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Antimicrobial Compound from *Streptomyces* Isolate Characterized Using HPLC

Nupur Mathur[a]*, Anoop Paliwal[a], Pratibha Sharma[a], Manish Kumar[a], Pradeep Bhatnagar [b]

[a] Env ironmental Molecular Microbiology Unit, Department of Zoology, University of Rajasthan, Jaipur 302004,
[b] Department of Life Sciences, International College for Girls, IIS University , Jaipur 302020, Rajasthan, India

***Corresponding author: E-mail: nupurmathur123@rediffmail.com**

Abstract:

Need for novel, safe and more efficient antibiotics is a key challenge to the pharmaceutical industry today. 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 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. In this research we evaluate the potential of antibiotic production and characterize 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. Then, following the extraction of secondary metabolite, the HPLC analysis was carried out for characterization of various extracts. Considering the coordinate analysis of HPLC pattern, isolate A4 was found to be a potential producer of an antibiotic 'Monensin'. The results highlight the importance of *Streptomyces* isolates in antibiotic and antifungal production. HPLC confirmed the production when compared with standards.

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

1.0 Introduction:

Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes (Okami and Hotta, 1988). Actinomycetes are abundant in terrestrial soils, a source of the majority of isolates shown to produce a number of bioactive compounds. The result of intensive screening program carried out over the past several decades is that there is a growing problem of rediscovery of already known bioactive compounds (Dhanasekaran *et al.,* 2009). An approach to address this problem is to expand the source of actinomycetes by carrying out ecological assessment of environments other than usual soils.

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 (Berdy, 1995; Baltz, 1998).

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.
Besides, to identify the potential of these selected actinomycetes isolates to produces antibiotics, antimicrobial screening was done by streak plate

method (Brock *et al*., 1994). Further, the identified antagonistic actinomycetes were characterized based on morphological, biochemical, cultural and physiological characteristics. The antibacterial compounds were characterized using HPLC.

2.0 Materials and Methods:

2.1 Bacterial strain and growth condition:

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 upto 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 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 (Seong *et al.*, 2001). 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. Thus isolated colonies were preserved in Glycerol based media and stored at - 70°C (Ozgur *et al.,* 2008).

2.2 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.

2.3 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. The antimicrobial producer isolates inhibited the growth of test microorganisms and were selected for further experiments.

2.4 Isolation of antibacterial metabolites

The selected isolates were cultured in Nutrient broth and incubated at 28°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°C.

2.5 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 (2000) 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.

2.6 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. Residues were collected in standard vials and stored in refrigerator at 4^0C till further use.

2.7 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). Column L1 was used for analysis of samples and the mobile phase was also prepared accordingingly.

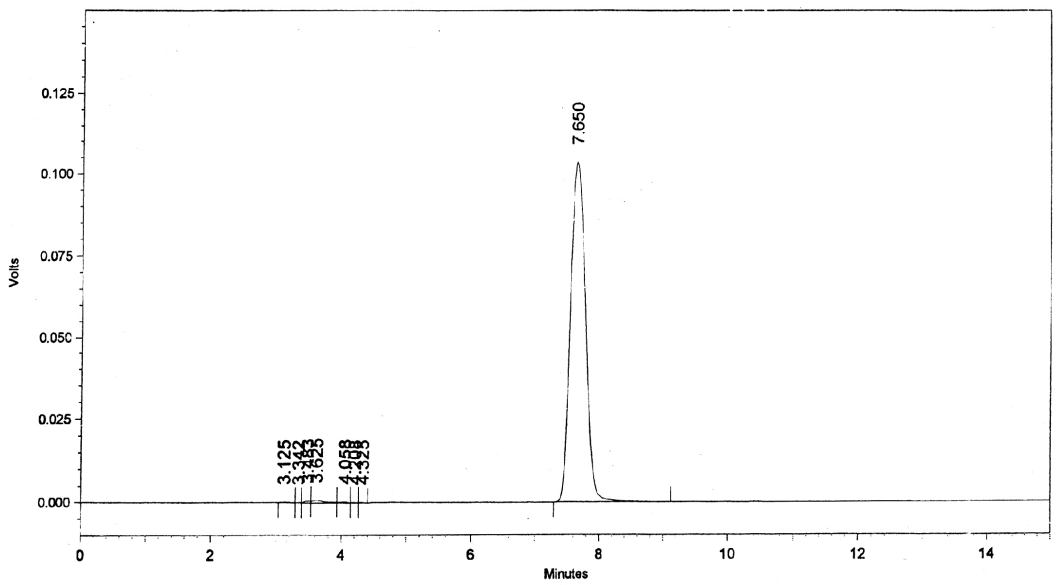
3.0 Results and Discussion:

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 (Baltz, 2008).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 (Das *et al*., 2006 ; Hop *et al*., 2011).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. This work was carried out in the course of a screening program for specific bioactive substances that demonstrated inhibitory affects against prokaryotic and eukaryotic microorganisms from actinomycetes. *Streptomyces* was most prevalent genera of isolated actinomycetes with 73%, followed by *Nocardia* about 24% and 3% *Micromonospora*. 1 out of these isolates showing antimicrobial activity on Nutrient Agar plate; was taken for further study.

Isolate A4 showed antibacterial activity against Gram negative bacteria such as *E. coli, P. vulgaris* and *P. aeruginosa*. Isolate showed a powdery colony on Nutrient Agar plate; colony colour was white; aerial mycelium with highly branched and nonfragmenting and spiral chains containing around 50 spores 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. This 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 A4 also failed to grow at high temperature such as 40^0C and at low temperature such as 10^0C but 27^0C 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.

Besides, an attempt was made to identify the antibacterial & antifungal compounds being produced by actinomycetes from an unusual ecological site. In current study we used Shimadzu LC 500 for the analysis of unknown compound against the reference antibiotics mentioned in USP 34. For identification of active compound in extract of A4 isolate, Column L1 was used with flow rate 0.7 ml/min and injection volume 200µl. Peak was observed at retention time of 7.650 minutes and run time was 15 minutes (Fig.1) .The active actinomycetes isolate A4 showed 8 peaks in HPLC graph, with retention times of 3.1 min, 3.3 min, 3.5 min, 3.6 min, 4.1 min, 4.2 min, 4.3 min and 7.65 min. Results indicate that 8^{th} peak showed resemblance with standard of Monensin and thus isolate A4 may be producing Monensin with many other compounds. In the present study, the actinomycetes isolated from soil samples of green forest cover of Jaipur, Rajasthan. The result presented in this investigation could explain the ability of the *Streptomyces* sp. to produce antibiotics and further evaluates the antimicrobial activity of the various isolates through the HPLC. Besides, there is a demanding need for new and more effective antibacterial for use in more economical uses through industries. Considering the results on antibiotic production potential of A4 isolate, it might be cited that *Streptomyces* potential in antibacterial production could possibly meet the demand.

Further, Technical Research Centre of Finland and the University of Turku have demonstrated that antibiotic Monensin prevents the growth of Prostate cancer cells. It also works against free merozoites (Ketola *et al*., 2010).



Detector A (318nm)

Pk #	Name	Retention Time	Area	Area Percent
1		3.125	1267	0.07
2		3.342	345	0.02
3		3.483	3700	0.22
4		3.625	11717	0.69
5		4.058	3710	0.22
6		4.208	1295	0.08
7		4.325	1256	0.07
8	MONENSIN	7.650	1683977	98.64
Totals			1707267	100.00

Fig. 1: HPLC chromatogram of extract of actinomycetes isolates A4

4.0 Acknowledgment:

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