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ISOLATION AND CHARACTERIZATION OF NOVEL ACTINOMYCETES STRAINS FROM THE SOIL OF DARJEELING TEA GARDEN

[1]*Swagata Chakraborty and [2]Dr. Maitree Bhattacharyya

[1]Department of Biotechnology, Techno India University, Salt Lake, EM-4, Sector V, Kolkata- 700091, West Bengal, India.
[2]Department of Biochemistry, University of Calcutta, 51/1, Hazra Road, Kolkata- 700019, West Bengal, India.

Corresponding Author: Swagata Chakraborty
Department of Biotechnology, Techno India University, Salt Lake, EM-4, Sector V, Kolkata- 700091, West Bengal, India.

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ABSTRACT

The organism Actinomycetes was isolated from the soil of the Darjeeling tea garden for carrying out the research. The organism Actinomycetes is a sub-group of Actinobacteria which are Gram- positive and are generally rod-shaped or filamentous in nature. They are generally found in soil and also in the urogenital tract, gums and in the digestive system of humans. The bacteria was grown in starch-caesin media followed by the genomic DNA isolation. PCR was carried out for the amplification of the 16s rRNA gene which is unique in almost all bacterial communities and thus acts as a historical or chronological clock, followed by agarose gel electrophoresis. For bacterial identification Biochemical tests were carried out. The actinomycete strains were further screened for the production of secondary metabolites by cross-streak. Furthur purification was carried out and sequencing was done which was then matched with nucleotide sequences from a number of databases.

KEYWORDS: Actinomycetes, gram positive- bacteria, PCR, 16s r RNA, Biochemical tests, secondary metabolites.

INTRODUCTION

The Darjeeling tea garden is a home to variety of soil micro-organisms. Darjeeling tea is mainly grown in the Darjeeling district and the Kalimpong district. Unlike most Indian trees, Darjeeling tea is normally made from the small-leaved Chinese variety of Camellia sinensis. Traditionally Darjeeling tea is black, however Darjeeling oolong and green tea are becoming much more easier to find or produce. However the Darjeeling estates are producing white tea as well.

Geography- Darjeeling is located in 27.0360 ℕ and 88.2627 ℍ

Soils of Darjeeling

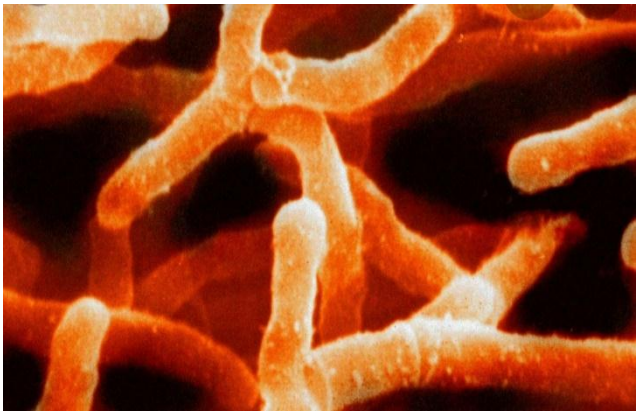
The soils in the Kalimpong area are predominantly red in colour. All the soils are acidic in nature with the tendency to slightly increase slightly in depth, in most cases indicating the lacking of bases from surface and accumulation in lower horizons. The basic soil types are yellow, red brown and deep brown forest soil.

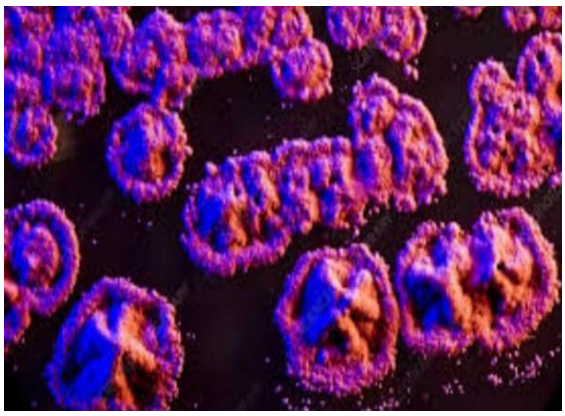
Figure 1: Shows Darjeeling soil.

