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Evaluation of Antibacterial Activity of Actinobacteria Isolated from Soil Sample

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Abstract

The present study is carried out by isolation and characterization of soil microflora which are responsible for production of secondary metabolites in the form of antibacterial compound. Soil samples were collected from various places in Lucknow. The bacterial cultures were isolated through serial dilution method, further cultures were identified through Bergey's manual. The results obtained in the form of 1 gram negative cocci and 1gram positive cocci. Several tests were performed like endospore forming, catalase test, glucose test and nitrate reduction test that confirms *Neisseria mucosa* and *Streptococcus equisimilis* spp. of the bacteria according to the Bergey's manual. To check the activities of antibacterial compounds against various pathogens, Antibiogram analysis was done against 3 pathogens *E. coli*, *S. aureus* and *P. aeruginosa*. *N. mucosa* gives 32.5 mm zone of inhibition against *E. coli* and *S. equisimilis* gives 27.5 mm against *E. coli*. Production of antibiotics was done by using production media which was kept in shaker for incubation for 3-4 days. Antibiogram analysis test for intra-extracellular components of bacteria against various pathogens was done, they inhibit the bacterial growth. By Antibiogram analysis of intra and extra cellular against various pathogens and by comparing with tetracycline, intracellular component showed better result than tetracycline against *P. aeruginosa*. Intracellular give 29.5 mm of zone of inhibition and tetracycline give 26 mm.

Key words: *Endospore, Antibacterial, Antibiogram, Extracellular, Intracellular.*

Introduction

The *Actinomycetes* are gram positive, high G+C (>55%) organisms that tend to grow slowly as branching filaments. Many *Actinomycetes* will grow on the common bacteriological media used in the laboratory, such as nutrient agar, trypticase soy agar, blood agar, and even brain-heart infusion agar. *Actinomycetes* encompass a wide range of bacteria.They can be terrestrial or aquatic. *Actinobacteria* is one of the dominant phyla of the bacteria.*Actinomycetes* are best known for their ability to produce antibiotics and are gram positive bacteria which comprise a group of branching unicellular microorganisms. *Actinobacteria* are well known as secondary metabolites producers and hence of high pharmacological and commercial interest. In **1940 Selman Waksman** discovered the soil bacteria. Antibiotics compounds are produced by microorganisms that are able to inhibit the growth of other microorganisms. Antibiotics are medicinal products that have an anti-bacterial effect, they either kill bacteria in the system or keep away them from reproducing, allowing the infected body to heal by producing its own defenses and overcome the infection[1]. Screening of antibiotics has been widely performed for aboutlast 50 years and new antibiotics are still being found. In screening of new antibiotics, new approaches are required and following three factors must be considered[2] i.e. detection of antibiotic producingmicroorganisms, selection of producing microorganisms andcultivation methods. These are closely related to each other and their efficient combination is essential for successful screening of anantibiotic. The most of widely known antibiotic is penicillin, famously made from mold. The term "Antibiotic" was coined by **Selman in 1942**. Antibiotics were referred as antibiosis. It is a chemical substance derived from microorganism which has the capacity of inhibiting growth and even destroying other organism in dilute solution. Antibiotics, also known as antimicrobial drugs, are drugs that fight against infection caused by bacteria. Antibiotics are low molecular-weight (non-protein) molecules produced as secondary

metabolites, mainly by microorganisms that live in the soil. Most of these microorganisms form some type of a spore or other dormant cell and there is thought to be some relationship (besides temporal) between antibiotic production and the processes of sporulation [3,4,5,6]. Soil are cheap sources of antibiotics, many bacteria are present in soil. Many antibiotics produced via microorganisms.
The present study is carried out by isolation, production and characterization of anti-bacterial compound from soil microbes of the soil sample.

Methodology
Collection of soil sample

Soil samples were collected from Garden soil of different places in Lucknow.

- Indra Nagar
- Aliganj

Isolation of antibacterial compound producing bacteria from soil

Serial dilution:

Serial dilution agar plating method or viable count method is one of the most commonly used procedures for the isolation and enrichment of the most prevalent micro-organism such as fungi, bacteria. This method is based on the principle that when sample containing the micro-organism is cultured, each viable micro-organism will develop into a colony; this method is used for reduced bacterial colonies in order to get pure cultures.

Mixed culture- A culture plate containing two or more micro-organism is known as mixed culture.

Procedure- Based on the work done by Jeffery[7].

Primary screening

- After incubated overnight four types of colonies were present in the plates.
- Zone of inhibition around the colonies were observed.
- Colonies showing zones of inhibition were selected for streaking and secondary screening.

