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Isolation, Molecular Identification, Characterization of Actinomycetes and Study on its Antimicrobial and Antioxidant Activity

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Abstract

Actinomycetes, Gram-positive, filamentous bacteria, thrive in diverse environments and are prolific antibiotic producers. Streptomyces species, a key genus, produce around 80% of recognized secondary metabolites, including glycopeptides, beta-lactams, and aminoglycosides. This study aimed to produce and characterize bioactive compounds from Actinomycetes and screen their antioxidant properties. Biochemical tests confirmed the isolate's properties, showing positive results for starch, casein, gelatin, and cellulase hydrolysis, urea hydrolysis, nitrate reduction, catalase, H_2S production, indole, MR, Voges-Proskauer, and citrate tests, indicating diverse enzymatic and metabolic capabilities. The bioactivity of isolates was confirmed against *P. aureus, E. coli, Salmonella*, and *Proteus*, with significant inhibition zones. The crude compound was purified via thin layer chromatography (TLC) with a retention factor of 0.83 and exhibited antimicrobial activity. The isolate was identified as *Streptomyces longisporoflavus* through 16s rRNA analysis.

***Key words*:** *Actinomycetes*, Secondary metabolites, Antimicrobial activity, Antioxidants, 16S rRNA

Actinomycetes are a diverse group of Gram's positive, free living, saprophytic, filamentous bacteria and are a major source for the production of antibiotics [1]. They belong to the order Actinomycetales (Super kingdom: Bacteria, Phylum: Firmicutes, Class: Actinobacteria, Subclass: Actinobacteridae). They are found in soil, fresh water and marine water environments [2]. They have high G+C (>55%) content in their DNA. They are the best common source of antibiotics and provide approximately two-third of naturally occurring antibiotics, including many of medical importance [3]. *Actinomycetes* are the most economically and biotechnologically valuable prokaryotes and are responsible for the production of about half of the discovered secondary metabolites. In the recent times, they have been exploited successfully for their biologically potential secondary metabolites. They produce diverse group of antimicrobial metabolites notably glycopeptides, beta lactams, aminoglycosides, polyenes, polyketides, macrolides, actinomycin's and tetra cyclins.

Streptomyces spp. have the ability to produce many different biologically active secondary metabolites such as antibacterial [4-5]. Marine *Streptomyces* have a unique secondary metabolites variety by producing new natural antimicrobials. About 2/3 of the famous antibiotics was produced by the genus *Streptomyces* and about 75% of useful antibiotics are produced by the *Streptomyces spices*. There are about 23,000 recognized secondary metabolites and around 80% of which are produced by *Streptomyces species* [6-8] which are primary antibiotic-producing organisms exploited by the pharmaceutical industry [9-10].

Antimicrobial resistance (AMR) is the emergent universal threaten which accounts for nearly 700,000 annual deaths in the world [11]. In addition, the wide spreading of infectious diseases from various sources which increases the clinical burden. The multi-drug resistance demands the world to explore novel antimicrobial drugs which are effective against drug resistant pathogenic microorganisms. Accordingly, searching and generating unique antimicrobial drugs, as well as combined antibiotic treatment were shown to delay the emergency of microbial resistance and can also produce desirable synergistic effects in the treatment of diseases caused by microorganisms.

In developing countries, discovery and production of new and effective antimicrobials will make a voluminous impact on the clinical insight and can save millions of lives across the world. It is better known that soil microorganisms serve as a better source for the separation and identification of therapeutically active products. Among those, the predominant is *Actinomycetes*, a group of saprophytic bacteria known to generate a broad range of secondary metabolites, bioactive compounds and antibiotics that control microbial growth. It is estimated that 70-80 % of secondary metabolites and one-third

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of antibiotics available commercially till date have been obtained from *Actinomycetes* [12].

Mostly, *Streptomyces* genus was reports for approximately 80% of the natural products documented to date [13]. But the risk factor in discovering and producing new bioactive compounds is that it might be a commercially existing compound from known species. Thus, a group of actinobacteria from unexamined habitats could be sources of novel biologically active compounds [14]. Many pathways are related with secondary metabolites produced by the genus *Streptomyces*, these include antimicrobial, antitumor and enzyme inhibitors, compared to terrestrial species, marine *Streptomyces* are essential sources of unique antibiotics. Therefore, the marine *Streptomyces* are investigated to extract secondary metabolites and there are many secondary metabolites have been screened recently [15].

