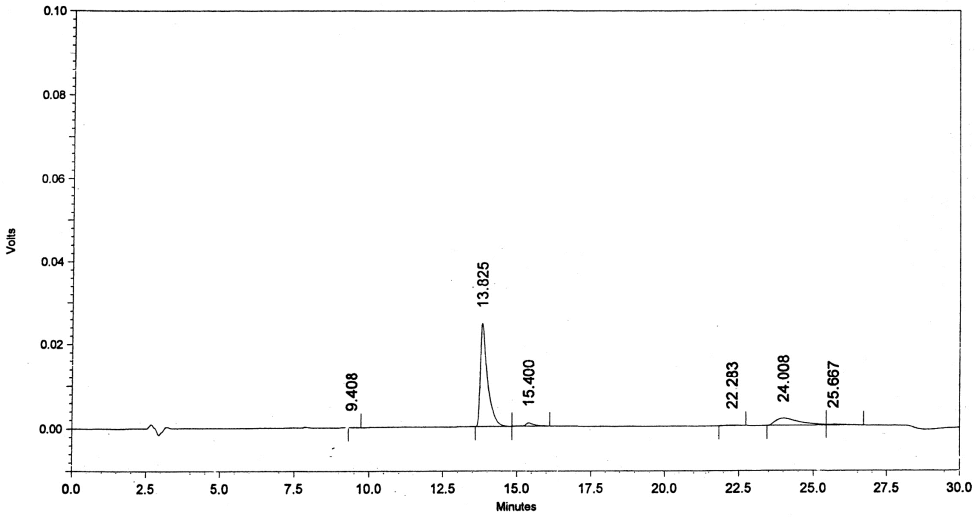


Detector A (235nm)

Pk #	Name	Retention Time	Area	Area Percent
1		4.600	211	0.04
2	CAPREOMYCIN IA	10.133	529169	95.37
3	CAPREOMYCIN IB	12.517	25508	4.60
Totals			554888	100.00

Fig. 3: HPLC chromatogram of extract of actinomycetes isolates A2

Shimadzu CLASS-VP V6.14 SP1 **Acquired: 11/10/2010 11:49:00 AM**
Method Name: C:/CLASS-VP/METHODS/CLAVULANIC **Run Time : 30 min**
Data Name: C:/CLASS-VP/DATA/2011/NOV/CLAVULANIC ACID/V004 **Vial No.: 10004**
User: System **Injection Volume:20µL**
Sample ID: CLAVULANIC SAMPLE **Flow Rate: 2.0 ml/min**
Column Name: LICROSPHERE BXU L1 **Column No: L34**



Detector A (278nm)

Pk #	Name	Retention Time	Area	Area Percent
1		9.408	760	0.14
2	CLAVULANIC ACID	13.825	403620	76.35
3		15.400	15146	2.87
4		22.283	3423	0.65
5		24.008	95438	18.05
6		25.667	10245	1.94
Totals			528632	100.00

Fig. 4: HPLC chromatograms of extract of actinomycetes isolate A3

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 (Table 3).

Ramya and Vijaykumar (2008)[11] used high performance liquid chromatographic (HPLC) for separation of antimicrobial compound on a LC-10 AT vp model HPLC using 250 x 4.60 mm Rheodysne Columnn (C-18). The solvent system methanol and water (HPLC grade) was used in the ratio of 88:12. The operating pressure was 114 kgf, at a flow rate 0.8 ml/min and the temperature was set at 30^0C. The UV-Vis (SPD-10 Avp) detector was set at 210 nm. They reported cephalaxine and cephalosporin C in their study.

In the present study, extract of isolate A1 showed two peaks in HPLC graph, first peak at retention time of 2.3 min and second peak at retention time of 3.9 min (Fig. 2). After comparison of theses peaks with standards, it was

concluded that second peak resembled with the standard peak of Rifampin, so it may be said that sample A1 is producing Rifampin with some other compounds. Rifampin is an antibiotic used against many pathogens, but is best known for activity against *Mycobacterium* Strains, such as tuberculosis and Hansen's disease (Leprosy). Sample extract of A2 showed three peaks in HPLC graph, first peak at retention time of 4.5 min, second peak at 10.1 minute and third peak at 12.5 min (Fig. 3). Comparison of these peaks with standard revealed that second peak resembled with the standard peak of Capreomycin IA and third peak showed resemblance with Capreomycin IB. So isolate A2 might be potential producer of Capreomycin IA and Capreomycin IB. Capreomycins are among the most effective antibiotics against multidrug-resistant tuberculosis.

