ISOLATION AND MOLECULAR CHARACTERIZATION OF STREPTOMYCES SPECIES OF ACTINOMYCETES

Submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Sathyabama Institute of Science and Technology

by

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BONAFIDE CERTIFICATE

This is to certify that this Project report is the bonafide work of **R.JEEVIKA (39230018)** who carried out project entitled **"ISOLATION AND MOLECULAR CHARACTERIZATION OF STREPTOMYCES SPECIES OF ACTINOMYCETES"** under my supervision from **October 2022 to April 2023.**

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DECLARATION

I **R. JEEVIKA (39230018)** hereby declare that the Project Report entitled **ISOLATION AND MOLECULAR CHARACTERIZATION OF STREPTOMYCES SPECIES OF ACTINOMYCETES** done by me under the guidance of **Dr. C. VALLI NACHIYAR** (Internal guide) and **Dr.R. RAJESH KANNAN** (External Guide) at **IRC** (International Research Centre) in Sathyabama Institute of Science and Technology is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

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ABSTRACT

Streptomyces species are a type of *actinomycetes*, which are gram-positive, filamentous bacteria that are commonly found in soil. They are known for producing a wide variety of bioactive compounds, including antibiotics, anti-tumor agents, and immunosuppressants.*Streptomyces* species are important members of the *Actinomycetes* group, known for their ability to produce a vast array of secondary metabolites with diverse biological activities.In this study isolation and characterization of the *Streptomyces* species of *Actinomycetes* was carried out.

Actinomycetes have provided many industrially important bioactive compounds having great economic importance and have long been recognized as prolific producers of enzymes, antibiotics, anticancer agents. Numerous methods have been advocated for isolation of *actinomycetes* to facilitate the discovery of natural compounds especially antibiotics.*Actinomycetes* have been isolated from a wide range of soil types collected from disparate geographical locations.For the isolation of *actinomycetes*, various methods can be performed on the basis of different sources and media.Further characterization can be performed to study the different strains of *actinomycetes*This species have several applications in medicine, bioremediation, agriculture, industrial biotechnology, and drug discovery.

The plating(methods)technique and media uses are one which ensures the development of most microorganisms as surface colonies, thus greatly expediting their tentative identification and comparison. Fifteen-ml aliquots of an appropriate medium were poured into Petri dishes and allowed to harden to form a basal layer.In serial dilution method,the dilution count provides an estimate of the number of living organisms in a sample which are capable of multiplying in a given liquid medium.Also, the cultural characteristics of the species be examined,to evaluate the biochemical test of the species,to characterise the species by sequencing and antimicrobial activity of the species can be evaluated for further more detailed information about the species

Keywords- *Actinomycetes,* isolation, bioactive compounds,.

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1.INTRODUCTION

In earlier times the *Actinomycetes* were designated as fungi because of their morphological appearance and the development like fungi of true mycelium.Therefore they were at first called "ray fungi". However recent exhaustive studies give support to the opinion that the *Actinomycetes* are more closely related to bacteria than to fungi.The *Actinomycetes* are well known as a group of filamentous, Gram-positive bacteria that produce many useful secondary metabolites, including antibiotics and enzymes. Although they have been intensively studied for both theoretical and practical objectives, there is much scope for developing our basic knowledge of the means of detection and isolation of these microbes.Many of these problems could be solved by improved techniques and a broad based assessment of the roles of *actinomycetes* in the natural environment and their potential for production of metabolites in vitro.

Actinomycetes have been extensively studied for their ability to produce pharmaceutically useful compounds, however, the rate of discovery of novel compounds from these bacteria has significantly decreased. New Species of *actinomycetes* have attracted considerable attention as one of the most important resources for new bioactive compounds, but there is much controversy regarding whether these species of *actinomycetes* will produce new compounds with high frequency.

To investigate the problem, the isolated new and known species of *actinomycetes* from a variety of substrates are examined the secondary metabolites produced, including potential novel compounds.Taken together, the percentage of new compound producers to new species was 6.6%, new compounds to new species was 9.4%, new compound producers to known species was 5.4%, and new compounds to known species was 10.4%.

On the other hand, *Streptomyces* species that have high similarity to known species produced compounds with diverse structures. Because recent advances in genome research have revealed that the ability of *actinomycetes* to produce secondary metabolites has been underestimated due to the presence of cryptic biosynthetic gene clusters, we attempted to culture these strains with several production media, but the production of new metabolites by these strains was not remarkably improved by changing production media.In conclusion, *actinomycetes*, especially *Streptomyces,*should still be considered attractive sources of bioactive compounds, and it is important to isolate actinomycetes from a wide variety of environmental substrates by using various isolation methods for obtaining new bioactive compounds.

Actinomycetes are widely distributed in nature. They are found in virtually every natural substrate and are the most prolific producer of 80% of known antibiotics.*Actinomycetes* are prokaryotes with extremely various metabolic possibilities.The G+C content of DNA of *Actinomycetes* ranges from 57-75 %. The genus *Streptomyces* represented in nature by the largest number of species and varieties among the family *Actinomycetaceae*. They differ greatly in their morphology, physiology and biochemical activities, producing the majority of known antibiotics.

Antibiotics have been isolated from almost all the suborders of *actinomycetes*. Despite increase in antibiotic resistance to commonly used drugs, there is still a steady supply of novel antimicrobial agents from *actinomycetes* isolated from the natural environment.*Actinomycetes* play a vital role in the soil such as mineralization of organic matters, immobilisation of nutrients, antibiosis and production of plant promoters.The term *actinomycete* has no taxonomic validity since these organisms are classified as bacteria in a strict sense. It belongs to the order *Actinomycetales*, but not all genera of the *Actinomycetales* are considered to be actinomycetes in common parlance. The *actinomycetes* are microorganisms that produce slender, branched filaments, which develop into a mycelium in all soil genera except for the genus *Actinomyces*.