Actinomycetes

Actinomycetes are Gram-positive bacteria having a complex cell wall(containing muramic acid) that makes Gram staining unsuitable. They have prokaryotic nuclei and are susceptible to antibiotics. They generally form ring or chain like structures which are viewed under microscope.

This type of bacteria is mostly found in soil or in decomposing matter and they form symbiotic nitrogen fixing associations with almost 200 species of plants. Apart from plants they can also be found on the human urogenital tract as well as the digestive system. Compared to the DNA of the organisms, the actinomycetes have a high percentage of guanine-cytosine bases. In growth, this genus generally resembles the fungi but they are much smaller than the fungi. They are highly beneficial in human medicine, as half of the antibiotics such as chloromycetin, kanamycin, streptomycin etc came from soil actinomycetes.





(2) **(3)**

Figure 2 and 3 shows actinomycetes bacteria.

16s rRNA

16s r RNA or 16s ribosomal RNA is the component of the 30S small subunit of a Prokaryotic ribosome that binds to the Shine-Dalgarno sequence. The genes coding for it are referred to as 16s RNA gene. The 16s rRNA genes are ubiquitous i.e the ribosomes cannot translate mRNA without the 16s RNA component. The conserved regions allows the primer to be designed to target the bacteria, but they can amplify the 16s gene through a variable region in which differences in the sequence of the bases allow for the determination of various species. 16s rRNA genes are important because the phylogenetic trees are well developed and taxonomic information is readily

AIMS AND OBJECTIVES

1. To determine the uniqueness of the actinomycetes species
2. To determine the type of species
3. To determine the size of the DNA
4. 16S RNA sequencing for identification of the species
5. Biochemical Test to differentiate the bacterial type for determination of secondary metabolite synthesis
6. How anthropogenic influence shapes the characteristic of the bacteria
7. Characterization
8. Bioactive molecule screening

Literature Review

1-Definition

Actinomycetes- They are a order of actinobacteria which are Gram positive bacteria and generally aerobic in nature which can be mainly found in soil

Ref- Actinomycetales- List of prokaryotic names with standing in nomenclature

Origin

The word actinomycetes were derived from a Greek word mukes or muketes. Actinomycete is a modern latin word.

Ref- Wikipedia

2-Definition

16s r RNA gene- 16s rRNA is the component of the 30S small subunit of a prokaryotic ribosome that binds to Shine-Delgarno sequence and the genes coding for it are referred to as 16s rRNA genes.

Ref- Wikipedia

Origin

The large ribosomal subunit and the peptidyl transferase centre reconstructed the ancestral sequences of the tRNA's and they showed similarity between concatamers of proto-tRNAs with modern PTC.

Ref- Article in origin of the 16s Ribosomal molecule from ancestor tRNAs.

The gram positive bacteria actinomycetes are slow growing filamentous bacteria that are mainly found in the soil. Apart from this they are also found in the human's urogenital tract and their digestive tract. 16s rRNA genes are mainly found in all the bacterial species. Thus this gene acts as an evolutionary clock, the

sequencing of which will aid in the identification of the organism. The 16s rRNA PCR and sequencing was done.

Ref- Identification by 16s rRNA Gene sequencing of Actinomycetes; NCBI

Analysis of Actinomycete communities by specific amplification of genes encoding 16s rRNA and gel electrophoretic separation in denaturing gradient; Heuner, Krsek, Baker, Smalla, Wellington; American society for Microbiology journal.

However, microbial pathogens are becoming increasingly resistant to available treatment and thus new antibiotics are needed, against antibiotic resistant strains of the organisms. The Actinomycetes provide a variety of antibiotics which are diverse in nature. The organism is needed to produce a variety of bioactive compounds and the 16s rRNA gene sequencing is unique to the bacterial community.

Ref- Diversity and Versaltility and its role in antibiotic production; Journal of applied pharmaceutical science.

MATERIALS AND METHODS

Isolation

Cultivable strains of bacteria were isolated using initial screening in normal saline solutio0.9%). Serial dilution was done(10 fold dilution) and the saline suspensions were spread spread on casein-starch medium containing starch, casein, potassium nitrate, potassium dihydrogen phosphate, sodium chloride, magnesium sulphate, calcium carbonate, ferrous sulphate with suitable antibiotics. The plates were incubated at 28 ℃ for 5-7 days. Single colonies were selected and further streaked on casein-starch medium. The process was repeated 5-6 times to facilitate enrichment of pure cultures.