It is commonly acknowledged that new antibiotics are urgently required, and that the most promising sources are natural habitats. The marine environment is largely unexploited source for new antibiotics, in a view of the enormous diversity of microorganisms-producing secondary metabolites [16]. The production of secondary metabolites from the genus *Streptomyces* can be influenced by optimization of the nutritional requirements and cultural conditions. These conditions play an important role in the production of these secondary metabolites [17]. Because of their useful biological activities, microbial secondary metabolites have received considerable attention especially in the beneficial effects of human health. Biosynthesis of these secondary metabolites through metabolic engineering and industrial biotechnology offers significant advantage over conventional methods for extraction from biomass.

Together with antibacterial activity, gene-based approach can be applied for efficient screening of isolated strains of pharmaceutical value and related compounds [18]. Mostly, *Streptomyces* genus was reports for approximately 80% of the natural products documented to date [12]. But the risk factor in discovering and producing new bioactive compounds is that it might be a commercially existing compound from known species. Thus, a group of actinobacteria from unexamined habitats could be sources of novel biologically active compounds [13]. The increase of bacterial resistance to antibiotics is due to the concurrent usage of existing antibiotics, the search for new potent antibiotic to control these resistant pathogens, new antibiotics are in need and should be developed [14]. Therefore, screening, isolation, and characterization of promising strains of *Actinomycetes* producing potential antibiotics and other therapeutics have been a major part of research. Recent studies are focusing on the response of antioxidant system of bacteria, which is important in terms of biotechnology, such as *Streptomyces* growth in various oxidative stress conditions [19]. Searching for unique actinomycete that metabolized an essential component in natural product-based drug is becoming more and more interesting and meaningful.

Antioxidants play an important role in inhibiting and scavenging free radicals, thus providing protection to humans against various infections and degenerative diseases. Either increased free radicals or decreased antioxidant can lead to oxidative stress, which signifies the identification of natural antioxidative agents. There are certain naturally occurring antioxidants that can give protection against oxidative stress induced damage in human cells. Modern research is now directed towards natural antioxidants from plants and microorganisms which serves as safe therapeutics. Therefore, the main objective of this work is the production and characterization of bioactive compound from *Actinomycetes* and to screen their antioxidant properties.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were aseptically collected from agricultural land of Bhavani region, Tamil Nadu, India. The soil samples were obtained from 10 to 15 cm below the soil surface with a clean, dry bag by using sterile spatula and transferred to lab. The air-dried soil samples were pulverized using a sterilized mortar and pestle. Then the soil samples sieved to take off the unwanted debris and finely sieved soil samples were kept in a dry sterile container for *Actinomycetes* isolation.

Pretreatment of sample

The soil samples were given a various physical and chemical pretreatment methods in order to facilitate the isolation of *Actinomycetes*. In physical treatment, 1 gram of soil sample was mixed with sterile (9.0 ml) distilled water, and exposed at high temperature at 70℃ for 15 minutes to kill the contaminants. In 250 ml conical flask, 5 ml of the pre-treated soil sample was added to 50 ml of the *Actinomycetes* isolation broth medium. The medium was supplemented with Streptomycin (50µg/ml) and Cycloheximide (25µg/ml) for prevention of fungal contamination [20-21]. The flask was incubated at 30℃ for 7 days.

Isolation and maintenance of actinomycetes

Enumeration and isolation of *Actinomycetes* from soil samples were performed with serial dilution methods [22]. Concisely, 1 gram of soil sample was serially diluted with 9 ml of sterile saline water up to 10^7dilutions. Then 0.1 ml aliquot of diluted sample was used for plating with starch casein nitrate agar (SCN) and the petri plates were incubated at 30°C for 5 days. Finally, purified isolates were sub-cultured with SCN agar. All the isolates were stored in SCN agar slants at 4°C.

Identification and characterization of perspective isolate
Colony and spore morphology

The colony morphology, colour and pigment production of the *Actinomycetes* isolates were considered by growing them on ISP medium. The ISP plates were inoculated with isolates and incubated at 30℃ for 3 days and the growth of isolates were observed at regular intervals. The nature of the specific colony, colour and pigmentation were recorded. Spore morphology was observed by cover slip method described by Williams and Davies [23]. Briefly, sterilized coverslip was placed at an angle of 45° in a petri plate containing ISP4 agar medium. A loop of inoculum was streaked along the cover slip line and then the plates were incubated at 30℃ for 3 days. Later, the coverslip was carefully withdrawn from the medium and examined their morphological features of aerial mycelium, substrate mycelium, spores and sporangia were examined with light microscopy.

Micro-morphology

To study the aerial mycelium and its sporulation characteristics, two methods such as direct and inclined coverslip methods were used. Gram's staining was performed.