The most powerful approaches to taxonomy are through the study of nucleic acids. Analysis of the 16S rDNA begins by isolating DNA and amplifying the gene coding for 16S rRNA using the polymerase chain reaction. The purified DNA fragments are directly sequenced. The sequencing reactions are performed using DNA sequencer in order to determine the order in which the bases are arranged within the length of sample, and a computer is then used for studying the sequence for identification using phylogenetic analysis procedures.The sequence of the 16S rRNA gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny), but more recently it has also become important as a means to identify an unknown bacterium to the genus or species level.The most common phylogenetic marker for microbial community diversity studies is the 16S ribosomal RNA gene and in the last 10 years the field has moved from sequencing a small number of amplicons and samples to more complex studies where thousands of samples and multiple different gene regions are interrogated. Two approaches have been widely used in practice to describe microbial community structure, 16S rRNA gene profiling and shotgun metagenomic sequencing.Ribosomal RNA genes are highly conserved and evolutionarily stable but differ in their hypervariable region, these features have made them the ideal tool for phylogenetic studies. Several papers have described the natural variation using in silico analyses and genome sequencing have boosted our knowledge of the biological world improving ribosomal gene databases which support phylogenetic studies. These improvements have made the 16S rRNA gene the ideal marker for the characterization of microbial community diversity. The 16S rRNA gene compromises 9 hypervariable regions, which differ in length, position and taxonomic discrimination.The 16S rRNA is suitable for this purpose for several reasons. The gene is universally distributed, allowing the analysis of phylogenetic relationships among distant taxa. As a functionally indispensable part of the core gene set, the 16S rRNA gene is expected to be only weakly affected by horizontal gene transfer, which further supports its use for phylogenetic studies. Despite the above,16S rRNA is still subject to variation, especially in certain variable regions.

Likewise, the traditional methods of species classification and the identification of the organism(s) are mainly based on morphological, physiological, biochemical, developmental, and nutritional characters, and it is not adequate, and warrants for the use of molecular level approaches for assigning accurate taxonomic classification. Hence, precise assignment of taxonomic status to the novel bioactive microbial isolates through existing predictive bioinformatics methods and tools are very essential and aid in chemical characterization of the active molecules.With the variation of type of soil, according to the geographical changes, soil provide very complex habitat to the microbes residing in it. Due to this intricate environment, the soil microbes play an important role in the isolation of novel drugs. Among soil microbes, the members of Streptomyces sp. of actinomycetes, have been widely exploited for the production of commercially important secondary metabolites and enzymes. In the present study, the soil samples from **Sathyabama University** have been screened-out to isolate *Streptomyces* sp. of *Actinomycetes* possessing molecular, cultural and biochemical characterization.

2.REVIEW OF LITERATURE

Actinomycetes have been recognized for over a hundred years primarily on morphological criteria. They are usually considered to be bacteria with the ability to form branching hyphae at some stage of their development. This attribute can be difficult to detect and is not always sufficient to distinguish actinomycetes with a transient mycelium from some other gram-positive bacteria. Indeed, the exact composition and boundaries of the order Actinomycetales remains open to question and to modification from the continued application of new taxonomic methods.Actinomycetes exist in their natural habitats in states either metabolically active or relatively inactive.

The actinomycetes,particularly species from the genus Streptomyces, have proved to be a tremendous high-impact source of valuable chemicals. They have yielded many clinically essential antimicrobial compounds, including streptomycin, actinomycin, and streptothricin.Approximately two-thirds of all known antibiotics are produced by actinomycetes, predominantly by Streptomyces.It is believed that the actinomycetes are the source of some 61% of all microorganism-derived bioactive substances so far discovered with 16% of the total originating from the “rare actinomycetes”, mostly from the Micromonosporaceae, with additional smaller contributions from the Pseudonocardiaceae and Thermomonosporaceae. This suggests that rare actinomycetes are a valuable source of novel compounds, and that improved isolation strategies are required to increase the frequency in which they are isolated.

Natural products have been the source of the world’s most important bioactive compounds and microorganisms are a primary resource driving drug discovery.Although numerous actinomycetes have been isolated, and used as producer of key drugs and biomedical agents, it has lately become increasingly difficult to find novel compounds as well as to avoid time and resource consuming repeated detection of known compounds.

2.1. BIOACTIVE COMPOUNDS

Because of their useful biological activities, microbial secondary metabolites have received considerable attention especially in the beneficial effects of human health. actinomycetes also play an extensive role in the pharmaceutical and medical industry for their capacity to produce secondary metabolites with diverse chemical structures and biological activities.Thousands of bioactive compounds have been isolated and characterized,many of which have been developed into drugs for treatment of wide range of diseases in human, veterinary,and agriculture sectors.Hence, the actinomycetes are considered to be the most potent source for the production of secondary metabolites, antibiotics, and other bioactive compounds. It is well established that each actinomycete strain has probably genetic potential ability to produce 10–20 secondary metabolites.Therefore, screening, isolation, and characterization of promising strains of actinomycetes producing potential antibiotics and other therapeutics have been a major part of research.

2.2. APPLICATIONS OF BIOACTIVE COMPOUNDS OF ACTINOMYCETES

2.2.1. ENZYME PRODUCTION FROM ACTINOMYCETES

Actinomycetes secrete amylases to the outside of the cells to carry out extracellular digestion. α amylase starch degrading amylolytic enzymes is of great significance in biotechnological applications such as food industry, fermentation and textile to paper industries.Lipase is produced from a variety of actinomycetes, bacteria, and fungi.Lipases havebroad applications in the detergent industries, foodstuff, oleochemical, diagnostic settings and also in industries of pharmaceutical fields.Enzyme inhibitors are finding possible uses in cancer treatment. e. g. revistin, an enzyme inhibitor from Streptomyces species inhibit reverse transcriptase.α-Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry.

These enzymes account for about 30 % of the world‟s enzyme production.Lipases are part of the family of hydrolases that act on carboxylic ester bonds. The natural function of lipases is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol.α -Amylases are the enzymes first to be commercially produced and marketed. Dr. J.Takamine established the first industrial production of α -amylase from A. oryzae known as “Taka diastase”, which was used as a digestive aid.The world production of α- amylases from B. licheniformisand Aspergillus sp. was about 300 tons of pure enzyme protein per year.The bulk uses of alkaline proteases in industrial sectors are Food and feed industry. Traditionally, microbial proteases have been exploited in the food industries in many ways. Alkaline proteases have been used in the preparation of protein hydrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks.