Sub culturing: Subculturing was done by streaking method.
Pure culture: A pure culture is usually derived from a mixed culture (containing many species) by methods that separate the individual cells so that, when they multiply, each will form an individually distinct colony, which may then be used to establish new cultures with the assurance that only one type of organism will be present. Pure cultures may be more easily isolated if the growth medium of the original mixed culture favors the growth of one organism to the exclusion of others.

Staining and biochemical characterization of sample

Gram staining was performed to identify the culture according to Bergey's manual [8].

Secondary screening
Activity of biological compounds agent pathogen an important task of the clinical microbiology laboratory is the performance of antibacterial susceptibility testing of significant bacterial isolates. The goals of testing are to detect possible drug resistance in common pathogens and to assure susceptibility to drugs of choice for particular infections.
Endospore test and Catalase test were performed to identify the culture through Bergey's manual [9, 10].
Screening test- for *Streptococcus*
6.5% NaCl test, Acid production from glycerol test, OF glucose test, Nitrate test were performed to identify the culture and methods were used as par Aneja.

Production of antibacterial compound by shake flask fermentation

Fermentation- The process by which complex organic compounds, such as glucose, are broken down by the action of enzymes in to simpler compounds without the use of oxygen.

Extraction of antibacterialcompound from Production media

Centrifugation- It is a process that involves the use of the centrifugal force for the sedimentation of mixtures with a centrifuge, used in industry and in laboratory settings. More-dense components of the mixture migrate away from the axis of the centrifuge, while less-dense components of the mixture migrate towards the axis. Chemists and biologists may increase the effective gravitational force on a test tube so as to more rapidly and completely cause the precipitate (pellet) to gather on the bottom of the tube. The remaining solution properly called the supernatant or supernatant liquid. The supernatant liquid is then either quickly decanted from the tube without disturbing the precipitate, or withdrawn with a pipette.

Procedure:

After incubation production media is transferred to 6 centrifuge tubes (5 ml each). The tubes were centrifuge at 10,000 rpm for 10 min. After centrifugation, store supernatant and pellets. Supernatant is extracellular component and pellet is intracellular component.

Extraction of intracellular compound

Solvent extraction is a method for processing materials by using a solvent to separate out variables components with a material sample.

Extraction of extracellular compound by solvent extraction method

Antibiotic sensitivity test

Antibiotic sensitivity test is done to check the activity of antibiotic against pathogens.

Antibiotic: chemical compounds produce by microorganisms or a similar product produce wholly (synthetic) or partially (semi-synthetic) by chemical synthesis that inhibit growth or kill other microorganisms.

Antibiotic sensitivity: is a term used to describe the susceptibility of bacteria to antibiotics. Antibiotic sensitivity testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection in vivo.

Study of growth kinetics

Growth is an orderly increase in the quantity of cellular constituents. It depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosome, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division. This asexual process of reproduction is called binary fission.

Lag Phase, Log Phase, Stationary Phase and Death Phase.

Antibiogram analysis

An antibiogram is the result of a laboratory testing for the sensitivity of an isolated bacterial strain to different antibiotics. Once a culture is established, there are two possible ways to get an Antibiogram: **Kirby-Bauer antibiotic testing** (KB testing or disk diffusion antibiotic sensitivity testing)[11] is a test which use antibiotic impregnated wafers to test whether particular bacteria are susceptible to specific antibiotics. A known quantity of bacteria is grown on agar plates in the presence of thin wafers containing relevant antibiotics. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the water where bacteria are not capable of growing(called a zone of inhibition).

Procedure:

Prepare 50ml NA media and autoclave, after autoclaving of glass wear and media are taken in LAF. The media is poured in 3 petriplates and left for solidification, after solidification of media, spread 50µl of 3 pathogens (*E. coli, S. aureus & P. aeruginosa)* in three petriplates. Then prepare the wells with the help of sterile borer and load the first well by extracellular component, second well by intracellular component and third well by tetracycline. Incubate at 37^0C for overnight.

Results

Isolation and screening of antibiotic producing microorganism

Microbes from soil were isolated by serial dilution method and mixed culture was obtained by spreading as shown in figure:

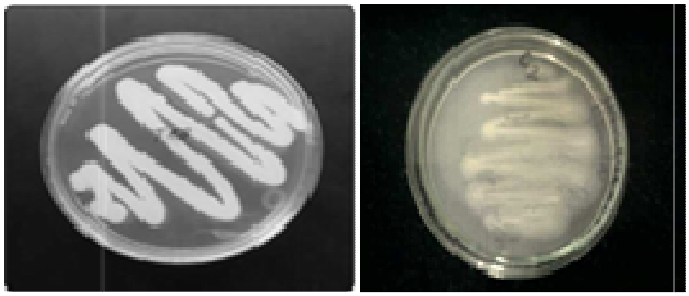
Figure 1: Mixed colonies in spread plate.

