Screening for Actinomycetes from Government Science College Campus and Study of their Secondary Metabolites

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Abstract:- Actinomycetes are a group of organisms which have characteristics of both bacteria and fungi, hence, they are also called as 'Actinobacter' and 'Ray fungi'. Actinomycetes are known for producing novel secondary metabolites like enzymes, anti-biotics, anti-cancerous agents and play major role in recycling of organic matter. In this present research study, actinomycetes were isolated from 11 different soil samples from different places from college campus by serially diluting and spread plate technique on SCA media. 22 actinomycete isolates were obtained, which were identified by gram staining and biochemical tests using Bergey's manual. The secondary metabolites of the isolated actinomycetes were screened for anti-microbial activity against 8 clinical pathogens by perpendicular streak method (primary screening) and agar well diffusion method (secondary screening). 2 actinomycetes showed mild anti-microbial activity against *Proteus vulgaris* and *Staphylococcus aureus*. By using lysozyme method, DNA from selected 8 actinomycetes was isolated and on study of effect of lysozyme concentration on the quantity of DNA using 3 isolates and it was found that .5mg/1ml (15mg/10ml) lysozyme concentration yields highest amount of DNA. Also RAPD PCR was performed on isolate number 4 and its DNA was successfully amplified.

Keywords:- *Actinomycetes, Bergey's manual, anti-microbial activity, DNA, lysozyme, nanodrop, RAPD PCR.*

I. INTRODUCTION

Actinomycetes are known for decomposing resistant organic materials such as chitin, a complex sugar found in the outer skeleton of insects and elsewhere are common soil microorganisms generally called "thread or ray bacteria." They are gram positive, have a high G+C base composition, and are mycelium forming organisms. Nucleic materials consist of a helical structure with a sugar backbone attached through the bases adenine – thymine (A+T) and guanine – cytosine (G+C). Compared with the DNA of other organisms, actinomycetes have a high percentage of guanine – cytosine bases i.e., upto 70.80%. In growth habit, many actinomycetes resemble fungi but are smaller. "The most common genus of actinomycetes in soil is Streptomyces that produces straight chains or coils of spores or conidia. More than one-half of the antibiotics used in human medicine, including aureomycin, chloromycetin, kanamycin, neomycin, streptomycin, and terramycin, have been produced from soil actinomycetes." (Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A and Tripathi CKM, 2016). The smell of freshly turned soil is due to metabolic end products called geosmins that are produced by these organisms and move through soil as unseen volatiles.

"Actinomycetales can be found mostly in soil and decaying organic matter, as well as in living organisms such as humans and animals. They form symbiotic nitrogen fixing associations with over 200 species of plants, and can also serve as growth promoting or biocontrol agents, or cause disease in some species of plants. Actinomycetales can be found in the human urogenital tract as well as in the digestive system including the mouth, throat, and gastrointestinal tract in the form of Helicobacter without causing disease in the host. They also have wide medicinal and botanical applications, and are used as a source of many antibiotics and pesticides." (Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A and Tripathi CKM, 2016).

➢ *Characteristics of Actinomycetes*

The Actinomycetes have a hyphal diameter of approximately 1µm.These organisms reproduce by asexual spores called conidia when they are naked or sporangiospores when enclosed in a sporangium. Although these spores are not heat-resistant, they are resistant to desiccation and aid survival of the species during periods of drought. These filamentous bacteria are mainly harmless soil organisms, although a few are pathogenic for humans

(***Streptomyces somaliensis*** causes actinomycetoma of human), other animals (***Actinomyces bovis*** causes lumpy-jaw disease of cattle), or plants (***Streptomyces scabies*** causes common scab in potatoes and sugar beets). In soil they are saprophytic and chemoorganotrophic, and they have the important function of degrading plant or animal resides.

➢ *Economic Importance of Actinomycetes*

"Actinomycetes have gained the greatest importance in recent years as producers of therapeutic substances. Many of the Actinomycetes have the ability to synthesize metabolites which hinder the growth of bacteria; these are called antibiotics, and, although harmful to bacteria are more or less harmless when introduced into the human or animal body. Antibiotics have great therapeutical and industrial value. The past decade has seen considerable interest in the Actinomycetes as producers of antibiotic substances. The successful use in chemotherapy of streptomycin, chloromphenicol aureomycin and terramycin all metabolites of the Actinomycetes, has stimulated the search for new Actinomycetes and new antibiotics among the Actinomycetes."

(https://www.biologydiscussion.com/bacteria/actinomycetes-economic-importance-and-reproduction/58664)

➢ *Distribution and Mode of Nutrition of Actinomycetes*

The Actinomycetes are essentially mesophilic and aerobic in their requirements for growth and thus resemble both bacteria and fungi. They along with other microorganisms, form the soil microflora and produce powerful enzymes by means of which they are able to decompose organic matter.

The Actinomycetes grow slowly and on artificial media produce hard and chalky colonies. They are particularly abundant in forest soil because of the abundance of organic matter. They occur mainly in soils of neutral pH, although some prefer acidic or alkaline soil. The Actinomycetes can grow in soils having less water content than that needed for most others bacteria. They are capable of utilizing a large number of carbohydrates as energy sources.

Most of the Actinomycetes attack proteins and polypeptides, and are also able to utilize nitrates and ammonia as sources of nitrogen. Nearly all synthesize vitamin B12 when grown on media containing cobalt salts, and many are able to synthesize rather complex organic molecules which have antibiotic properties. The Actinomycetes grow slowly and on artificial media produce hard and chalky colonies. They are particularly abundant in forest soil because of the abundance of organic matter. They occur mainly in soils of neutral pH, although some prefer acidic or alkaline soil. The Actinomycetes can grow in soils having less water content than that needed for most others bacteria. They are capable of utilizing a large number of carbohydrates as energy sources (Berdy, 2005; Singh and Tripathi, 2011).