2.2.2. Human Health Importance

Actinomycetes are produced many **antibiotics**, that are best recognized and most valuable. These antibiotics include **amphotericin, nystatin, chloramphenicol, gentamycin, erythromycin, vancomycin,tetracycline, novobiocin, neomycin,** etc.In these antibiotics some are targeted bacterial ribosome‟s and are used in treating respiratory infections, for example in treating the Legionnaires‟ disease used tetracycline and erythromycin.e. Amphotericin is one of the minority antibiotics that attack fugal membranes. These antibiotics usually do not influence human cells and for that reason have fewer side effects.Actinomycetes produce a variety of antibiotics with diverse chemical structures such as polyketides, b-lactams and peptides in addition to a variety of other secondary metabolites that have **antifungal, anti-tumor and immunosuppressive activities.**

2.2.3. BIOREMEDIATION

Actinomycetes are abundant in soil,and are responsible for much of the digestion of resistant carbohydrates such as chitin and cellulose.They are liable for the pleasant odor of freshly turned soil.

Several actinomycetes and other actinobacteria are renowned as degraders of toxic materials and are used in **bioremediation**. They are significantly well adapted to survival in harsh environments. Some are able to grow at elevated temperatures (>50°C) and are essential to the composting method.Actinomycetes possess many properties that make them good candidates for **application in bioremediation** of soils contaminated with organic pollutants.They play an important role in the recycling of organic carbon and are able to degrade **complex polymers**.Some reports indicated that Streptomyces flora could play a very important role in degradation of hydrocarbons.Actinomycetes species have the capability to live in an **oily environment**. So we can apply these microorganisms in Bioremediation to deduct oil pollutants.

2.2.4. AGRICULTURE

Advances in our understanding of the Systems responsible for plant growth improvement is a first logical step in opening the way to improving these bacterial strains through genetic engineering, and creating more interest in their progress for widespread commercial **use for both biocontrol and plant growth promotion**. The isolates used *S. pulcher, S. canescens, and S. citreus fluorescens* as a seed-coating, all three of the strains were effective at variable levels in **controlling the test pathogens**. In addition, tomato growth was observed to be significantly improved with the antagonistic *Streptomyces* spp. as a seed-coating.The culture filtrates alone of two different *Streptomyces* spp.(S. olivaceoviridis (Preobrazhenskaya and Ryabova) Pridhamet al. and S. rochei Berger et al.) was found to significantly **increase the shoot**.This demonstrated that selected *Streptomyces* spp. produces at least one class of compounds that directly influence **plant growth**.As the environmental contamination by toxic chemicals increases, different approaches for **controlling pest populations** became analysis priorities. These have enclosed biological or ecological management strategies for limiting the harmful impacts of pest populations, particularly in agriculture.**Chitinase** is originally an enzyme used by insects to degrade the structural polysaccharide “chitin” during the moulting process (Zhang et al. 2002).

The largest chitinase activity among bacteria has been determined in species of *Streptomyces*, Serratia, Vibrio and Bacillus (Reguera and Leschine 2001). Chitinase enzyme is extremely necessary within the **biological control of insects** (Reguera and Leschine 2001) and plant pathogenic fungi Species of *Streptomyces* showed high multiplicity of chitinase genes (Williamson et al. 2000, Saito et al. 2003), as in the case of *Streptomyces coelicolor* and *Streptomyces griseus* (Itoh et al. 2003).In addition, several agroactive effectors have been commercially produced from *Streptomyces* sp., with a large spectrum of action against plant diseases, and these treatments are reported to reduce plant disease severity by 95%.Some antimicrobial metabolites were released by Actinomycetes, which were active against plant viruses. For example, Actinomycete-derived ε-poly-lysine was active against tobacco mosaic virus by acting as a curative and protective agent.Work on novel formulation and delivery strategies will provide enhanced biocontrol and plant growth-promoting products for sustainable agriculture.

3.AIM AND OBJECTIVE

3.1. AIM

The aim is to isolate and study the characterization of *Streptomyces* species of *Actinomycetes*.

3.2. OBJECTIVES

- ➢ To isolate the *Streptomyces* species of *Actinomycetes*.
- ➢ To examine the cultural characteristics of the isolated *Streptomyces* species.
- ➢ To evaluate the biochemical and physiological characterization of the isolated *Streptomyces* species.
- ➢ To characterize the *Streptomyces* species by 16S rRNA sequencing.

4.METHODOLOGY

4.1 MATERIALS AND METHODS

4.1.1. Isolation of actinomycetes

1g of pre-treated sponge sample was serially diluted in sterile seawater and spread plated by using L-rod, over the medium containing soluble starch 20 g, KNO3 1 g, NaCl 0.5 g, K2HPO4 o.5 g, MgSO4 0.5 g, FeSO 20 µM and agar 20 g in 1L of seawater with 100 µl of 0.22µm filtered sponge extract. Plates were incubated at 28 °C for 7-14 days. Dry colonies of actinomycetes were selected and isolated. All the colonies growing on the Petri plates were purified by repeated streaking on an actinomycetes isolation medium (Iniyan et al., 2017). Actinomycetes isolation medium (AIM) slants were used for short time preservation of purified actinomycetes. For long-term preservation, the purified actinomycetes were inoculated in AIM broth and were incubated at 28 °C for 7-14 days. From this broth 20% glycerol stocks were prepared and stored at -20°C.

4.2. Biochemical test

4.2.1. Gram's staining

A loop full of smear culture was taken and straked in a clean glass slide and heated slightly over the flame. The smear was covered with a thin film of crystal violet for 1 minute and washed gently in de-ionized water. Gram's iodine solution was flooded over the smear for 1 minute and washed with tap water gently. Acetone was used to decolorize the smear until the violet colour ceased to flow away. The slide was washed with water and the counter with safranin was flooded over the smear for 2 minutes. Then the slide was washed, drained, air dried, and viewed under oil immersion at 100 x in the microscope. The culture retaining the violet colour indicated Gram-positive organism while the pink colour indicated gram-negative organisms.

4.2.2. Indole Production test

Tryptone broth tubes were prepared and sterilized at 121°C for 15 minutes. The culture was inoculated and incubated at 37°C for 24 hours. Following incubation, a few drops of Kovac's reagent were added. After a few seconds, tubes were observed. Development of the cherry red color ring indicated a positive result. The absence of color change was considered a negative result.

4.2.3. Methyl Red production test

MR-VP broth tubes were prepared and sterilized at 121 °C for 15 minutes. The culture was inoculated and incubated at 37 °C for 24 hours. After overnight of incubation, 5 or 6 drops of MR indicator solution were added. Tubes developed stable red colour which indicated sufficient acid production and was recorded as a positive result. A weakly positive result was red-orange and yellow-orange colour indicated negative result.