Table 1: Colony morphology of culture from sample C1, C2, C3 and C4.

Colony morphology	Culture C1	Culture C2	Culture C3	Culture C4
Shape	Circular	Circular	Circular	Circular
Margin	Entire	Entire	Entire	Entire
Opacity	Translucent	Translucent	Opaque	Opaque
Pigmentation	White	Off white	Off white	Off white

Sub Culturing

The cultures C1, C2, C3 and C4 obtain through primary screening were purified by zigzag or simple streaking was shown in fig. 2:



C1 **C2**

C3 **C4**

Fig 2: Pure colonies obtained through simple streaking

C3 and C4 pure colonies obtained through Quadrant Streaking.

C3 **C4**

Fig 3:- Pure colonies obtained through Quadrant streaking.

Gram's Staining Result

After gram's staining slide were observed under microscope and some colonies (C1, C2, C3) get pink colour and some get purple colour (C4). The colonies showing pink colour are gram negative cocci and colony which shows purple colour are gram positive cocci bacteria.

Antibiogram Analysis

Antibiogram of purified cultures C1, C2, C3 and C4 was performed against various pathogens (*E. coli, P. aeruginosa and S. aureus*). There were zones of inhibition obtained in each culture. Each cultures show positive result against pathogens.

E. coli

S. aureus

P. aeruginosa

Fig 4: Antibiogram analysis of cultures against various pathogens.

Table 2: Antibiogram of isolated cultures against various pathogens

S. No.	Pathogens	Culture C1 Diameter (mm)	Culture C2 Diameter (mm)	Culture C3 Diameter (mm)	Culture C4 Diameter (mm)
1	*E. coli*	10	10	0	8
2	*S. aureus*	10	0	9	0
3	*P. aeruginosa*	10	10.5	0	9

Endospore test result:

The pink colour was observed for Cocci under microscope, which confirms that these are non-endospore forming bacteria.

Catalase test:

In this test bubble formation was not observed. By this we confirmed that the bacteria are from group *Streptococcus.*

6.5% NaCl test:

No growth was observed in 6.5% NaCl. Therefore, further glycerol test was done to confirm the species of Streptococcus.

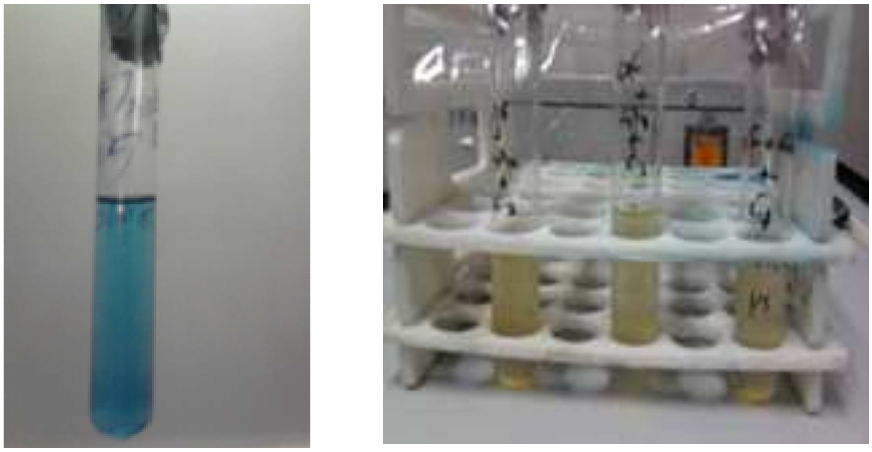
Acid from glycerol test:

After 72 hrs appearance of yellow halo surrounding the streak was appeared showing that these gram positive bacteria are producing acid, which confirmed *Streptococcus equisimilis* species.

Fig 5:- Gram positive bacteria are producing acid

OF glucose test:

After performing this test blue colour of the solution changes to yellow due to glucose fermentation and confirm *Neisseria* group.



Blank **Test sample**

Fig 6:- Showing glucose fermentation

Nitrate test:

This test is done to confirm the species of *Neisseria*. In this test the red colour of solution was observed which shows that nitrate was reduced and confirmed *Neisseria mucosa* species.