➢ *Somatic Structures of Actinomycetes*

"They begin their development as unicellular organisms but grow into branched filaments or hyphae which grow profusely by producing further branches constituting the mycelium. The width of thehyphae is usually 1 µm. The delicate mycelia often grow in all directions from a central point and produce an appearance that has been compared with the rays of sun or of a star."

"Therefore, the Actinomycetes are also called 'ray fungi'. They are Gram-positive. The protoplasm of the young hyphae appears to be undifferentiated, but the older parts of the mycelium show definite granules, vacuoles and nuclei. Many Actinomycetes at first produce a very delicate, widely branched, mycelium that may embed itself into the soil, or, if grown in culture, into the solid medium. This kind of mycelium is therefore called the 'substratum or primary mycelium'."

"After a period of growth, hyphae of a different kind develop, which raise themselves up from the substratum mycelium and grow into the air. These are called aerial hyphae, and the corresponding mycelium is the aerial or secondary mycelium. The aerial mycelium may be white yellow, violet, red, blue, green, or grey and many form pigments that are excreted into the medium. Therefore, the Actinomycetes are also called 'ray fungi'. The protoplasm of the young hyphae appears to be undifferentiated, but the older parts of the mycelium show definite granules, vacuoles and nuclei."

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"The aerial mycellium is usually slightly wider than the substratum mycelium. The aerial hyphae possess an extra ceil wall layer (sheath). The hyphal tip undergoes septation within this sheath to form a chain of conidia. Conidial cell contains a plump, deeply staining, oval or rod-shaped nuclear body."(https://www.biologydiscussion.com/bacteria/actinomycetes-economic-importance-and-reproduction/58664)

➢ *Isolation of genomic DNA from Actinomycetes*

Actinomycetes produce important drugs such as antibiotics, immunosuppressants, and antitumor compounds. "The isolation of genomic DNA is imperative for the understanding of the biosynthesis of these compounds and has led to the rational design of new analogs. Actinomycetes are Gram-positive bacteria, making DNA isolation difficult due to their resistance to cell lysis. Most methods use lysozyme and sodium docecyl sulfate (SDS) for cell disruption. To further increase lysis, glycine is often incorporated into media to minimize peptidoglycan cross-linking; muramidases such as mutanolysin or grinding of mycelia are also commonly used. Compared with DNA

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isolation methods for Escherichia coli, most methods are time-consuming or low yielding, or give low-quality DNA. An improved method for DNA isolation from Streptomyces species using achromopeptidase, lysozyme, and SDS for cell lysis that results in higher yield compared with current standard methods. When incubated simultaneously with lysozyme disrupting glycosidic linkages in the polymer, the resulting bacterial structures are more susceptible to SDS lysis. The increase in cell lysis would lead to an increase in DNA concentration for purification in the later stages of the protocol." (Jasmina Nikodinovic, Kevin D. Barrow and Jo-Anne Chuck, University of New South Wales, Sydney and University of Western Sydney, Penrith South, Australia, BioTechniques 35:932-936, November 2003).

II. MATERIALS and METHODS

❖ *Collection of Soil Sample*

The soil sample was collected from eleven different places from the campus of Government Science College in sterile petri pates at a depth of 1-2 cm below soil surface with sterile spatula and forceps.

❖ *Places of soil sample collection*

- Opposite to KV hall
- Opposite to chemistry lab
- Biotechnology garden
- Near canteen
- Near library
- Central flag area
- College ground
- Earthworm casts 1
- Earthworm casts 2
- Botany garden
- Gate garden pots

❖ *Serial Dilution of Soil Samples*

1g of all soil samples was weighed and dissolved in 10 ml of sterile distilled water and was serially diluted upto 10^{-6} dilution using 9ml of sterile distilled water.

❖ *Spread Plate Technique*

Media used for culturing Actinomycetes
STARCH CASEIN AGAR
Soluble Starch – 10.0g
Casein – 0.30g
KNO_3 – 2.00g
$MgSO_4 .7H_2O$ – 0.05g
K_2HPO_4 – 2.00g
NaCl – 2.00g
$CaCO_3$ – 0.02g
$FeSO_4 .7H_2O$ – 0.01g
Agar – 20g
Distilled water – 1000ml
At p.H-7.0

Approximately 20 ml of sterile media was poured into the sterile plates and allowed to solidify. Once the media gets solidified 0.1 ml of 10^{-2}, 10^{-4} and 10^{-6} dilution of soil sample was pipetted on the center of the agar plate. Dip a L-shaped glass spreader into alcohol and then flame the glass spreader over a bunsen burner. Then by using the sterile glass spreader the sample was evenly spread on the agar surface by carefully rotating the petri dish underneath at an angle of 45° at the same time. For each dilution of every soil sample duplicates of agar plates were made. The plates were incubated at 28-30^{0}C for 48-72hrs. After the incubation the plates were observed for growth of colonies of actinomycetes.

❖ *Pure Culture*

The isolated colonies of actinomycetes were pure cultured on slants of starch casein agar using sterile inoculaton loop and then further subcultured on plates for subsequent steps of experiments.

❖ *Bio-Chemical Tests*

Tests were carried out for identification of the isolates (Williams et al., 1989; Holt et al., 1994). Catalase Test

- Transfer a small amount of bacterial colony to a surface of clean, dry glass slide using a loop or sterile wooden stick
- Place a drop of 3% H_2O_2 to the slide and mix.
- A positive result is the rapid evolution of oxygen (within 5-10 sec.) as evidenced by bubbling.
- A negative result is no bubbles or only a few scattered bubbles.
- Dispose of your slide in the biohazard glass disposal container.

Oxidase Test

Requirements for Oxidase test : Moist filter paper with the substrate (1% tetramethyl-p-phenylenediamine dihydrochloride), or commercially prepared paper disk, wooden wire or platinum wire.

- Take a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride.
- Moisten the paper with a sterile distilled water.
- Pick the colony to be tested with wooden or platinum loop and smear in the filter paper.

- Observe inoculated area of paper for a color change to deep blue or purple within 10-30 seconds.