4.2.4. Voges-Proskauer test

MR-VP broth tubes were prepared and sterilized at 121 °C for 15 minutes. Culture was inoculated and tubes were incubated at 37 °C for 24 hours. After overnight incubation 3 ml of 5% Barrit's reagent 'A' and 1 ml of 40% Barrit's reagent B were added into the broths. Development of red colour indicated positive result, absence of red colour indicated negative result.

4.2.5. Citrate utilization test

Simmon's citrate agar was prepared, dispensed in test tubes and sterilized at 121°C for 15 min,and allowed to set as slope.The culture was streaked into the Simmon's citrate agar slant and incubated at 37 °C for 48-72 hr. After the incubation period, the development of blue colour from the original green colour of the medium indicated the ability of the organisms to utilize citrate as a carbon source and it's considered a result. The absence of blue colour indicated a negative reaction.

4.2.6. Catalase test

A loop full of culture was taken from the slant and it was dipped in 1ml of H2O2 solution. Evolution of bubbles due to the presence of catalase enzyme indicates positive result.

4.2.7. Urease test

5ml of distilled water was taken in tubes and sterilized after sterilization, urease broth was added into the sterilized distilled water. Culture was inoculated into the urease broth and incubated at 37 °C for 24 hours. Production of enzyme urease was detectable by change in the colour of the medium from yellow to purple.

4.2.8. Hydrogen sulphide (H2S) production test

Triple sugar iron slants were prepared and the culture was inoculated. Test tubes were incubated at 37 °C for overnight. After incubation, the growth of organisms on the TSI slant indicated the type of sugar fermented (dextrose, sucrose, and lactose) and also identifies the production of hydrogen sulfide (H2S). With acid production, the color of the phenol red indicator turned yellow. An alkaline reaction of the medium was indicated by the purple color. Production of hydrogen sulfide was indicated by the formation of black color, as hydrogen sulfide combines with ferrous ammonium sulphate. Splitting of the agar in the butt indicated gas production. Absence of black colour indicated negative result.

4.2.9. Oxidase test

Nutrient agar was prepared and the media was sterilized at 121 °C for 15 minutes. After sterilization, media was proud on sterilized plates. Media was allowed to solidify. Single line streak of test organisms was made in the center of plates. Plates were incubated at 37 °C for overnight. After incubation, cytochrome oxidase disc was overlaid on 5 days old culture and then plates were observed. Positive result was indicated by the purple or violet colour of the cytochrome oxidase disc within 5 sec. In a negative result no colour change of cytochrome oxidase disc was observed.

4.2.10. Casein hydrolysis test

Casein is a complex protein which is responsible for the fair colour of milk.The milk agar medium was autoclaved and poured in petri-plates.Inoculation of the sample as a single streak was done and incubated at 37°C for 48hrs. The results were noted.

4.2.11. Lipid hydrolysis Test

This test determines the capacity of the particular organism to produce the lipase enzyme in the medium. The Tributryin agar medium was pre sterilized and poured in petri plates at the required rates. The samples were streaked on the medium and incubated for four days at room temperature.Results are observed.

4.2.12. Starch hydrolysis Test

A clear zone around the organism will be seen while the other area were covered with iodine solution.This is because, the organism was surrounded by the amylase enzyme produced by them. The amylase was produced in order to degrade the starch present in the medium.

4.2.13. Gelatin hydrolysis Test

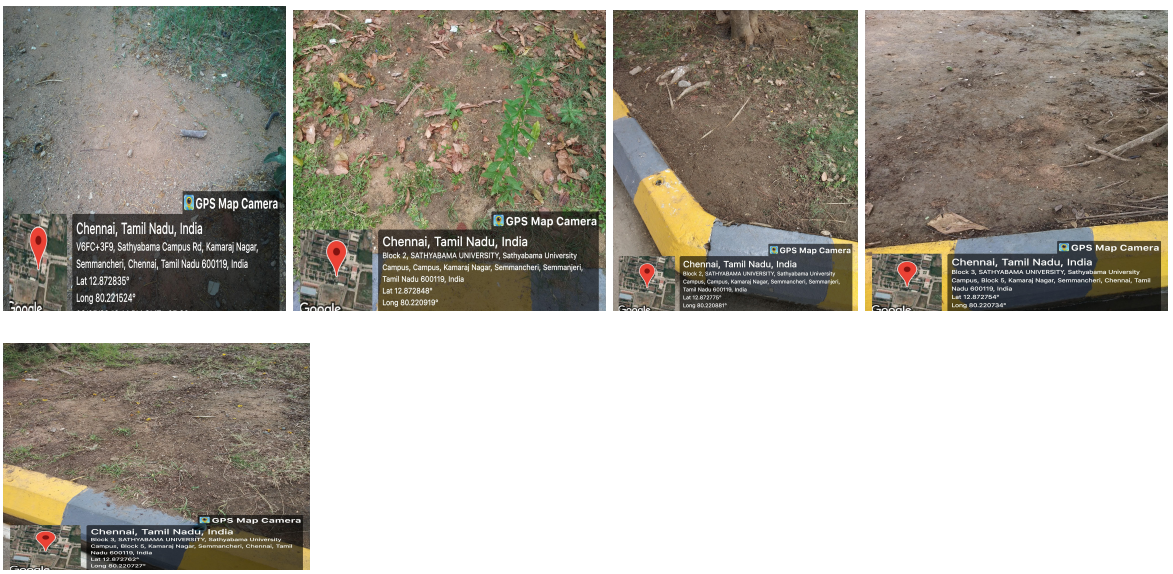
Gelatin is composed of short amino acid polymers and their derivatives. They are considered as nitrogen and carbon source for a wide variety of microbes. Nutrient gelatin agar medium were sterilized and inoculated by the sample organism and incubated at 35° C for ten days.After incubation the tubes were kept in ice to check liquefaction of the medium.

4.3. Morphological Characterization

Macroscopically, the actinomycetes isolates were differentiated by their colony characters, e.g., size, shape, colour, etc. Cultural characteristics of isolated strains were examined by the visible observation of 14-day old culture grown on AIM medium. Micromorphology, spore chain morphology and sporulation were observed under light microscope by cover slip culture method (Shinobu and Kawato, 1959). After incubating at 28°C for 7 days, 14 days and 21 days intervals. Colours of aerial and substrate mycelia were determined and recorded.