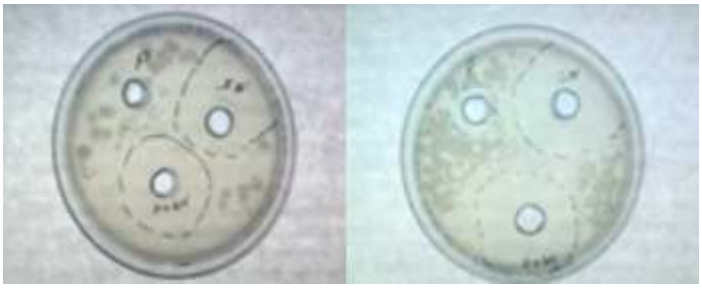
Initial colour **Final colour showing nitrate reduced**

Fig 7:- Showing Nitrate reduction.

Antibiogram of intracellular and extracellular antibiotic extract:
Antibiogram analysis of intra-extracellular from sample C3 and C4 was performed against various pathogens.

Table 3: Antibiogram analysis of intra-extracellular antibiotic extract from *N. mucosa*

S. No.	*Pathogens*	*Intracellular Diameter (mm)*	*Extracellular Diameter (mm)*	*Intra + Extra Diameter (mm)*
1	*E. coli*	0	32.5	27.5
2	*S. aureus*	14.5	32.5	29
3	*P. aeruginosa*	14.5	29	29



E. coli ***S. aureus***

P. aeruginosa

Fig 8: Antibiogram of intracellular and extracellular antibiotic extract from *N. mucosa*

For *S. equisimilis*

Table 4: Antibiogram analysis of intra-extracellular antibiotic extract from *S. equisimilis*

S. No.	*Pathogens*	*Intracellular Diameter (mm)*	*Extracellular Diameter (mm)*	*Intra + Extra Diameter (mm)*
1	*E. coli*	27.5	0	22
2	*S. aureus*	18	0	12.5
3	*P. aeruginosa*	19	0	20

E. coli ***S. aureus***

P. aeruginosa

Fig 9:- Antibiogram for of intracellular and extracellular antibiotic extract from *S. equisimilis*

Table 5: Growth Kinetics of *S. equisimilis*

Days	O.D of the Culture	Growth Phase
1	0.77	Lag
2	1.03	Log
3	1.05	Log
4	1.03	Stationary
5	1	Stationary
6	0.89	Decline
7	0.84	Decline
8	0.5	Decline

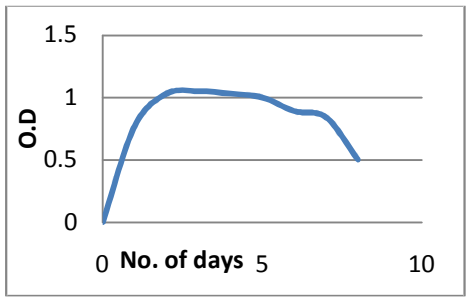


Fig 10: Growth Kinetics of *S. equisimilis*

Table 7: Growth Kinetics of *N. mucosa*

Days	O.D of the Culture	Growth Phase
1	0.24	Lag
2	0.57	Log
3	0.95	Log
4	0.94	Stationary
5	0.93	Stationary
6	0.87	Decline
7	0.83	Decline

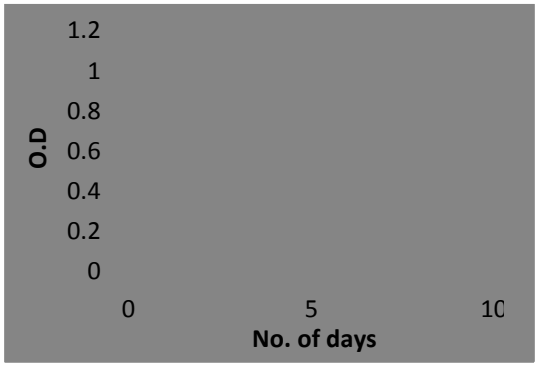
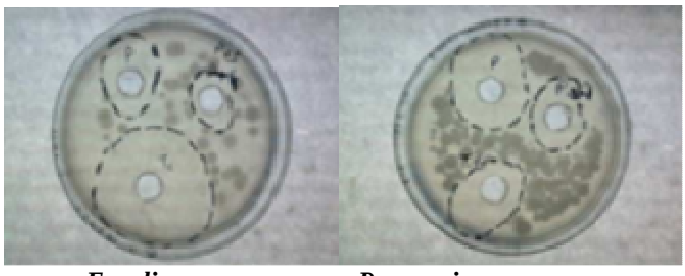