After isolation starch-caesin media was prepared again for the growth of the bacteria. The growth of the bacteria was determined in two ways: 1) growth in plate and 2) growth in broth.

Media preparation and components for plates

Starch-10gm
Caesin-1gm
KNO3-2gm
KH2PO4-2gm
NaCl-2gm
MgSO4-0.5gm
CaCO3-0.02gm
FeSO4.7H2O-0.001
Agar-18gm

The pH was checked and maintained between 7.0-7.4(calibrated with 2M NaOH)

Pour plate method

The media was autoclaved and it was carefully poured into the plates. They were kept overnight to solidify.

Quadrant plate streaking

The rules for quadrant streaking was followed and the plates were streaked for isolating single bacterial colonies. The plates were kept at 28 ℃ for 5-7 days as this bacterial species is slow growing in nature.

Media preparation and components for broth
Starch-10gm
Caesin-1gm
KNO3-2gm
KH2PO4-2gm
NaCl-2gm
MgSO4-0.5gm
CaCO3-0.02gm
FeSO4.7H2O-0.001
pH was measured and kept between 7.0-7.4(calibrated with 2M NaOH)

The agar plates on which the bacteria has grown for several days are taken out. The broth media and toothpicks are autoclaved. The work was carried out in a laminar air flow chamber. Sterile toothpicks were used to pick up the bacterial colonies from the plate and put in the broth. After the procedure was over, the broth tubes were kept in 28 ℃ for the next 5-7 days. The clear solution turned turbid or yellowish which confirmed the growth of the bacteria.

Genomic DNA Isolation

2ml culture was taken and centrifuged at 14,000 rpm to collect the pellet. The culture media was removed and discarded
It was then resuspended into 300µl lysis buffer.

2µl RNAase solution was added and incubated at room temperature for 2mins. This helps in removing the RNA from the mixture.

20µl Proteinase K solution was added which helps in inhibiting the protein. The solution was mixed and the resuspended cells were transferred to the HiBead Tube and incubated for 30mins at 55 ℃.

The HiBead Tubes were horizontally secured on a flat bed vortex pad with tape. It was then vortexed at maximum speed for 5-7 mins.

The mixture was then incubated for 10mins at 95 ℃ followed by pulse vortexing.

The tubes were centrifuged at 13,000rpm for 1min at room temperature.

The supernatant was then transferred to clean capped 2.0 ml collection tube.

200µl lysis buffer was added and vortexed thoroughly (15secs), followed by incubation for 10mins at 55 ℃.

200µl ethanol was added to the lysate and mixed

thoroughly by vortexing for 15 secs.

The lysate was the loaded in HiEulute Mini prep spin column (capped) and centrifuged at 10,000rpm for 1min. The flow through was discarded and the spin column was placed in the same 2.0ml collection tube.

Prewash: 500µl of prewash solution was added to the column and centrifuged at 10,00rpm for 1min. The flow through was was discarded and thee same collection tube with the column was reused.

Wash: 500µl of wash buffer was added to the column and centrifuged for 3mins at 14,000rpm. The flow through was discarded the same collection tube was used.

Additional 1 min of centrifugation was done for presence of ethanol in column.

DNA Elution: The flow through was discarded and the column was transferred to an uncapped collection tube. At first 70µl of Elution buffer was added directly to the centre of the column and incubated at room temperature for 1 min. It was then centrifuged at 10,00rpm for 1min. Now 30µl Elution buffer was added and the same procedure was followed as above.
The DNA was stored at -20℃.

16S r DNA PCR
Primer designing was done to determine the suitable primers
PCR reagents and amounts
Water – 16.25µl
Buffer – 2.5µl
Forward primer -1µl
Reverse primer -1µl
dNTP-1µl
Taq pol -0.25µl
Template -3µl
Total- 25µL

PCR conditions
The V1-V9 region of the 16s rRNA gene was amplified using the standard PCR protocol. The PCR conditions are mentioned below;

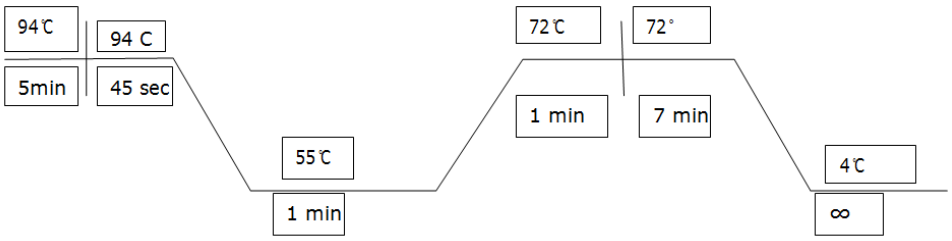


Figure 4: shows the PCR conditions.