4.4. Serial dilution procedure

The five soil samples were collected from five different locations of **Sathyabama University.**The images of location of the soil sample collected were attached below.The samples were collected in the sterile small plastic tubes and properly labelled indicating the date of collection and the depth.The collected soil samples were dried in a hot air oven at 60-65°C for about three hours.This was done to reduce the number of bacteria in the soil other then *streptomyces*.Six sterile test tubes with (9 ml) of sterile distilled water were labelled as 1,2,3,4,5 and 6..One gram of dried soil sample was added into the test tube 1 and mixed well. One ml of soil solution from tube 1 was transferred to tube 2 using a 1 ml micropipette and mixed well.This serial dilution was done up to tube 6. 1ml solution was discarded from tube 6. Thus the concentrations of tube 1 to 5 were obtained.0.1 ml of the solution was transferred onto the respective labelled Petri dishes from the diluted sample. Immediately after the transfer each soil solution was spread on the agar medium using a sterile glass spreader. Then, the plates were incubated at 30°C for 5 days.After the incubation period the plates were examined for typical *streptomyces* colonies.The colonies on the dilution plates were then counted and recorded.



4.5. 16S rDNA sequencing and phylogenetic analysis

16S rDNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between bacteria, and to identify bacteria from various sources, such as environmental or clinical specimens. This technology is used today in clinical laboratories for routine identifications, especially for slow-growing, unusual or fastidious bacteria, but also for bacteria that are poorly differentiated by conventional methods; however, it provides no information about antibiotic resistance.To assign the strain to the species level, a widely used molecular biological method,sequencing of 16S rDNA was performed to build the phylogenetic tree, was used in this study.

5.RESULTS AND DISCUSSION

5.1. Isolation of actinomycetes from the soil samples of Sathyabama University

The homogenized sponge tissue serial dilutions resulted in the appearance of several morphotypes observed in the culture media supplemented with sponge extract. From three days of incubation at 28 °C, actinomycetes colonies were appeared and the plates were kept up to 14 days at 28 °C for the isolation of slow growing actinomycetes. The actinomycetes strains were isolated based on their morphological appearances.

5.1.1. Growth at different media composition

The antagonistic strain Streptomyces was observed for its suitable growth in different media composition such as AIM medium (soluble starch 20 g, KNO3 1 g, Nacl 0.5 g, K2HPO4 0.5 g, MgSO4 0.5 g, FeSO4 20 µM and agar 20 g in 1L of seawater), ISP2 (International Streptomyces Project-2 Medium) (Yeast extract 4.0 g, Malt extract 10.0 g, Dextrose 4.0 g, Agar 20.0 g, and Distilled water 1l), ISP5 medium (L- asparagine anhydrous basis 1.0 g, Glycerol 10.0 g, K2HPO4 (anhydrous basis) 1.0 g, Trace salts solution 1ml/lit, Agar 20.0 g, and Distilled water 1l), Raffinose Histidine agar (Raffinose 1.0 g, histidine 1.0 g, MgSO4 0.5, FeSO4 0.01 g, and NaCl 20 g), Starch casein agar (Soluble starch 10 g, Casein (Vitamin Free) 0.30 g, KNO3 2 g, MgSO4.7H2O 0.05 g, K2HPO4 2 g, NaCl 2 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, Agar 18 g). The strain Streptomyces was streaked in different mediums and observed the growth pattern the suitable well growth medium was ISP2, Starch casein agar, and Aim medium was observed in the optimum temperature as 28 °C.

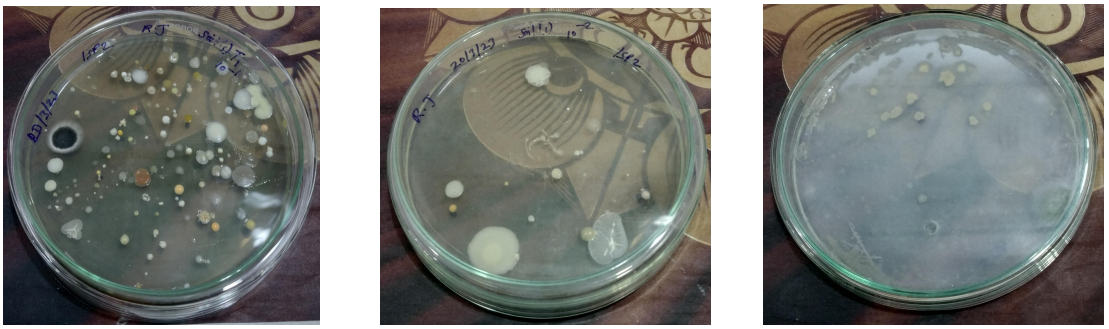


Fig.1. colonies obtained from soil sample(1)