Biochemical characteristics

Biochemical characteristics of the selected strain were performed to identify and confirm the genus and species level of the organism according to the procedure of Cappuccino and Sherman [24]. The different biochemical tests viz., Indole test,

MR-VP test, citrate test, starch hydrolysis test, casein hydrolysis test, gelatin liquefaction test, H_2S production, urease test, nitrate reduction test, cellulase test and catalase test were performed [25].

Indole production test

The test was performed by inoculating the cultures into tubes containing tryptone broth incubated at 30 ± 0.10°C for 96 h. After inoculation, Kovac's reagent was added (1:1 by volume) and mixed to check for indole production which was indicated by a pink ring at the interface of the two solutions.

Methyl red test

Production of acid lowers the pH of the medium below 4.2 which is detected by the pH indicator methyl red. *Actinomycetes* were inoculated into tubes containing Methyl Red-Voges Proskauer (MRVP) broth and incubated at 30 ± 0.10°C for 96 h. After incubation alcoholic methyl red indicator was added. Positive reaction was indicated by change of colour of medium to red.

Voges-Proskauer test

The isolates were tested on MR-VP broth medium to detect their ability to produce neutral products like acetoin (acetyl methyl carbinol) during metabolism of glucose present in the medium. After incubation, 0.6 ml of 5% naphthol was added followed by 0.2 ml of 40% KOH to 1 ml of broth culture. The solution was allowed to stand for 30 minutes. A change in colour of the medium to wine red was as positive reaction while copper colour indicates a negative result.

Citrate utilization test

The capability of the isolates to utilize citrate as the sole source of carbon and energy was studied on Simmons citrate agar medium. Colour change of the slant from green to royal blue was considered as positive result while no change in colour was taken as negative.

Extracellular enzymatic activity

The activities of various extracellular enzymes produced by the isolates were studied by the following tests [26].

Starch hydrolysis test

Capacity of the organisms to hydrolyze starch into simple substances like dextrin, glucose, maltose etc. by amylase enzymes was detected by spot inoculating the cultures on NA plates containing 1% soluble starch. After incubation for 96 hrs at 30 ± 0.10°C, all the plates were then exposed to iodine vapour for 5 to 10 minutes. Starch hydrolysis was noted from a clear zone formed around the colonies. Reddish-brown area around the colonies indicated partial hydrolysis of starch.

Gelatin hydrolysis test

Gelatin liquefaction was tested by spot inoculating the Actinobacteria on SCN plates containing 1% gelatin followed by incubation at 30 ± 0.10 °C for 4-5 days. The plates were flooded with acidic mercuric chloride solution (15%), waited for 5-10 min, the excess solution was decanted off and appearance of a clear zone around the colonies was indicative of hydrolysis of gelatin by the enzyme gelatinase. The clear zones depicted the activity levels of the organisms. Otherwise, unhydrolyzed and continuous opaque zone around the actinobacterial growth i.e., white opaque precipitate was taken as no gelatinase enzyme production.

Casein hydrolysis test

Casein hydrolyzing activity of the bacteria was recorded from liquefaction of casein by the bacteria on SCN plates containing 1% casein, spot inoculated and incubated at 30±0.10°C for 96 h. The plates were flooded with acidic $HgCl_2$ (15%) or 1% tannic acid, excess solution was decanted off and clear zone formation was observed.

Cellulase test

Carboxy methyl cellulose (CMC) - SCN plates were prepared using SCN/5, 1% (W/V) CMC and 1.5% agar. All the isolates were spot inoculated on the plates and incubated at 37°C for 24 to 48 hours. All the plates were then exposed to 1% Congo red for 5 to 10 minutes, 1N HCl for 5 minutes and finally treated with 1N NaOH for 5 minutes respectively. A clear zone around a colony on a red background indicated positive cellulolytic activity of the isolate caused by the production of cellulase.

Catalase test

1-2 ml of hydrogen peroxide solution was taken in a test tube. Using a sterile glass rod 18-24 hours test organism was taken and immersed with hydrogen peroxide solution. Effervescence of oxygen indicate the positive reaction.

Urease test

To test for the presence of the enzyme urease in the isolates which split urea into ammonia and CO_2 was studied using Christensen's urea agar medium. Colour change of the slant from yellow to pink was considered as positive result while no change in colour was considered as negative.

Production of secondary metabolites from crude extract
Shake flask fermentation

Shake flask fermentation process was used for the production of bioactive compounds from selected potential strain ACT1F. A loopful of strain spore was inoculated into a four 500 ml flask each containing 250 ml of ISP broth medium and finally production medium volume was 1200 ml. All the flasks were incubated for 14 days at 30°C in a rotary shaker with 200 rpm [27].