Agarose gel electrophoresis
This is the most unique way of determining the size of DNA. The phosphate in the DNA is negatively charged so it will run towards the positive pole. The band was obtained in 1000bp range as compared with the DNA ladder.

PCR Purification
The protocol for PCR purification was followed and the products were purified for sequencing. The Qiagen gel extraction was used for purification.

Library Preparation
The amplicon library was purified and pooled amplicons were diluted as recommended and amplified by emulsion PCR on a thermal cycler.

Biochemical Test
Gram Staining
The Gram stain developed in 1884 by Danish physician Christian Gram is the most widely employed staining method in bacteriology. It is an example of differential staining because the use of Gram stain divides the bacteria into two classes- Gram positive and Gram negative.

The procedure is as follows
After heat fixation, Smear is stained with basic dye crystal violet, the primary stain

Treatment with iodine solution functioning as a mordant. The iodine increases the interaction between the cell and the dye so that the cell is stained more strongly

Smear is decolourised by washing with ethanol or acetone, this step generated differential aspect of Gram staining, the gram-positive bacteria wil retain the crystal violet dye whereas the gram-negative bacteria will become colourless.

Finally the smear is stained with a counter stain called safranin. The gram negative bacteria as a result becomes pink as it retains the safranin stain.

Secondary Metabolites
Screening of microbial secondary metabolites is an important method to identify biologically active compounds. The procedure is as follows;

At first media was prepared (LB Agar broth) and autoclaved.

Then the media was poured into the plates and kept to solidify in the LAF for about 1 hour.

Strains of Actinomycetes were taken and streaked into the LB agar plates in a perpendicular manner and incubated at 28℃.

Ater 7 days the growth was checked and it was streaked with other standard strains for determining the activity of secondary metabolites.

The secondary metabolites spreads into the middle of the plate to a particular distance. So upto that distance the other micro-organisms cannot grow which means that with respect to that organism, these secondary metabolites are potent antibiotics which inhibits their growth. So it can be said that the secondary metabolite synthesized from this compound is a potential source of new antibiotic.

RESULTS AND DISCUSSION

Quadrant Streak Plates

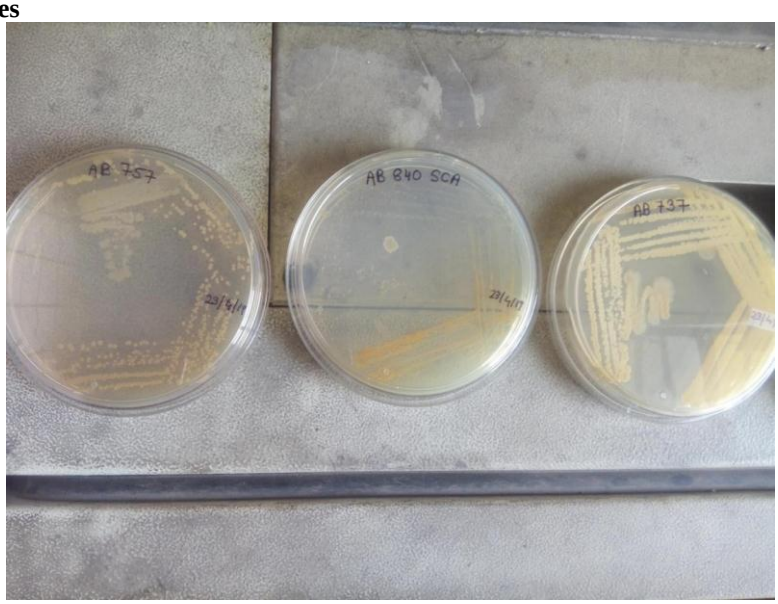


Figure 5: Shows the first three strains used for quadrant streaking.