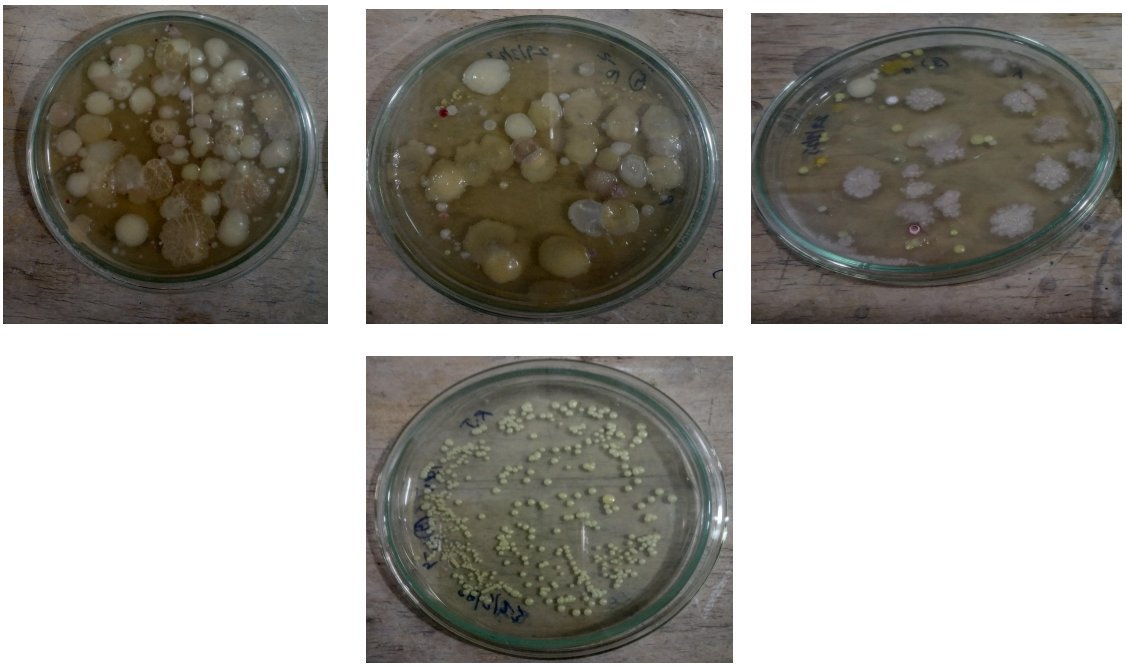
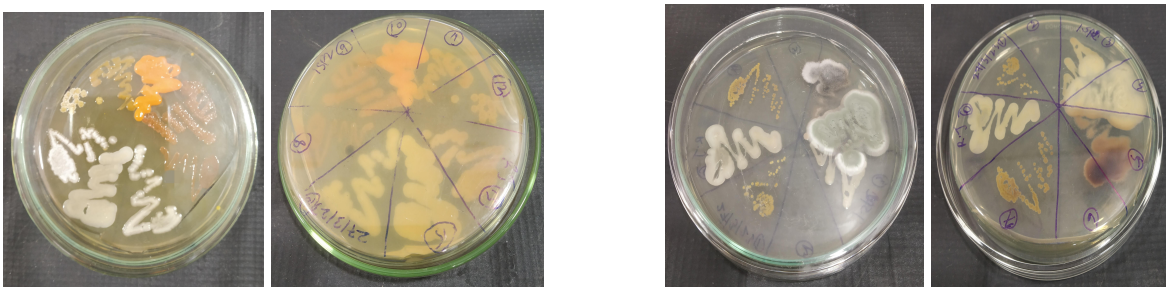
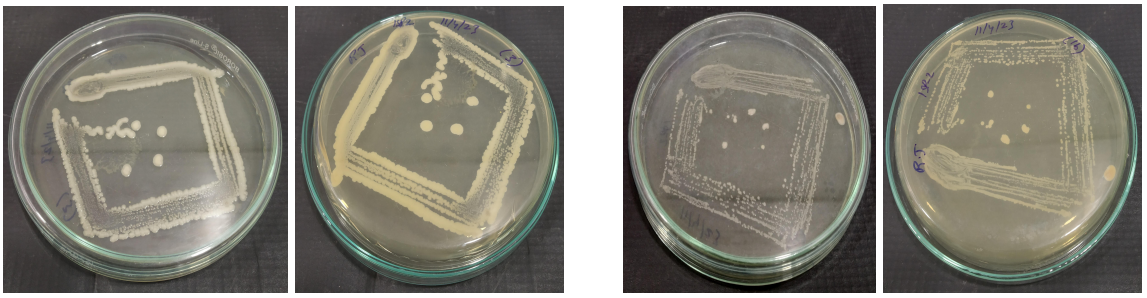


Fig.2. colonies obtained from soil samples (2),(3),(4),(5)



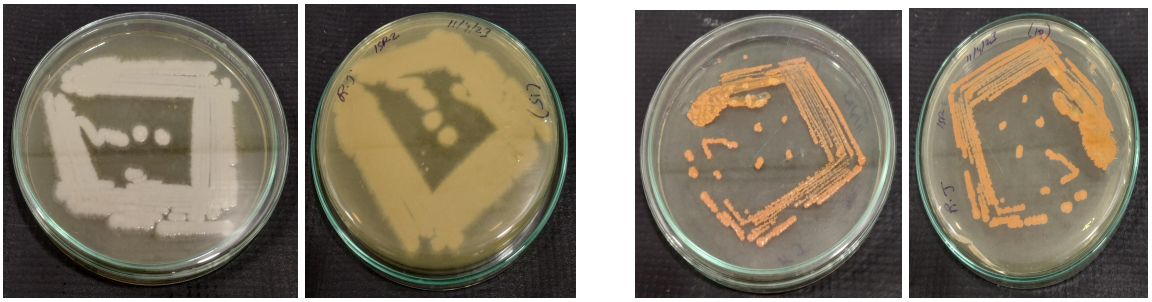
Front view. **Reverse view.** **Front view.** **Reverse view**

Fig.3. single streak of each colonies obtained from soil samples



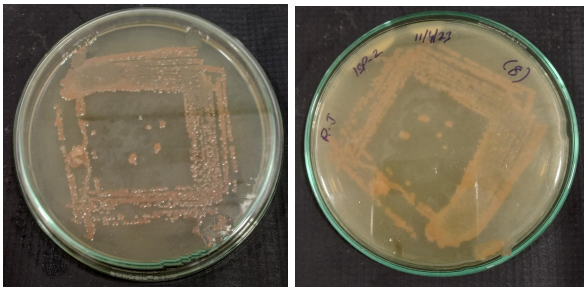
Front view. **Reverse view.** **Front view.** **Reverse view**