❖ *Screening of Secondary metabolite for Anti-microbial Property*

Primary Screening

Primary screening for evaluating the antimicrobial potential of the isolated actinomycetes was performed by perpendicular streak method against pathogenic bacterial strains. "Isolates were screened for antagonism studied by inoculating a single streak of the pure producer organism in the middle of the assay media (MHA media) plate. The plates were incubated for 4 days at 28° C and subsequently seeded with "test" organism by a single streak at a 90° angle to the steak of the "producer strain" and finally the plates were incubated for 1-2 days at 28° C. The microbial interactions were analyzed by determining the distance of

inhibition measured in mm.” (Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A and Tripathi CKM, 2016)

Clinical Pathogens (Test Organisms) used

- *Proteus vulgaris*
- *Staphylococcus aureus*
- *Pseudomonas aeruginosa*
- *Bacillus subtilis*
- *Escherichia coli*
- *Enterococcus aerogens*
- *Klebsiella pneumonia*
- *Candida albicans*

Secondary Screening

“Isolates showing “moderate” to “ good” inhibition activity were selected for secondary screening, which was performed by agar well method. Pure and active cultures of isolates selected for secondary screening experiment were grown in X-medium (soybean meal-10g/L, $CaCO_3$-g/L, $MgSO_4.7H_2O$-0.5g/L, $(NH_4)_2HPO_4$-0.5g/L, NaCl-3g/L, K_2HPO_4-1g/L, glycerol-15ml/L Ph-6.9 to 7.0),and incubated at 28°C for 3-5 days and cellular growth was confirmed by visible pellets, clumps, aggregates or turbidity in the culture broth. The culture broths were centrifuged separately and filtrates (100µl) were used to evaluate the antimicrobial activity against the above mentioned test micro-organisms by agar well diffusion method.”(Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A and Tripathi CKM, 2016).

- *Isolation Of DNA From Pure Culture*

- “The isolates , were cultured in 30ml of nutrient broth medium (beef extract-2g/L,yeast extract 2g/L,peptone-5g/L and sodium chloride-8g/L pH-7.1 after sterilisation)
- Cells were harvested by centrifugation (5min,4000xg), washed (2x10ml of 10% (w/v) dextrose.
- The pellet was resuspended in 10ml of lysis solution (0.3M dextrose,25mM EDTA,25mM Tris-HCl ,pH-7.5)
- Lysozyme (10mg) was added as crystalline solid (powdered form) to the bacterial suspension and incubated at 37^{0}C for 20 mins.
- For checking for variation in DNA concentration lysozyme concentration was varied (5mg, 10mg and 15mg).
- 12% SDS (1ml) was then added with further incubation at 55^{0}C for 1.5hr.
- After addition of 5M NaCl (3.6ml) and chloroform(15ml) ,the sample was rotated end-over-end for 20 mins at 6 rpm .
- After centrifugation (20mins,5000xg),the aqueous phase was transferred with a pipette into a clean tube .
- DNA was precipitated by addition of 1volume of chilled isopropanol and centrifuged for 10-20mins at 5000rpm.The pellet was rinsed with 1ml 70%(v/v)ethanol.
- The air dried DNA was dissolved in minimum volume of prewarmed buffer containing 10mM Tris;Hcl and 10mM EDTA at 60^{0}C,pH ;7.4.
- Quality of DNA were determined by agarose gel electrophoresis and quantified using Nanodrop.”

(Jasmina Nikodinovic, Kevin D. Barrow and Jo-Anne Chuck, University of New South Wales, Sydney and University of Western Sydney, Penrith South, Australia, BioTechniques 35:932-936, November 2003).

- *Agarose Gel Electrophoresis*

- 1 g of agarose is dissolved in 100 mL 1xTAE in Microwave for 1-3 min until the agarose is completely dissolved.
- Agarose solution is cooled to about 50 °C.
- Ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL is added .
- Agarose is poured into a gel tray with the well comb in place and let sit at room temperature for 20-30 mins, until it has completely solidified.
- Once solidified, it is placed in the electrophoresis unit and covered with 1xTAE buffer.
- Molecular weight ladder of 1kb is loaded into the first lane of the gel.
- Samples are loaded into the additional wells of the gel.
- The gel is run ar 50-100V until the dye line is approximately 75-80% of the way down the gel.
- The DNA fragments are visualized under UV light using UV transilluminator.

- *Quantification of isolated DNA by Nanodrop for study of lysozyme concentration variation*

Procedure

- Double click on the desktop NanoDrop™ 2000 software icon and select the application of interest. Follow the prompts for instrument initialization.
- Establish a Blank using the appropriate buffer. Pipette 1-2 µl of the blanking buffer onto the bottom pedestal, lower the arm and click the Blank button. The blank solution used is TE buffer.
- Wipe away the blank and enter the sample ID in the appropriate field. Pipette 1-2 µl of sample and hit Measure.
- Note the readout.

- *Amplification of isolated DNA by RAPD*

Protocol of RAPD

- Thaw the DNA, PCR master mix and primer vials completely on ice.
- Prepare a cocktail of the reaction components excluding template DNA samples in a vial as follows:

COMPONENTS	QUANTITY
Nuclease free water	35µl
2X PCR master mix	50µl
Template	-
Random Primer	5µl

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- Now aliquot 18µl of the above cocktail into a fresh vial for each sample.
- Add 2µl of the respective template DNA using fresh tip for each sample.
- Mix the contents and incubate in the thermal cycler after setting the following conditions.

PCR conditions:

94º c	2 minutes	Initial denaturation	
94º c	1 minute	Denaturation	40 cycles
45º c	1 minute	Annealing	
72º c	1 minute 30 seconds	Extension	
72º c	7 minutes	Final extension	

- After the reaction is complete, add 2µl of gel loading dye to each vial and load it onto 1.5% agarose gel.

III. RESULTS and DISSCUSION

- *Isolation of Actinomycetes from soil by spread plate method*

The media used for isolation of actinomycetes is SCA (starch casein agar) by using spread plate technique.