Quadrant Streak Plates

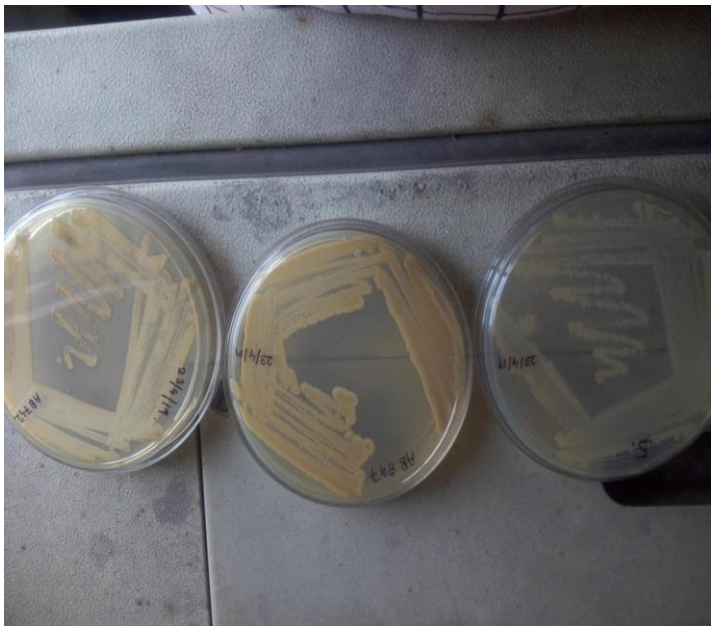


Figure 6: Shows the second part of the three strains used for quadrant streaking.

Gram-positive bacteria under microscope

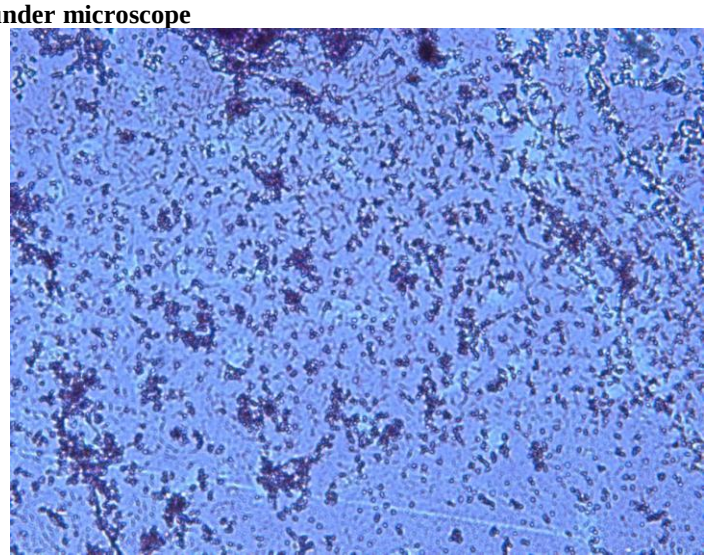


Figure 7: Shows Strain AB737 under microscope.

Gram positive bacteria under microscope

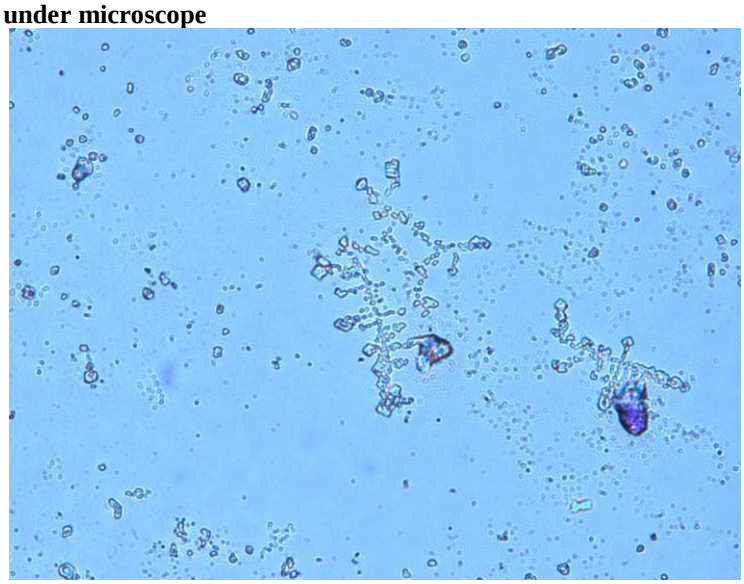


Figure 8: Represents Strain AB757 under microscope.