Fig 11: Growth Kinetics of *N. mucosa*

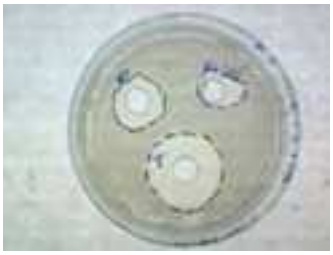
Antibiogram Analysis

Table 8: Antibiogram analysis of intra and extra cellular antimicrobial extract against various pathogens

S. No.	*Pathogens*	*Tetracycline (mm)*	*Intracellular (mm)*	*Intra-extra mixture (mm)*
1	*E. coli*	41	24	16.5
2	*P. aeruginosa*	26	29.5	22
3	*S. aureus*	35	25	23



E. coli ***P. aeruginosa***



S. aureus

Fig 12: Antibiogram analysis of culture sample against various pathogens

Discussion

Screening of antibiotics has been widely performed for about last 50 years and new antibiotics are still being found. In screening of new antibiotics, new approaches are required and following three factors must be considered i.e. detection of antibiotic producing microorganisms, selection of producing microorganisms and cultivation methods. Isolation of microbes by serial dilution method is done. Soil sample is collected from various places in Lucknow[12,13,14]. Gram's staining gives purple colour and pink colour bacteria and distinguish between gram positive and gram negative bacteria. Three gram negative cocci and one gram positive cocci are isolated. Several tests are done like Endospore forming, Catalase test , OF glucose test and Nitrate reduction test that confirms *Neisseria mucosa* and *Streptococcus equisimilis* spp. of the bacteria according to the Bergey's manual. To check the activities of antimicrobial compounds against various pathogens Antibiogram analysis is done. Pathogens used are *E. coli*, *S. aureus* and *P. aeruginosa*. Antibiogram analysis of antimicrobial compounds against various pathogens gives good result. *N. mucosa* gives 32.5 mm zone of inhibition against *E. coli* and *S. equisimilis* gives 27.5 mm against *E. coli*. Production of antibiotics was done by using production media which was kept in shaker for incubation for 3-4 days. Than Antibiogram analysis test for intra-extracellular components of bacteria against various pathogens is done. Antibiogram analysis of intra-extracellular component showed very good result, they inhibit the bacterial growth. The study of bacterial growth kinetics for isolated bacteria is done. After 3-5 days log phase is observed in every sample and after 5-6 days stationary and decline phase are found in bacterial growth. S shape curve (sigmoid) found in each samples.

By Antibiogram analysis of intra and extra cellular against various pathogens and by comparing with tetracycline, intracellular componentshows better result than tetracycline against *P. aeruginosa*. Intracellular give 29.5 mm of zone of inhibition and tetracycline give 26 mm.

Conclusion

Screening of antibiotics has been widely performed for about last 50 years and new antibiotics are still being found. In screening of new antibiotics, new approaches are required and following three factors must be considered i.e. detection of antibiotic producing microorganisms, selection of producing microorganisms and cultivation methods.

Finally it can be concluded that the *Actinomycetes* are the best source for antibiotic isolation.
In this work by Antibiogram analysis intracellular component shows better result than tetracycline against *P. aeruginosa*. Intracellular give 29.5 mm of zone of inhibition and tetracycline give 26 mm.

As the antibiotics are secondary metabolites, they are synthesized in trace amounts. Moreover the synthesis of antibiotic is regulated by tight metabolic and genetic regulation. Therefore it is the task to the biotechnologists to modify the wild type strain and to provide cultural conditions to improve the productivity of antibiotics. Improvement of the microbial strain offers the greatest opportunity for cost reduction without significant capital investment. The problem of the bacterial resistance to antibiotics had evolved and new compounds or derived from the known antibiotics had to be found to replace existing ones. Water and air microbes can also be used for the isolation, characterizing, purification and production of antimicrobial compound.

Future aspects

In this project further more studies can be done. Antimicrobial compounds producing microbes are isolated and characterized and for purification of the antimicrobial compounds several techniques like TLC (Thin layer chromatography), air exposure method and HPLC can be done. Before launching the product in the market, the minimum quantity at which the drug or product works better should be known. For this MIC is done. MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after incubation. Minimum inhibitory concentrations are important in diagnostic laboratory to confirm resistance of microorganism to an antimicrobial agent and also to monitor the activity of new antimicrobial agents.

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