Extraction of secondary metabolites

The spent media were spinning at 10000 rpm for 30 min to confiscate biomass and other cell debris. The solvent extraction method was employed to separate bioactive composites from the culture filtrates [28]. Concisely, culture filtrate was blended with butyl alcohol in the ratio of 1:1 (v/v) and dynamically shakes up for an hour. The aqueous and solvent phase was separated with separating funnel and solvent phase (butyl alcohol phase) comprises bioactive compounds was separated out from the aqueous phase. Then the phase was collected and concentrated with hot air oven at 40°C [29-30]. The concentrated, dried compounds were scrapped and dissolved in dimethyl sulfoxide.

Purification of secondary metabolites

The crude pigment was purified by thin layer chromatography. Commercially available Silica gel coated chromatography sheets were used. To separate the crude compound, the solvents chloroform: methanol (30:70) were used. The crude pigment was dissolved in 200 µl of ethyl acetate. With the help of capillary tube, the sample was spotted at the bottom of silica gel coated sheet and then it was placed in the developing beaker containing mobile phase, covered with the watch glass in order to prevent the evaporation of the solvents. The solvent was allowed to run till it reaches about

half a centimeter below the top of the plate. After running, the sheet was kept at room temperature for the complete drying of the plate. Then the sheet was kept in closed iodine chamber to visualize the separated compound as clear sports. Rf value of the spot separated on the TLC plate was determined. Rf value = movement of solute from the origin/movement of solvent from the origin [31].

Antimicrobial activity

The crude pigment was screened for biological activity against drug resistant pathogens of *E. coli, Proteus, salmonella, Pseudomonas aureus* by agar well diffusion method. Potato dextrose agar (PDA) plates were swabbed (sterile cotton swabs) with 24 hours old broth culture of bacteria. Wells (10 mm diameter and about 2 cm a part) were made in each of these plates using sterile cork borer. Stock solution of each extract was prepared at a concentration of mg/ml. About 100 µl solvent extracts were added by using sterile syringe into the wells and allowed to diffuse at room temperature. Then the plates were incubated at 37°C for 18-24 hours [32-33].

Antioxidant assay

Free radical scavenging ability of the extract was tested by DPPH radical scavenging assay. The hydrogen atom donating ability was determined by the decolorization of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH produces violet/ purple color in methanol solution and fades to shades of yellow color/ orange color in the presence of antioxidants. A solution of 0.1 mm DPPH in methanol was prepared, and 1 mL of this solution was mixed with 3 ml of extract in methanol at different concentrations (20-100 µg/mL). The reaction mixture was vortexed thoroughly and left in the dark at RT for 30 min. The absorbance of the mixture was measured calorimetrically at 517 nm [34-35]. Percentage DPPH radical scavenging activity was calculated by the following equation:

% DPPH radical scavenging activity = $\{(A_0 - A_1) / A_0\} \times 100\%$

Where A_0 is the absorbance of the control, and A1 is the absorbance of the extractives/standard. Then percentage of inhibition was calculated.

TLC bioautography

Ethyl acetate extract of ACT1F strain was subjected to thin layer chromatography analysis over analytical aluminium silica gel TLC plate for separation of metabolites to obtain Rf value of active fraction. The crude extract was dissolved in methanol was loaded on TLC plate which was developed with solvent system comprising of methanol and chloroform in the ratio 70:30. Separate bands were observed and their Rf values were calculated. To perform bioautography, 1.5 ml of 1% Mueller Hinton agar (MHA) was spread on the developed TLC plate under sterile environment and 50$\mu l$of log phase culture of S. aureus was spread with the help of sterile spreader. After incubation for 24 h at 30°C the plate was visualized by spraying iodonitrotetrazolium chloride solution pink color on plates signified cell growth whereas bands with clear zone indicated inhibition of cell growth [35]. Rf values of the bands with clear zone were recorded.

Molecular identification

DNA isolation and amplification

DNA isolation was done using the Expure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd. The 16s rRNA was amplified using with primer (27F 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492R 5'-TACGGYTACCTTGTTACGACTT-3') in BioRad T100 thermal cycler. The PCR conditions performed 35 cycles of 95°C for 2 min, 42°C for 30 s and 72°C for 4 min, and one additional cycle with 20 min for chain elongation. The resulting 16s rRNA region sequences were evaluated by comparing with those sequences with submitted sequence in NCBI databases using, Basic Local Alignment Search Tool (BLAST) and Ezbiocloud [36].