Gram positive bacteria under microscope

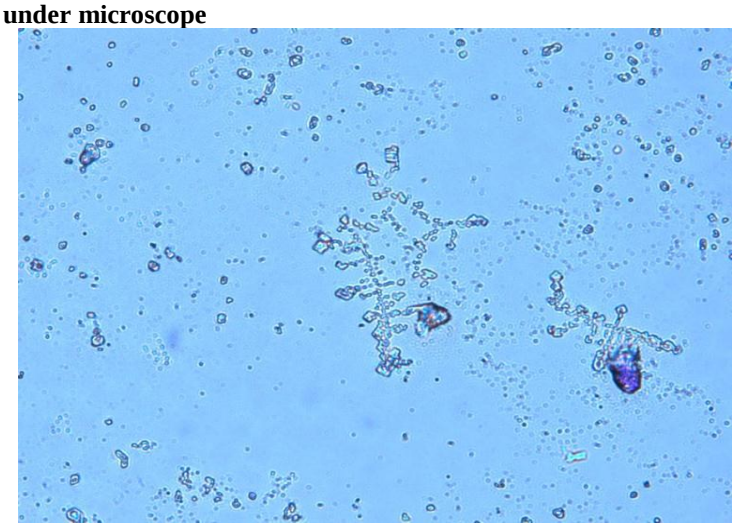


Figure 9: Represents Strain AB757 under microscope.

Gram-positive bacteria under microscope

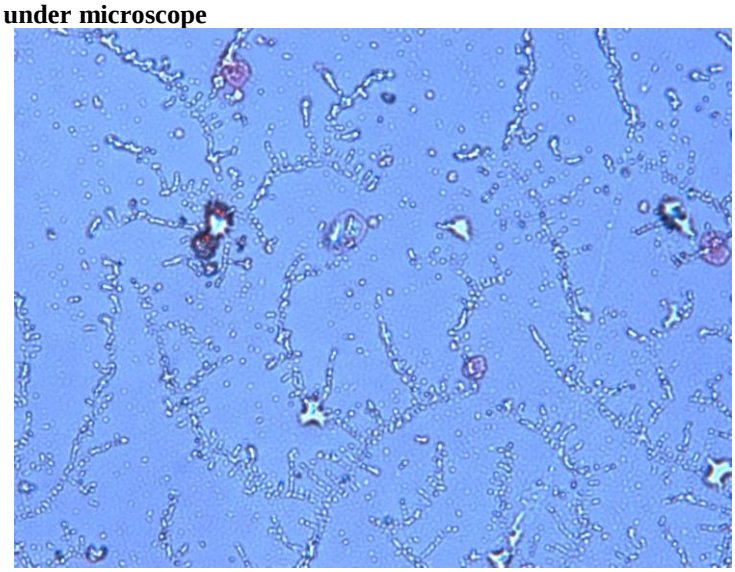


Figure 10: Shows Strain AB SCA5 under microscope.

Gram-positive bacteria under microscope

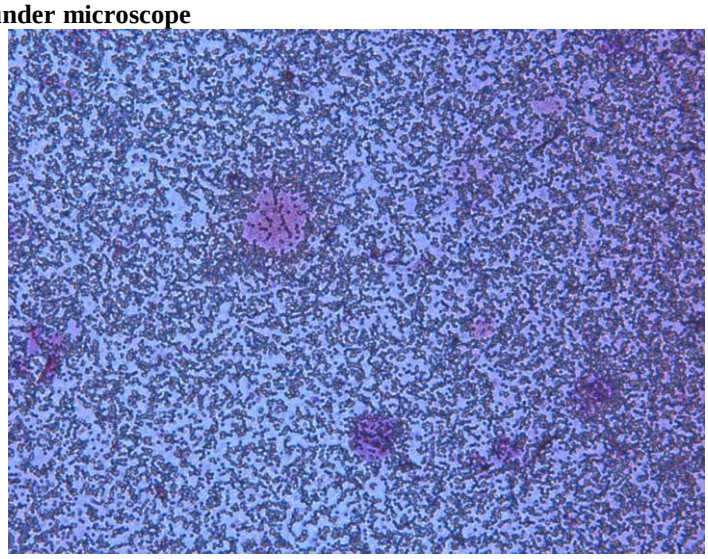


Figure 11: Shows Strain AB 742 under microscope.