Third isolate A3 showed six peaks in HPLC graph at the retention time of 9.4min, 13.8min, 15.4min, 22.2min, 24.0min and 25.6min (Fig. 4). When compared with standard, it was observed that second peak resembled with standard peak of Clavulanic acid, so isolate A3 is a potential producer of Clavulanic acid. Further this substance is also under investigation as a NAALADase inhibitor with purported antidepressant and aphrodisiac properties. Thus all the three actinomycetes isolates A1, A2 and A3 are producing commercially useful antibiotics (Table 4).

Table 4: Name of genera identified and antibiotic produced by actinomycetes isolates

Actinomycetes isolate	Related genera	Antibiotic produced
A1	*Streptomyces sp.*	Rifampin
A2	*Streptomyces sp.*	Capreomycin A & B
A3	*Streptomyces sp.*	Clavulanic acid

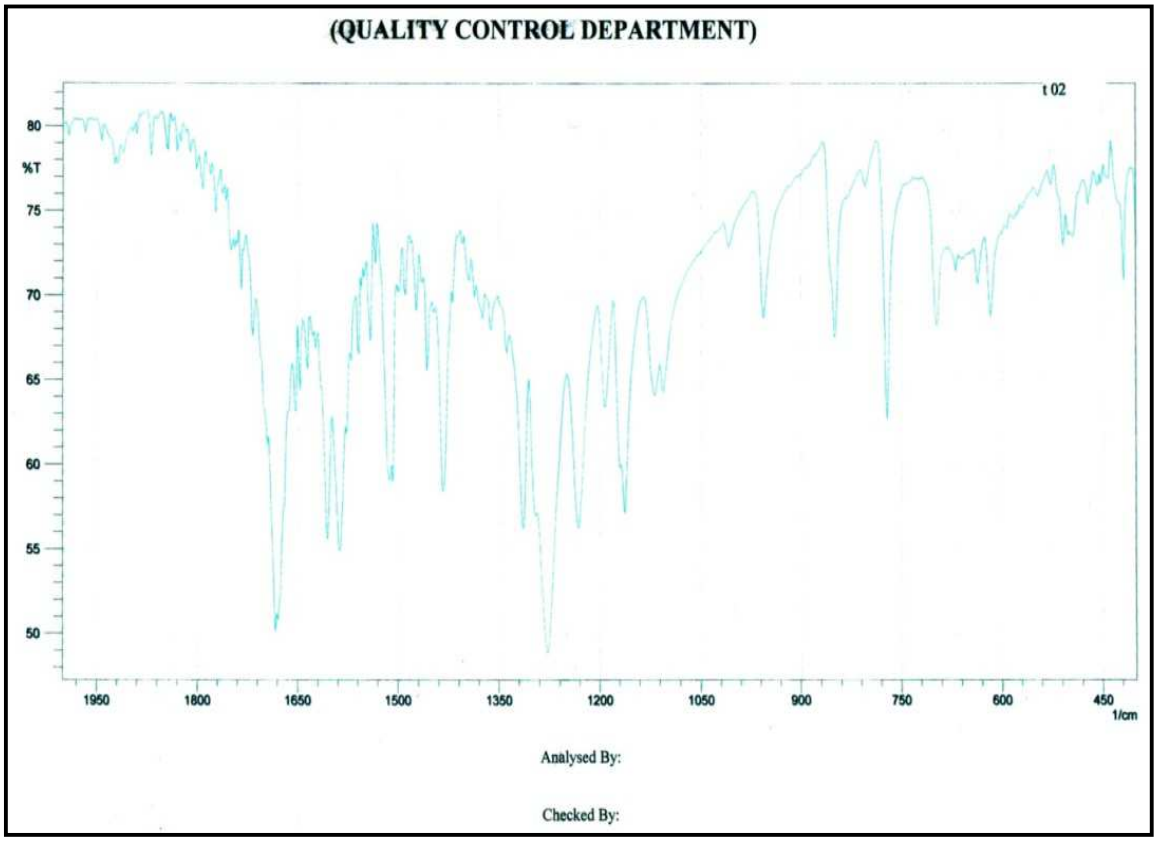


Fig. 5: IR spectrum of secondary metabolites from active actinomycetes isolates A3