Purification of PCR products

Purification of PCR Products removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing method

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequence similarities and phylogenetic analysis

The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of query sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. The program MUSCLE 3.7 was used for multiple alignments of sequences [37]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) [38-39]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering [40].

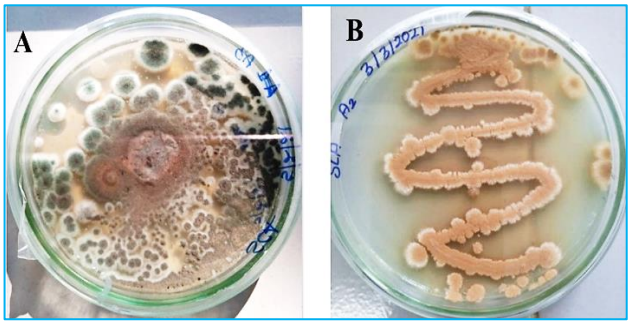

Fig 1 A) Different colonies on media and B) ACT1F isolate

RESULTS AND DISCUSSION

Isolation of actinomycete from soil sample

It was carried out by serial diluting the soil sample and inoculating on SCN and ISP media by spread plate technique. *Actinomycetes* strains around 15 colonies were observed with earthy odour and further sub cultured to identify their morphological, cultural, biochemical and antimicrobial activities. A characteristic rough colony with branched or aerial

mycelia and arrangement of spores on aerial mycelia were recognized as actinomycete species (Fig 1).

Identification and characterizations of perspective isolate
Cultural and colony morphology

The isolate ACT1F was cultured on different media such as SCN and ISP. It was developed as round, powdery, convex colonies with spreading edges. The ISP agar media was selected, because the medium suppressed the growth of bacterial and fungal colony and allowing the growth of *Actinomycetes*. The isolate ACT1F was extensively studied and it was gram positive with long filamentous mycelia and spores arranged as long chains (Fig 2).

Spore morphology

The spore chain morphology of strain ACT1F grown in cover slip was observed under high power and oil immersion objectives. The spore morphology of the isolated strain was observed as rectiflexibles (straight to flexuous) with simple branching and straight spore bearing hyphae.

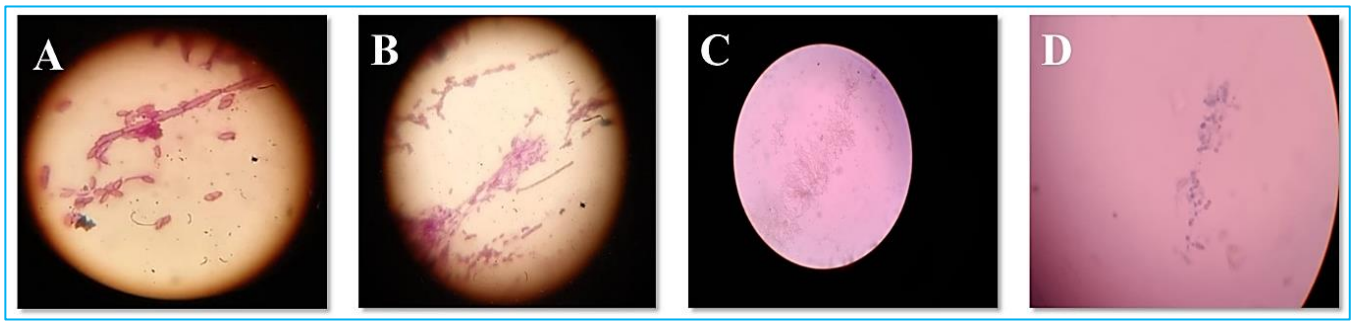

Fig 2 Microscopic images of isolate ACT1F where A and B- 100X and C and D- 40X magnification

Table 1 Cultural characteristics of the isolate *Streptomyces sp*. ACT 1F

S. No.	Characters	Act1f strain
1	Colony appearance	Mycelial (cottony)
2	Sporulation of aerial mycelia	Long chain
3	Colony color	Brown
4	Gram's staining	+ ve
5	Aerial mass color	Brownish
6	Reverse Side pigments	Yellow
7	Melanoid pigments	-ve
8	Starch hydrolysis	+ ve
9	Casein hydrolysis	-ve
10	Gelatin hydrolysis	+ ve
11	Urea hydrolysis	+ ve
12	Nitrate reduction test	+ ve
13	H_2S production test	+ ve
14	Amylase production test	+ ve
15	Protease production test	-ve
16	Cellulase production test	+ ve
17	Catalase production test	+ ve
18	Indole production test	+ ve
19	Methyl red test	+ ve
20	Voges Proskeuer test	+ ve
21	Citrate utilization test	+ ve
22	pH	7 to 8
23	Temperature	28℃ to 37℃