Highest number of actinomyetes were isolated from the soil sample collected from botany garden (6), then from the soil sample collected near KV hall (4) and no actinomycete was isolated from soil sample collected near chemistry lab.

Least number of actinomycetes were isolated near canteen (1), library (1), central flag area (1) and earthworm treated sample 1 (1).

The total number of isolated from 11 soil samples are 22. The results are tabulated in the table 1.

Table 1: Isolation of Actinomycetes from soil by spread plate method

Place of soil sample collected	Number of colonies isolated
Opposite to KV hall	04
Opposite to chemistry lab	00
Biotechnology garden	02
Near canteen	01
Near library	01
Central flag area	01
College ground	02
Earthworm casts 1	01
Earthworm casts 2	02
Botany garden	06
Garden pots	02

- *Organism Description (colony characteristics)*

On totally 22 isolates of actinomycetes were isolated from 11 soil samples which showed varied morphological variations from colony colour to colony features.

The various characters are tabulated in table 2.

Table 2: Organism Description

Organism number	Colony size in mm	Colony colour	pigmentation	Colony shape	Elevation	Margin	Consistency	Opacity	Gram character
01	4 mm	Pale yellow	Orangish brown	Irregular	Raised	Undulate	Hard	Opaque	Gram +ve
02	5 mm	Chalk white	Yellow	Circular	Flat	Irregular	Powdery	Opaque	Gram +ve
03	3 mm	Cream	Cream	Circular	Submerged	Curvy	Hard	Opaque	Gram +ve
04	2.5 mm	Cream	Purple	Circular	Flat	Undulate	Hard	Opaque	Gram +ve

05	4.5 mm	Chalk white	Yellowish slightly pink	Irregular	Raised	Undulate	Powdery	Opaque	Gram +ve
06	2 mm	Chalk white	Yellow	Circular	Flat	Entire	Powdery	Opaque	Gram +ve
07	3 mm	Chalk white	Yellow	Circular	Raised	Entire	Hard	Opaque	Gram +ve
08	2.5 mm	Creamish white	Light purple	Circular	Flat	Entire	Hard	Opaque	Gram +ve
09	3 mm	Grey	Creamish yellow	Circular	Raised	Entire	Powdery	Opaque	Gram +ve
10	1 mm	White	Cream	Irregular	Raised	Curvy	Powdery	Opaque	Gram +ve
11	6 mm	White	Yellow	Circular	Raised	Entire	Hard	Opaque	Gram +ve
12	4 mm	Creamish grey	Creamish yellow	Irregular	Raised	Undulate	Hard	Opaque	Gram +ve
13	5 mm	Creamish yellow	Brown	Circular	Raised	Entire	Hard slightly mucoid	Opaque	Gram +ve
14	4 mm	White	Creamish yellow	Circular	Flat	Entire	Hard	Opaque	Gram +ve
15	1 mm	Yellow	Light yellow	Circular	Raised	Entire	Hard	Opaque	Gram +ve
16	3 mm	Brick red	Maroon red	Circular	Raised	Entire	Hard	Opaque	Gram +ve
17	2 mm	Off white	Cream	Circular	Raised	Entire	Hard	Opaque	Gram +ve
18	3 mm	Golden yellow	Yellow	Irregular	Raised	Undulate	Hard	Opaque	Gram +ve
19	0.9 mm	Golden yellow	Creamish yellow	Irregular	Raised	Curvy	Hard	Opaque	Gram +ve
20	1 mm	Pinkish red	Pinkish yellow	Irregular	Flat	Undulate	Hard	Opaque	Gram +ve
21	3 mm	Grey	Light yellow	Irregular	Raised	Irregular	Hard	Opaque	Gram +ve
22	1 mm	Pink	Orangish red	Irregular	Flat	Curvy	Hard	Opaque	Gram +ve

❖ *Bio-chemial Tests*

Biochemical tests were performed on isolated actinomycetes for identification.

Actinomycetes showing positive catalase test were isolate number 1,4,7 and 8.Those showing negative catalase test were isolate number 2,3,5 and 6.

Actinomycetes showing positive for oxidase test were isolate number 1,3,5,6,7 and 8.Those showing negative for oxidase test were isolate number 2 and 4.

The results ar tabulated in table 3.

Table 3: Biochemical test results

Organism number	Catalase test	Oxidase test
01	+ve	+ve
02	-ve	-ve
03	-ve	+ve
04	+ve	-ve
05	-ve	+ve
06	-ve	+ve
07	+ve	+ve
08	+ve	+ve

❖ *Antimicrobial Screening*

➢ *Primary screening*

All 22 isolated actinomycetes were screened primarily by perpendicular streak method against 8 clinical pathogens. Out of the 22 isolates 2 isolates (9 and 14) showed inhibition activity against 2 clinical pathogens

(*Proteus vulgaris* and *Staphylococcus aureus*).
The results are tabulated in table no. 4

Table 4: Primary screening for antimicrobial activity

Isolate no.	*P. vulgaris*	*S. aureus*	*P. aeruginosa*	*B. subtilis*	*E. coli*	*E. aerogens*	*K. pneumonia*	*C. albicans*
01	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
02	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
03	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
04	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
05	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
06	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
07	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
08	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
09	+ve (4 mm)	+ve (2 mm)	-ve	-ve	-ve	-ve	-ve	-ve
10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
11	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
13	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
14	+ve (2 mm)	+ve (2 mm)	-ve	-ve	-ve	-ve	-ve	-ve
15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
18	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
19	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
20	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
21	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
22	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

➢ *Secondary Screening*

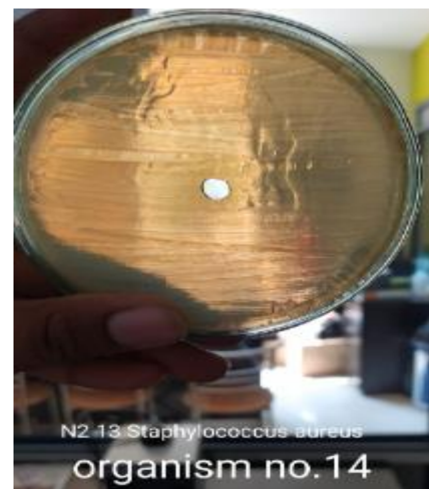
The isolates (9 and 14) that showed inhibition activity in primary screening were selected to perform secondary screening by agar well diffusion method against the clinical pathogens ahainst which the isolates showed inhibition activity.