For identification of fungal compounds produced by isolate A3, extract were send to the test house for FTIR. There has been only one previous study by Maleki and Mashinchian (2011)[12] in which the antimicrobial compound was finally identified using FTIR. They used UV, FTIR and HPLC for identification of isolated compound. In IR spectrum they reported that spectrum of ethyl acetate extracts of G614 C1exhibited absorption at 3411 cm-1, which

indicates hydroxyl groups, the absorption at 2856 and 2915 cm-1 indicating hydrocarbon chassis and the absorption at 1649 cm-1 indicating a double bond of polygenic compound More or less similar trend was observed by Augustine *et al* . (2005)[13], when they tested the FTIR spectrum of ethyl acetate extract of *S. albidoflavus*PU23 that exhibited absorption bands at 3296 and 1031.8 cm-1, which indicated hydroxyl groups and absorption at 1639 cm-1 indicating double bonding.

Many peaks were observed in FTIR of A3 active actinomycetes isolate and related groups were defined on the basis of peaks obtained (Fig. 5). Peak between 600-800 cm^{-1} showing Alkyl Halide, 1080-1360 cm^{-1} Amine group, 1400-1600 cm^{-1}Aromatic group, 1670-1820 cm^{-1}Carbonyl group and peaks between 1800-1830 cm^{-1} showing Anhydride group (Table 5).The result presented in this investigation could explain the ability of the *Streptomyces sp.* to produce antibiotics. Considering the results on antibiotic production potential of A1 and A2 isolate and antifungal production potential of A3 it might be cited that *streptomyces* potential in antibacterial production could possibly meet the future demand of antibiotics.

Table 5: IR Spectroscopical data and functional group identification of actinomycetes isolate A3

S. No.	Wave number (cm^{-1})	Functional Groups
1	600-800	Alkyl Halide (C-Cl)
2	1080-1360	Amine group (C-N)
3	1400-1600	Aromatic group (C=C)
4	1670-1820	Carbonyl group (C=O)
5	1800-1830	Anhydride (C=O)

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REFERENCES

[1] RH Baltz. *Curr. Opin. Pharmacol*., **2008,** 8(5), 557-63**.**
[2] M Hayakawa. *Actinomycetologica*, **2008,** 22(1), 12-19 **.**
[3] D Hop; Y Sakiyama; CT Binh; M Otoguro; DT Hang; S Miyadoh; DT Luong; K Ando. *J. Antibiot*, **2011,** 64(9), 599-606.
[4] SV Naikpatil; JL Rathod. *Journal of Ecobiotechnology*, **2011**, 3(10), 48-53 ().
[5] VP Agrawal. **2002**.
[6] CH Seong; JH Choi; KS Baik. *Journal of Microbiology,* **2001**, 17, 23-39.
[7] C Ozgur; O Gulten; U Aysel. Eurasia. *J. Bio. Sci.*, **2008**, 2, 73-82 ().
[8] M Athlete; J Lacey; M Goodfellow. *J. Appl. Bacteriol*., **1981**, 51, 289-229.
[9] Bergey’s manual of determinative bacteriology, Actinomycetales. 9th ed. **2000**.
[10] M Das; TV Royer; LG Leff. *Appl Environ. Microbiol*., **2006**, 73(3), 756–767 ().
[11] M Remya; R Vijayakumar. *Medicine and Biology*, **2008**, 15, 13-19.
[12] H Maleki; O Mashinchian. *BioImpacts*, **2011**, 1(1), 47-52 .
[13] SK Augustine; SP Bhavsar; BP Kapadnis. *Journal of Biosciences*, **2005**, 30(2), 201-211.