Biochemical test

Biochemical tests were performed to observe the activities of the isolated strain. The strain ACT1F showed diversified results and displayed in (Table 1). Different biochemical test of the selected isolate was performed. These test results confirmed about the properties of *Actinomycetes*. The isolated stain showed positive for starch hydrolysis (Fig 3) which indicates the production of amylase., casein hydrolysis (Fig 4) and cellulase production tests (Fig 5). The isolated strain showed a positive test for the hydrolysis of gelatin gives the result of either strong or weak positive i.e., liquefaction occurs within 3-4 days or negative which means no liquefaction even after 30 days (Fig 6A). It also shows the positive test for urea hydrolysis (Fig 6B) and nitrate reduction test (Fig 6C). Catalase test facilitate the detection of enzyme catalase in bacteria (Fig 6D). The positive reaction was evident by immediate bubble formation. No bubble formation represents a catalase negative reaction. The isolated stain showed positive for H_2S production (Fig 6E). Indole test reagents are used to detect the production of indole by bacteria growing on media containing tryptophan (Fig 6F). In case of MR test the culture medium turns red after addition of methyl red, because of a pH at or below 4.4 from the fermentation of glucose, the culture has a positive result for the MR test (Fig 6G). While negative results indicated by a yellow colour in the culture medium, which occurs when less acid is produced (pH is higher) from the fermentation of glucose. The isolated strain also showed positive for Voges Proskeuer test (Fig 6H). and citrate test (Fig 6 I).

The ACT1F strain showed positive for IMVIC test, citrate utilization, starch hydrolysis, casein hydrolysis, gelatin hydrolysis test, urease production, catalase, cellulase and nitrate reduction test.

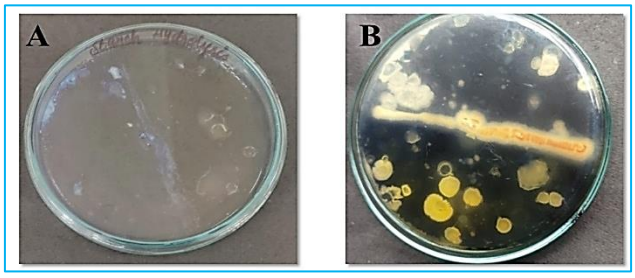

Fig 3 Starch hydrolysis test. A) control and B) ACT1F strain. ACT1F strain showed hydrolysis of starch indicates a positive result

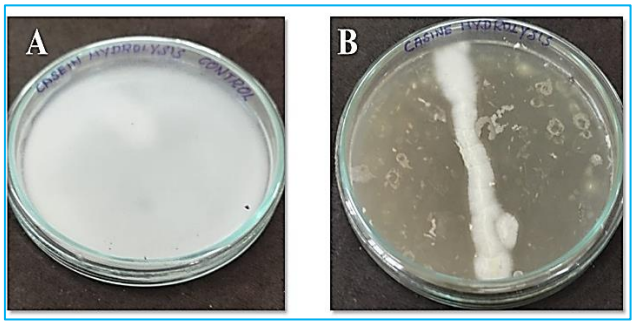

Fig 4 Casein hydrolysis test. A) control and B) ACT1F strain. ACT1F strain showed hydrolysis of casein indicates a positive result

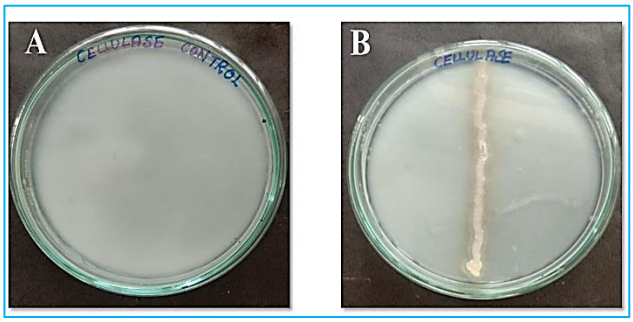

Fig 5 Cellulase production test
A) control and B) ACT1F strain. ACT1F strain showed cellulolytic activity by the production of cellulase