The isolates showed very less inhibition activity against the pathogens.

The results are tabulated in table no. 5

Table: 5 Secondary screening

Isolate number	Pathogen	Inhibition zone
09	P. vulgaris	> 1 mm
	S. aureus	-ve
14	P. vulgaris	> 1 mm
	S. aureus	-ve



- ❖ *Quantification of isolated DNA by Nanodrop for variation of lysozyme concentration results*

The isolated DNA from all the selected 8 organisms (isolate number 1,2,3,4,5,6,7 and 8) was run on 1% agore gel electrophorosis and from these 3 organisms (isolate number 4,6 and 8) were selected for testing the effect of lysozyme concentration to quantify the concentration of DNA and to standardize the protocol.

In isolate 04 organism isolated DNA concentration was seen maximum with lysozyme concentration 15mg and least with 10mg concentration.

In isolate 06 organism isolated DNA concentration was seen maximum with lysozyme concentration 5mg amd least with 15mg concentration.

In isolate 08 organism isolated DNA concentration was seen maximum with lysozyme concentration 15mg and least with 10mg concentration.

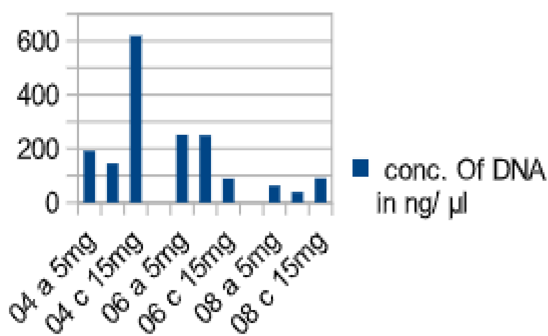
Therefore , maximum concentration of DNA was seen by the usage of 15mg lysozyme concentration.

The quantificaton of DNA was done by using Nanodrop technology and the hence results are depicted in the table 6 and are represented on a bar graph (figure 1).

Table 6: Nanodrop DNA Concentration Variation by varying lysozyme concentration results

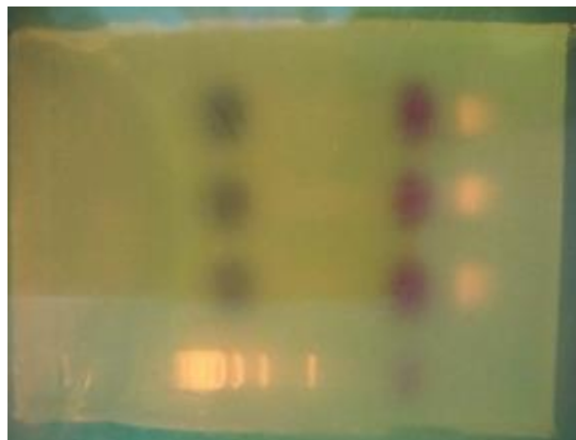
Organism	Place of isolation	Conc. Of lysozyme/ 10ml of lysis buffer	Conc. Of DNA in ng/ µl
04 a	Near KV Hall	5mg	192
04 b		10mg	144.7
04 c		15mg	618.5
06 a	Biotechnology garden	5mg	250.9
06 b		10mg	249.3
06 c		15mg	89.2
08 a	Near Library	5mg	63
08 b		10mg	38.8
08 c		15mg	90.5

FIGURE:01



Lane 1: ladder (1kb) (FROM DOWN TO TOP)
Lane 2: 8a (5mg)
Lane 3: 8b (10mg)
Lane 4: 8c (15mg)
Lane 5: 6a (5mg)
Lane 6: 6b (10mg)
Lane 7: 6c (15mg)
Lane 8: 4a (5mg)
Lane 9: 4b (10mg)
Lane 10: 4c (15mg)

- **Amplification of Isolate no. 4 varied DNA by change in lysozyme concentration by RAPD**



Lane 1: ladder (1kb) (FROM DOWN TO TOP)
Lane 2: amplified DNA of 4a (5mg)
Lane 3: amplified DNA of 4b (10mg)
Lane 4: amplified DNA of 4c (15mg)

- **Identification of isolated actinomycetes using Bergey's manual**

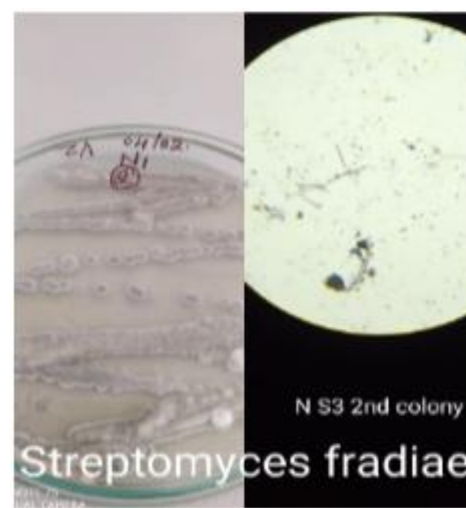
The isolated actinomycetes were identified based on their colony morphology and their microscopic morphology using Bergey's Manual of Systematic Bacteriology Volume five – The Actinobacteria.