Fig.4. pale and light white strain isolated from soil sample



Front view **Reverse view.** **Front view.** **Reverse view**

Fig.5. white and orange strain isolated from soil sample



Front view. **Reverse view**

Fig.6. pale orange strain isolated from soil sample

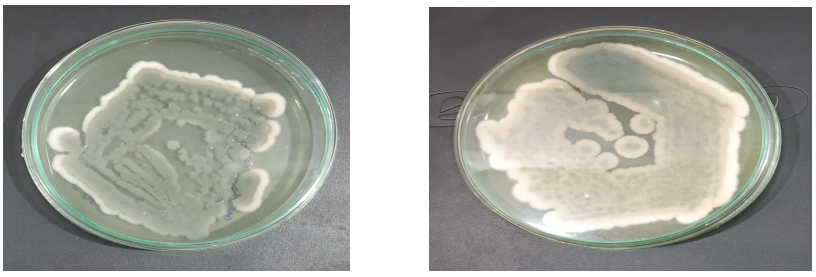


Fig.7. Isolated from obtained colonies *Streptomyces* strain1 and strain 2

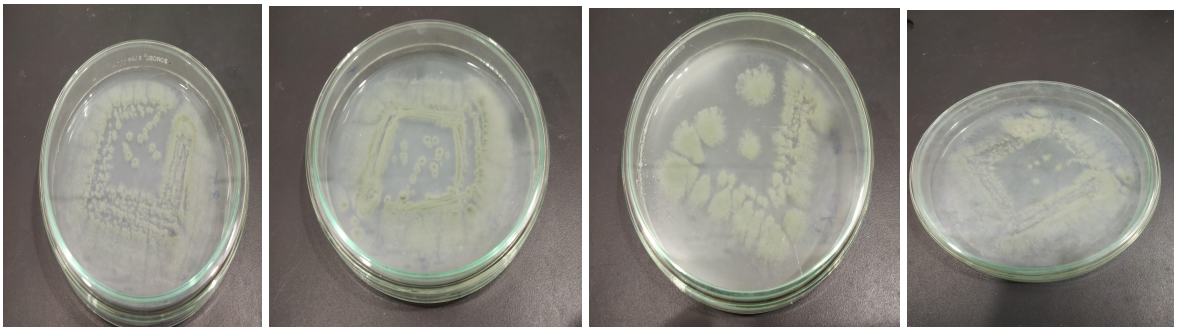


Fig.8. Type-1 strains obtained from subculture of dry colonies

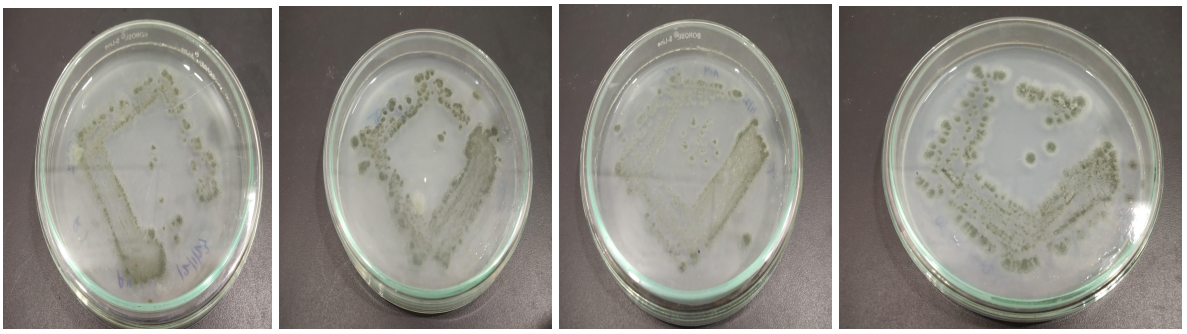


Fig.9. Type-2 strains obtained from subculture of dry colonies

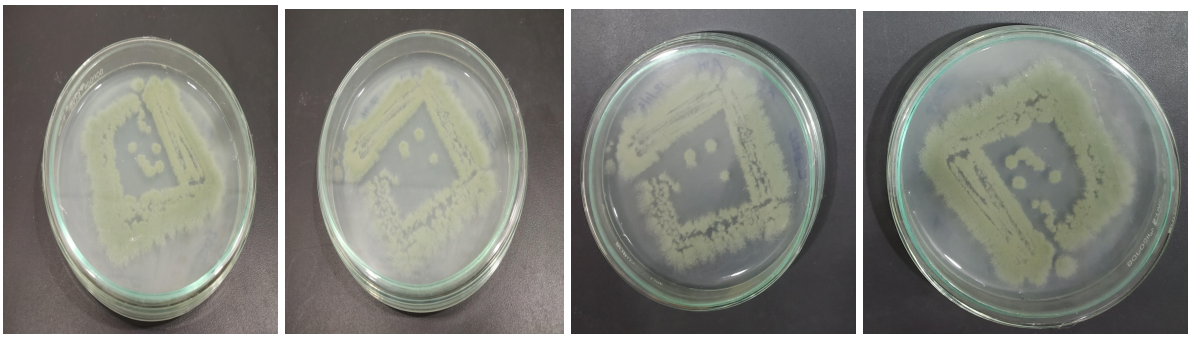


Fig.10 Type-3 strains obtained from subculture of dry colonies

5.2 Morphological characterization of active strain streptomyces

Morphological characteristics	Streptomyces
Colour of the colony	Grey
Pigmentation in the medium	Brown
Aerial mycelium	Present, Grey
Substrate mycelium	Present, highly branched
Melanin pigment	Nil
Metabolic Exudation	Nil
Spore chain	Open spiral
Spore surface	Spiny
Number of spores	30-50

Table 1. Morphological characterization of active strain *streptomyces*

5.2.1 Growth characterization of antibacterial active strain *Streptomyces*

Characteristics	Growth	Optimum growth
Different media		
Actinomycetes isolation medium	++	ISP2 & Starch casein agar
ISP2	+++	
Starch casein agar	+++	
Temperature		
28 °C	+++	28 °C
37 °C	++	

Number of days		
3	+	7
7	+++	7
14	++	7

Table 2. Growth characterization of antibacterial active strain *Streptomyces*

5.3 Biochemical test

The biochemical characteristics obtained for the pathogenic strains were depicted in table 1. The resistant pathogenic active strain of the present study Klebsiella pneumoniae, showed positive results in Voges Proskauer, citrate utilization, and E.coli shows Indole and Methyl red positive.

Table 3. Biochemical analysis of beta strain of *Streptomyces sp* SBRK127

S.No	**Test**	**Observation**	
		Strain 1	**Strain 2**
1	Gram's Staining	+	+
2	Indole	-	-
3	Methyl red	-	-
4	Voges Proskauer	-	+
5	Urease	-	-
6	H2S production	++	+
7	oxidase	+	-
8	Catalase	-	+

9	Citrate Utilization	+	+
10	Casein Hydrolysis	+	+
11	Lipid Hydrolysis	-	-
12	Starch Hydrolysis	+	+
13	Gelatin Hydrolysis	-	-

Note: - =Negative; + = positive.