Gram- positive bacteria under microscope

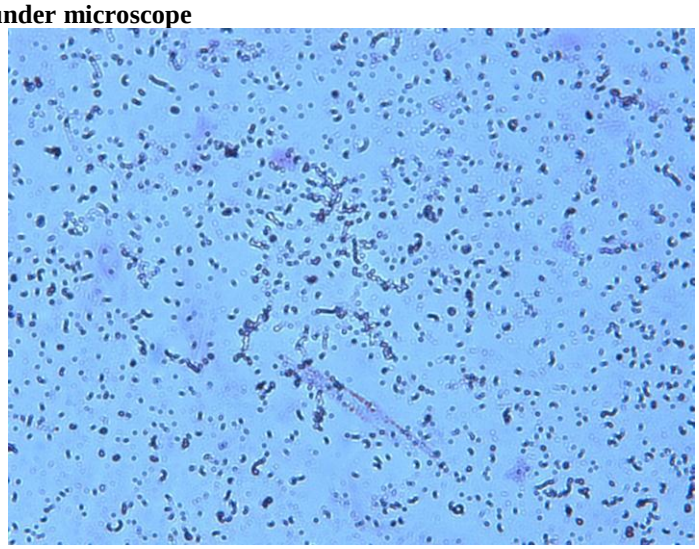


Figure 12: Shows Strain AB 847 under microscope.

Cross-streak for determining the activity of secondary metabolites

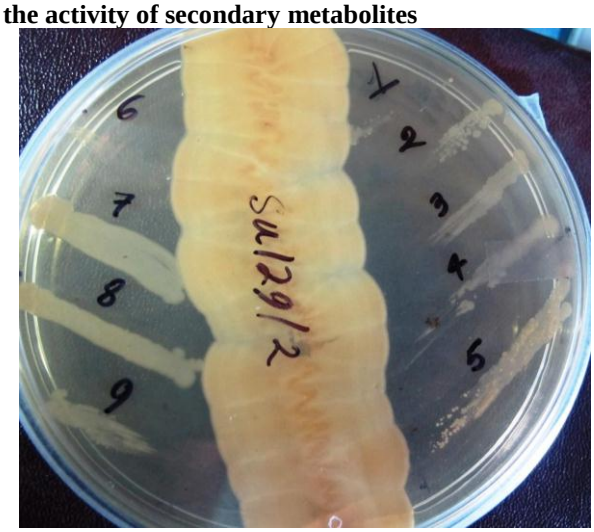


Figure 13: Shows the activity of the secondary metabolites. The mother strain streaked perpendicularly is the actinomycetes and the rest of the strains are as follows 1-E.coli, 2-B.subtilis, 3-P.aeruginosa 4-S.typhi 5-V.cholerae 6-E.aerogenes 7- S.aureus 8- S.typhimurium, 9- B.hemolyticus.

CONCLUSION

Actinomycetes are a group of gram positive bacteria which is hugely used in research due to its beneficial effect. These bacteria are a source of about 90% of potent antibiotics. Generally this group of bacteria uses variety of substrates found in soil and that is what makes it an important bacteria to study about. They are potent bio fertilizers and apart from being nitrogen fixing bacteria they produce geosmin which imparts earthy odor to soil.

In this project we mainly studied how actinomycetes behave as a potent source of antibiotics and how unique 16s r RNA genes are. These genes are ubiquitous i.e the cannot translate mRNA without their 16s RNA. This study clearly states how 16s r RNA acts as a signature sequence unique for a particular species thus giving us insight about the evolution of the organism through time.

Another main point of focus that the study iterates is the most challenging genre of research: antibiotic resistant. We used actinomycetes as the mother strain and streaked it against nine different strains to check the growth of potent secondary metabolite which will be a potent antibiotic with respect to that organism and thus we detected new strains which will be beneficial in the genre of antibiotic resistance.

More studies are done and are required because antibiotic resistance is a potential threat to mankind because there can be days when there will be no potent antibiotic to treat infections. In addition to this study, further studies are required on actinomycetes and anti-microbial resistance to further draw conclusion for antibiotic resistance which will most likely be another pandemic in near future and also for various reasons like bioremediation and natural resources.

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