The identified actinomycetes are tabulated in table number 7

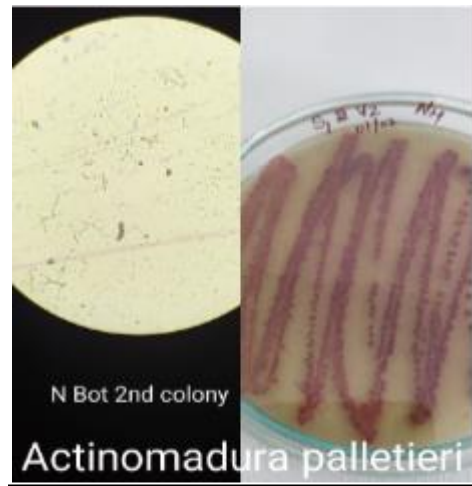
Table:7 Identification of Actinomycetes

ISOLATE NUMBER	IDENTIFIED NAME
01	*Streptomyces griseus*
02	*Streptomyces tandae*
03	*Streptomyces griseorubens*
04	*Streptomyces purpureus*
05	*Nacordiapis alba*
06	*Streptomyces lateritius*
07	*Streptomyces griseoauranticus*
08	*Actinomyces gerencseriae*
09	*Streptomyces fradiae*
10	*Actinomyces bovis*
11	Not identified
12	*Streptomyces viridochromogens*
13	*Gordonia amarae*
14	*Streptomyces bikiniensis*
15	*Actinomyces oris / cardiffensis*
16	*Actinomadura palletieri*

17	Streptomyces albogriseolus
18	Actinomyces turicensis
19	Micromonospora carbonacea
20	Streptomyces roseosporus
21	Actinomyces israelii
22	Streptomyces violaceoruber

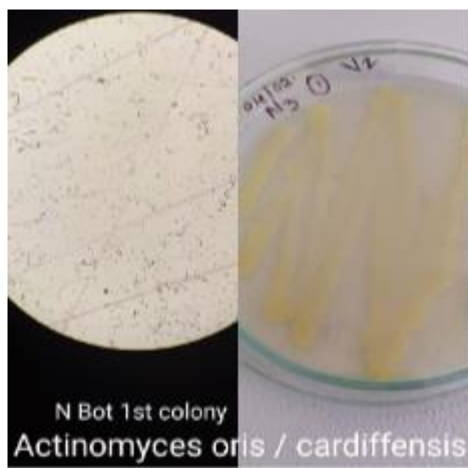


N M 8th colony
Streptomyces viridochromogenes


N Bot 2nd colony
Actinomadura palletieri

N M 13th colony
Streptomyces bikiniensis

N bot 13th colony
Streptomyces roseosporus


N Bot 1st colony
Actinomyces oris / cardiffensis

N bot 16 th colony
Actinomyces israelii

IV. CONCLUSION

The eleven soil samples collected from different places in the college campus were serially diluted to isolate Actinomycetes .As a result of which 22 Actinomycetes were isolated.

Antimicrobial study on all 22 isolates was done through primary (perpendicular streak method) and secondary (agar well diffusion method)) screening against 08 clinical pathogens. Two actinomycetes were found to show only mild anti-microbial property against *Proteus vulgaris*.

The genomic DNA of first 8 Actinomycetes was successfully isolated using lysozyme protocol. Their respective DNA band were viewed on agarose gel.The isolate 04, 06 and 08 were selected to study the effect of variation of lysozyme concentration (5mg,10mg and 15mg) on the amount of DNA .As a result we conclude that 15 mg lysozyme concentration yields the maximum amount of DNA (except for isolate 06). RAPD PCR was performed on all three variations of DNA isolated from isolate number 04 and it was successfully amplified.

Identification of Actinomycetes was done using Bergey's manual of systematic bacteriology volume 5- The Actinobacteria by studing their colony and microscopic morphology.

As a part of the project DNA sequencing of the two isolates that showed anti-microbial activity was supposed to be conducted and also BLAST and FASTA of DNA sequence was to be done. This was not conducted due to the pandemic conditions of COVID-19.

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REFERENCES

[1]. **Abd-Alla, M. H., El-Sayed, E. S. A., and Rasmey, A. H. M.** (2013). Biosynthesis of L-glutaminase by Streptomyces variabilis ASU319 isolated from rhizosphere of triticum vulgaris. Univ. J. Microbiol. Res. 1, 27–35. doi: 10.13189/ujmr.2013.010301.

[2]. **Abd-Alla, M. H., Rasmey, A. H. M., El-Sayed, E. S. A., El-Kady, I. A., and Yassin, I. M. (2016).** Biosynthesis of anti-inflammatory immunosuppressive metabolite by Streptomyces variabilis ASU319. Eur. J. Biol. Res. 6, 152–169.

[3]. **Aoyagi, T., Hatsu, M., Kojima, F., Hayashi, C., Hamada, M., and Takeuchi, T.** (1992). Benarthin: a new inhibitor of pyroglutamyl peptidase. i taxonomy, fermentation isolation and biological activities. J. Antibiot. 45, 1079–1083. doi: 10.7164/antibiotics.45.1079.

[4]. **Arishima, M., Sakamoto, J., and Sato, T.** (1956). An antibiotic Streptomyces no. 689 strain. I. Taxonomic studies. Nippon Nogei Kagaku Kaishi 30, 469–471. doi: 10.1271/nogeikagaku1924.30.8_469.

[5]. **Assaf, N.A. and W.A. Dick.** 1993. Spheroplast formation and plasmid isolation from Rhodococcus spp. BioTechniques 15:10101012.

[6]. **Baltz, R. H. and T. J. Hosted.** 1996. Molecular genetic methods for improving secondary-metabolite production in Actinomycetes. Trends Biotechnol. 14:245-250.

[7]. **Baltz, R. H.** 2001. Genetic methods and strategies for secondary metabolite yield improvement in Actinomycetes. Antonie van Leeuwenhoek 79:251-259.

[8]. **Berdy, J**. (2005). Bioactive microbial metabolites, a personal view. J. Antibiot. 58, 1–26. doi: 10.1038/ja.2005.1.

[9]. **Brockmann, H., and Musso, H.** (1954). Antibiotics from actinomycetes. XXIX. Geomycin 2. Chem. Ber. 87, 1779–1799. doi: 10.1002/cber.19540871128.