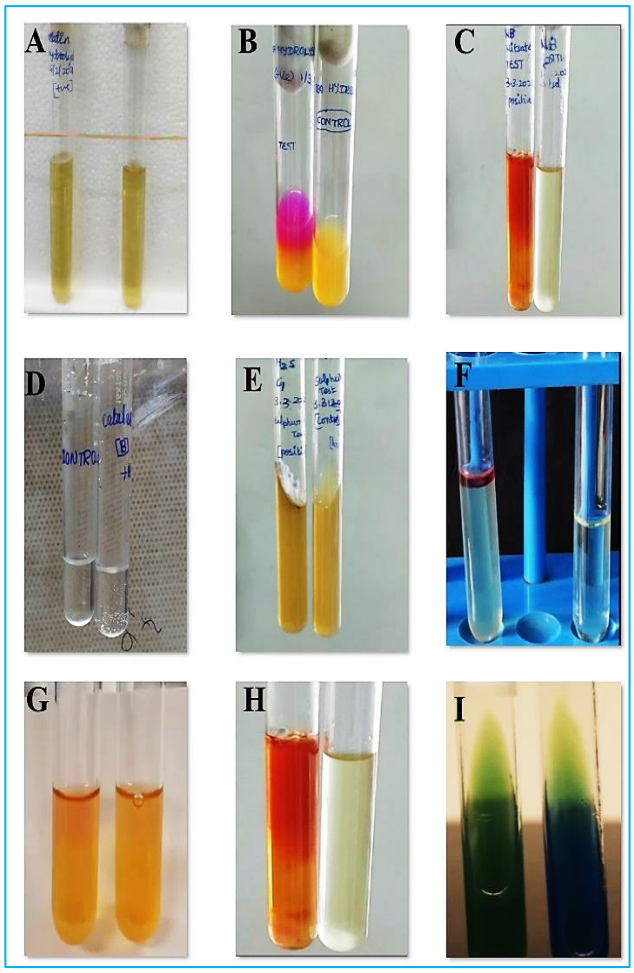

Fig 6 Biochemical test of *Actinomycetes* isolate ACT1F. A) Gelatin hydrolysis test, B) urea hydrolysis test, C) nitrate reduction test, D) catalase production test, E) H_2S production test, F) indole test, G) MR, H)VP test, and I) citrate test (right -test samples, ACT1F and right- control samples)

Fig 7 Production of isolated strain (left image) and culture filtrate (right image) of ACT1F

Production of bioactive compounds by shake flask fermentation

The production medium constituents and concentrations were nearly associated with the metabolic capabilities of the bioactive metabolites producing strain and also prominently influence the biogenesis of metabolic compounds. The isolated strain has owned its antimicrobial activities, in addition studies were provided appropriate cultural parameters for mass production (Fig 7).

Extraction and purification of bioactive compounds

Crude extract of bioactive strain was prepared through solvent-solvent extraction method (Fig 8). Bioactivity of isolates active against *P.aureus* , *Ecoli*, *Salmonella and Proteus* was confirmed by well diffusion assay. The isolate exhibited a prominent zone of inhibition against pathogens *P.aureus and Proteus* respectively, serving as a potential isolate for isolation of bioactive compounds. The purification and separation of crude compound was performed by TLC. The retention factors of moved spot was 0.83. The spot was scrapped and composed in a vial, then evaluated for its antimicrobial activity.

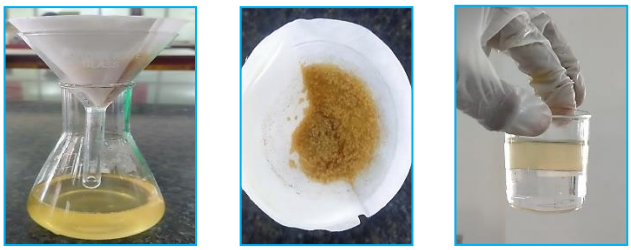

Fig 8 Filtration (left image), biomass cultivation (middle image) and extraction of secondary metabolite (right image) from the isolate ACT1F

Antimicrobial activity of crude extracts

The crude extract containing the secondary metabolite was examined for its antimicrobial activity alongside the bacterial test pathogens by agar well diffusion method. The inhibition zone was measured. The crude extract of ACT1F shown maximum antibacterial activity against *E. coil* and *Proteus* (Table 2, Fig 9).