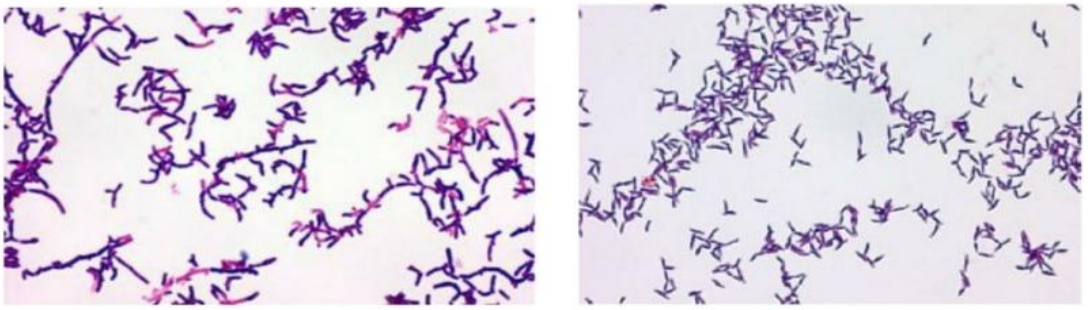
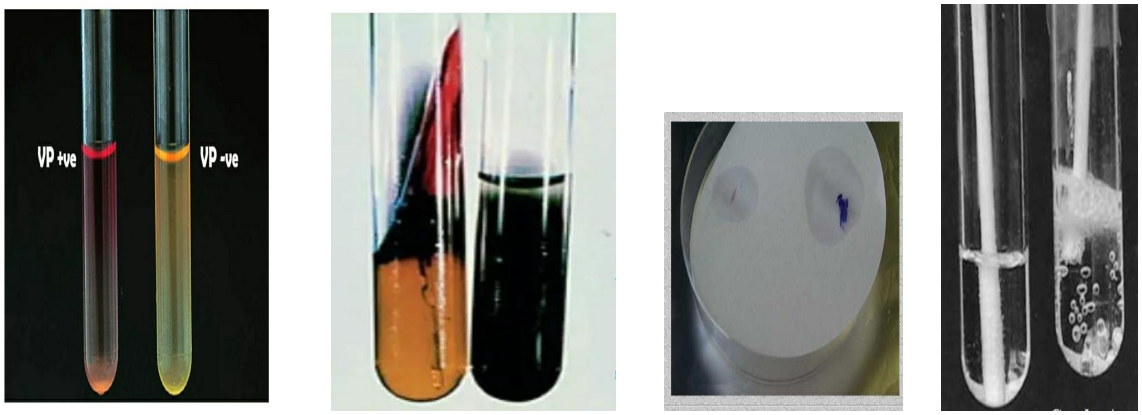


Fig.11. Gram straining of isolated Actinomycetes strains



(A) (B). (C). (D)

Fig.12. Biochemical tests for selected strain-1 **(A)**Voges Proskauer test **(B)** H2S production test**(C)**Oxidase test(**D)**Catalase test

(E). **(F).** **(G)**

Fig.13. Biochemical tests for selected strain-1 (**E)** Citrate utilization test (**F)**Casein hydrolysis test (**G)**Starch hydrolysis Test.

5.4 16S rDNA sequencing and phylogenetic analysis

To assign the strain to the species level, a widely used molecular biological method,sequencing of 16S rDNA was performed to build the phylogenetic tree,and the results were observed.The isolated species is **SBRK127** and the complete sequencing of the data and phylogenetic analysis is given.

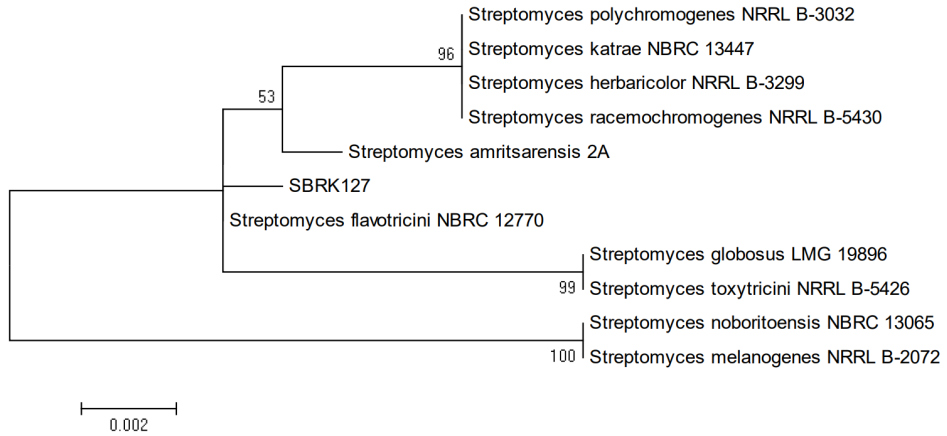


Fig.13. Phylogenetic tree analysis between the isolated strain and representative genus of the species on complete sequencing.

ACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCCTTCG
GGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACT
CTCGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACTGCGGAAGGCA
TCTTCCGTGGTGGAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGC
TTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAG
GGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTG
AGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAG
TGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGC
GGCTTGTCACGTCGGATGTGAAAGCCCGAGGCTTAACCTCGGGTCTGCATTCAT
ACGGGCTAGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGA
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CGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCGTG
TTGCTAGCATGCCCTTCGGGGTGATGGGGATCACAGGAGACCGCCGGGGTCAA
CTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGC
TGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTGGAG
CGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCAT
GAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCC
CGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGC
CGGTGGCCCAACCCTTGTGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGG
GACGAAGTCGTAACAAGGTAGCCGTACCGGAAGG

Fig.14. 16S rDNA sequence of the strain SBRK127

5.5 DISCUSSION

Actinomycetes are the most biotechnologically valuable prokaryotes responsible for the production of about half of the discovered bioactive secondary metabolites including antibiotics (Yuan et al., 2010). They are the main source of clinically important antibiotics. Among actinomycetes the genus Streptomyces is reported that a high proportion of organisms possessing antimicrobial activity by producing novel antibiotics (Demain and Sanchez, 2009). Marine habitat has been proven as an outstanding and fascinating resource for innovating new and potent bio actives producing microorganisms.

6.SUMMARY AND CONCLUSION

The present study was mainly focused on isolation and molecular characterization of actinomycetes from soil.. The morphological, biochemical, physiological, molecular characterization have been done.The current attempt for isolation and characterization of actinomycetes from soil will be favorable for the antimicrobial activity and identification of novel antibiotics in further studies.

In conclusion, Streptomyces species of Actinomycetes are an important source of bioactive compounds, including antibiotics, and have a wide range of potential applications in medicine, agriculture, biotechnology, and environmental science. The isolation and molecular characterization of Streptomyces species is crucial for the discovery of new bioactive compounds, understanding the biosynthesis of these compounds, bioremediation, and biotechnological applications.Therefore, continued research in the isolation and molecular characterization of Streptomyces species is necessary to understand and study the characterizations of the species for the development of new drugs, production processes, and environmental solutions.

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