[10]. **Cho, H., Beale, J. M., Graff, C., Mocek, U., Nakagawa, A., Omura, S., et al.** (1993). Studies on the biosynthesis of the antibiotic reductiomycin in Streptomyces xanthochromogenus. J. Am. Chem. Soc. 115, 12296–12304. doi: 10.1021/ja00079a009.

[11]. **de los Reyes-Gavilan, C.G., J.F. Aparicio, C. Barbes, C. Hardisson, and J. Sanchez.** 1988. An exocytoplasmic endonuclease with restriction function in Streptomyces antibioticus. J. Bacteriol. 170:1339-1345.

[12]. **Ezaki, T. and S. Suzuki.** 1982. Achromopeptidase for lysis of anaerobic gram positive cocci. J. Clin. Microbiol. 16:844-846.

[13]. **Griffiths, P. J. F., and Ellis, G. P.** (1972). Benzopyrones—VI1: the ultraviolet absorption spectra of chromone and 2-substituted chromones. Spectrochim. Acta A Mol. Spectros. 28, 707–713. doi: 10.1016/0584-8539(72)80039-4.

[14]. **Hayakawa, M.** (2008). Studies on the isolation and distribution of rare actinomycetes in soil. Actinomycetologica 22, 12–19. doi: 10.3209/saj.SAJ220103.

[15]. **Hayakawa, M., Sadakata, T., Kajiura, T., and Nonomura, H.** (1991). New methods for the highly selective isolation of Micromonospora and Microbispora from soil. J. Fermen. Bioeng. 72, 320–326. doi: 10.1016/0922-338X(91)90080-Z.

[16]. **Hazato, T., Naganawa, H., Kumagai, M., Aoyagi, T., and Umezawa, H.** (1979). Beta-Galactosidase-inhibiting new isoflavonoids produced by actinomycetes. J. Antibiot. 32, 217–222. doi: 10.7164/antibiotics.32.217.

[17]. **Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T., and Williams, S. T.** (1994). Bergey's Manual of Determinative Bacteriology, 9th Edn. Baltimore, MD; Philadelphia; Hong Kong; London; Munch; Sydney; Tokyo: William and Wilkins.

[18]. **Hopwood, D. A., and Wright, H. M.** (1973). A plasmid of Streptomyces coelicolor carrying a chromosomal locus and its inter-specific transfer. J. Gen. Microbiol. 79, 331–342. doi: 10.1099/00221287-79-2-331.

[19]. **Hunter, I.S.** 1985. Gene cloning in Streptomyces, p. 19-44. In D.M. Glover (Ed.), DNA Cloning, A Practical Approach, Volume II. IRL Press, Oxford.

[20]. **Jasmina Nikodinovic, Kevin D. Barrow and Jo-Anne Chuck**, University of New South Wales, Sydney and University of Western Sydney, Penrith South, Australia, BioTechniques 35:932-936, November 2003.

[21]. **Kang, M. J., Strap, J. L., and Crawford, D. L.** (2010). Isolation and characterization of potent antifungal strains of the Streptomyces violaceusniger clade active against Candida albicans. J. Ind. Microbiol. Biotechnol. 37, 35–41. doi: 10.1007/s10295-009-0641-9.

[22]. **Kekuda, P. T. R., Onkarappa, R., and Jayanna, N. D.** (2014). Characterization and antibacterial activity of a glycoside antibiotic from Streptomyces variabilis PO-178. Sci. Technol. Arts Res. J. 3, 116–121. doi: 10.4314/star.v3i4.17.

[23]. **Kieser, T., M.J. Bibb, M.J. Buttner, K.F. Chater, and D.A. Hopwood.** 2000. Practical Streptomyces Genetics. The John Innes Foundation, Norwich, England.

[24]. **Kim, C., Lee, K., Kwon, O., Park, D., and Shimazu, A.** (1995). Isolation of rare actinomycetes on various types of soil. Kor. J. Appl. Microbiol. Biotechnol. 23, 36–42.

[25]. **Kim, C., Lee, K., Kwon, O., Yoo, I., and Shimazu, A.** (1994). Selective isolation of actinomycetes by physical pretreatment of soil sample. Kor. J. Appl. Microbiol. Biotechnol. 22, 222–225.

[26]. **Kurtböke, D. I., Chen, C. F., and Williams, S. T.** (1992). Use of polyvalent phage for reduction of streptomycetes on soil dilution plates. J. Appl. Bacteriol. 72, 103–111. doi: 10.1111/j.1365-2672.1992.tb01810.

[27]. **Kutchma, A.J., M.A. Roberts, D.B. Knaebel, and D.L. Crawford.** 1998. Small-scale isolation of genomic DNA from Streptomyces mycelia or spores. BioTechniques 24: 452-456.

[28]. **Lee, Y.K., Kim, H.W., Liu, H.K. and H.K. Lee.** 2003. A simple method for DNA extraction from marine bacteria that produce extracellular materials. J. Microbiol. Methods 52: 245-250.

[29]. **Leonard, R.B. and K.C. Carroll.** 1997. Rapid lysis of gram positive cocci for pulse field electrophoresis using achromopeptidase. Diagn. Mol. Pathol. 6:288-291.

[30]. **Li, S., S. Norioka, and F. Sakiyama.** 1997. Purification, staphylolytic activity, and cleavage sites of a-lytic protease from Achromobacter lyticus. J. Biochem. 122:772-778.

[31]. **Madigan, M. T., Martiko, J. M., and Parker, J.** (1997). "Antibiotics: isolation and characterization," in Brock Biology of Microorganisms, 8th Edn., ed M. T. Madigan (New Jersey: Prentice-Hall International Inc.), 440–442.

[32]. **Marques, D. A. V., Santos-Ebinuma, V. D. C., de Oliveira, P. M. S., de Souza Lima, G. M., Araújo, J. M., Lima-Filho, J. L., et al.** (2014). Screening of wild type Streptomyces isolates able to overproduce clavulanic acid. Braz. J. Microbiol. 45, 919–928. doi: 10.1590/S1517-83822014000300022.

[33]. **Mordarska, H., S. Cebrat, B. Blach, and M. Goodfellow.** 1978. Differentiation of nocardioform Actinomycetes by lysozyme sensitivity. J. Genet. Microbiol. 109:381-384.

[34]. **Ogawa, H., S. Imai, A. Satoh, and M. Kojima.** 1983. An improved method for the preparation of Streptomycetes and Micromonospora protoplasts. J. Antibiot. 36:184-186.

[35]. **Ohba, K., Nakayama, H., Furihata, K., Shimazu, A., Endo, T., Seto, H., et al.** (1987). Nitropeptin, a new dipeptide antibiotic possessing a nitro group. J. Antibiot. 40, 709–713.

[36]. **Okami, Y., and Hotta, K.** (1988). "Search and discovery of new antibiotics," in Actinomycetes in Biotechnology, eds M. Goodfellow, S. T. Williams, and M. Mordarski (London: Academic Press), 37–67.

[37]. **Onda, M., Konda, Y., Hinotozawa, K., and Omura, S.** (1982). The alkaloid AM-6201 from Streptomyces xanthochromogenus. Chem. Pharmaceut. Bull. 30, 1210–1214. doi: 10.1248/cpb.30.1210.

[38]. **Otake, N., Seto, H., Nakayama, H., Endo, T., Oba, K., Iwata, M., et al.** (1988). Antibiotic 6257 manufacture with Streptomyces for Pyricularia oryzae infection control in rice. Jpn. Kokai Tokkyo Koho. JP 63126495 (Accessed May 30, 1988).

[39]. **Praveen, V., Tripathi, D., Tripathi, C. K. M., and Bihari, V.** (2008). Nutritional regulation of actinomycin-D production by a new isolate of Streptomyces sindenensis using statistical methods. Indian J. Exp. Biol. 46, 138–144.

[40]. **Ramalingam, V., and Rajaram, R.** (2016). Antioxidant activity of 1-hydroxy-1-norresistomycin derived from Streptomyces variabilis KP149559 and evaluation of its toxicity against zebra fish Danio rerio. RSC Adv. 6, 16615–16623. doi: 10.1039/C5RA22558B.

[41]. **Rao, R.N., M.A. Richardson, and S. Kuhstoss.** 1987. Cosmid shuttle vectors for cloning and analysis of Streptomyces DNA. Methods Enzymol. 153:166-198.

[42]. **Rodriguez, E. and R. McDaniel.** 2001. Combinatorial biosynthesis of antimicrobials and other natural products. Curr. Opin. Microbiol. 4:526-534.

[43]. **Sanchez, J., C. Barbes, A. Hernandez, C.R. de los Reyes-Gavilan, and C. Hardisson.** 1985. Restriction-modification systems in Streptomyces antibioticus. Can. J. Microbiol. 31:942-946.

[44]. **Shibamoto, N., Terasawa, T., Okamoto, R., Shin, T., and Murao, S.** (1993). Novel postproline endopeptidase and its manufacture with Streptomyces species. Jpn. Kokai Tokkyo Koho. Patent No. JP 05244947, Indexing: Fermentation and Bioindustrial Chemistry (Section 16-4).

[45]. **Shirling, E. B., and Gottlieb, D.** (1966). Methods for characterization of Streptomyces sp. Int. J. Syst. Bacteriol. 16, 313–340. doi: 10.1099/00207713-16-3-313.

[46]. **Singh, V., and Tripathi, C. K. M.** (2011). Olivanic acid production in fed batch cultivation by Streptomyces olivaceus MTCC 6820. Res. J. Pharmaceut. Biol. Chem. Sci. 2, 726–731.

[47]. **Singh, V., Praveen, V., Banga, J., and Tripathi, C. K. M.** (2009). Antimicrobial activities of microbial strains isolated from soil of stressed ecological niches of Eastern Uttar Pradesh, India. Indian J. Exp. Biol. 47, 298–303.

[48]. **Singh V, Haque S, Singh H, Verma J, Vibha K, Singh R, Jawed A and Tripathi CKM** (2016) Isolation, Screening, and Identification of Novel Isolates of Actinomycetes from India for Antimicrobial Applications. Front. Microbiol. 7:1921. doi: 10.3389/fmicb.2016.01921.

[49]. **Takahashi, Y., and Omura, S.** (2003). Isolation of new actinomycete strains for the screening of new bioactive compounds. J. Gen. Appl. Microbiol. 49, 141–154. doi: 10.2323/jgam.49.141.

[50]. **Tanaka, Y., and Omura, S.** (1990). Metabolism and products of actinomycetes. An introduction. Actinomycetolgica 4, 13–14. doi: 10.3209/saj.4_13.

[51]. **Williams, S. T., Goodfellow, M., and Alderson, G. (1989).** “Bergey's manual of systematic bacteriology,” in Genus Streptomyces Waksman and Henrici 1943, 339AL, Vol. 4, eds S. T. Williams, M. E. Sharpe, and J. G. Holt (Baltimore, MD: Williams and Wilkins), 2452–2492.

[52]. **Wu, R. Y.** (1984). Studies on the Streptomyces SC4. II Taxonomic and biological characteristics of Streptomyces strain SC4. Bot. Bull. Acad. Sin. 25, 111–123.

[53]. .**Yanagida, T. and H. Ogawara.** 1980. Deoxyribonucleases in Streptomyces. J. Antibiot. 33:1206-1207.

[54]. **Zhang, L., Zhang, J., Yang, W., and Bai, G.** (2008). Classification of Streptomyces strain Z314 and purification of its product pravastatin. Wei Sheng Wu Xue Bao 48, 33–37.

[55]. https://www.biologydiscussion.com/bacteria/actinomycetes-economic-importance-and-reproduction/58664.