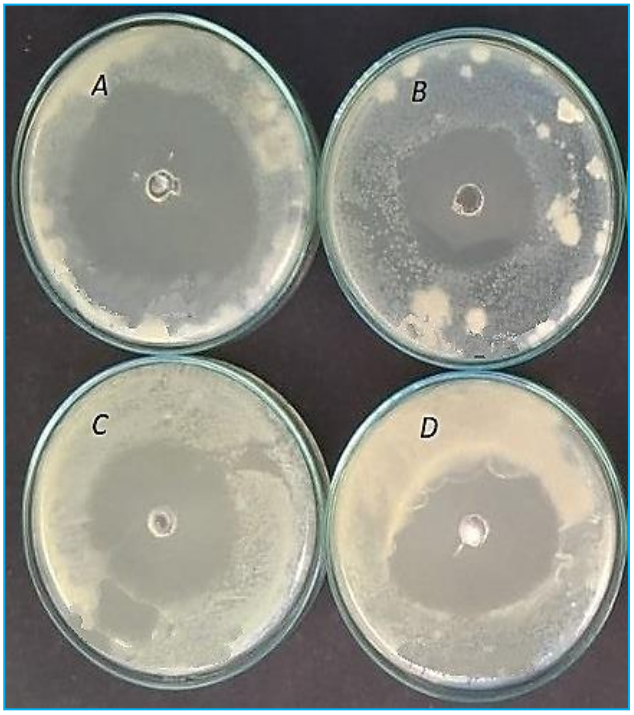

Fig 9 Microbial activity of ACT1F isolate against
A) *E. coli* B) *P. aureus* C) *Salmonella* D) *Proteus*

Table 2 Microbial activity of ACT1F isolate against selected pathogens

Pathogens	Zone of inhibition (mm)
E. coli	24
P. aureus	15
S. typhi	18
Proteus bacilli	21

TLC bioautography

In order to identify bioactive metabolites, TLC plate was developed to obtain 1-2 bands were observed and to identified as bioactive metabolites. Developed TLC plate was overlaid with *S. aureus* suspension on 1% agar, displayed clear zone with no cell growth against pinkish background at Rf between 0.12 to 0.41 (Fig 10).

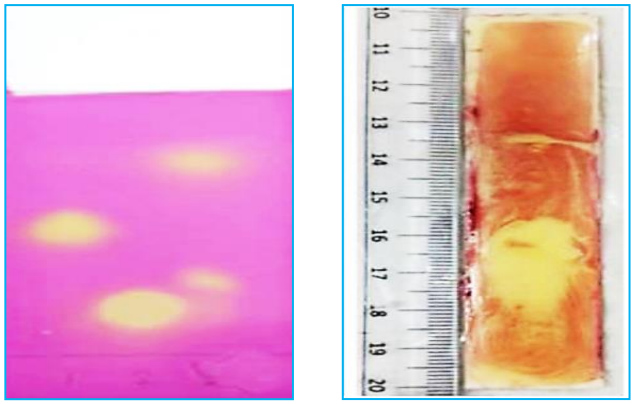

Fig 10 TLC bioautography plate of crude extract of ACT1F strain. Pink area represent cell growth and clear zone area depicts presence of bioactive compound at that region

Table 3 The radical scavenging activity of crude extract of the isolate ACT1F

Concentration (µg/mL)	OD value at (517 nm)	Percentage activity
20	0.089	24
40	0.082	30
60	0.081	31
80	0.079	33
100	0.086	27

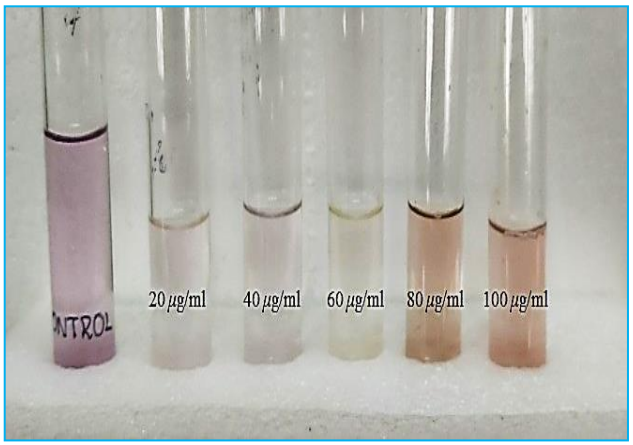

Fig 11 Free radical scavenging activity of crude extract at different concentration ranges from 20 µg/mL to 80 µg/mL

Free radical scavenging activity by DPPH

The colour spots indicated the presence of antioxidant nature. The results of radical scavenging effect and ascorbic acid have exhibited dependent scavenging activity of DPPH radicals. Though the fractions were able to scavenge DPPH and convert it into DPPH, the scavenging effect of the fractions was lesser than that of ascorbic acid. The radical scavenging effect of 80 µg/ml concentration was greater than all fraction (Table 3, Fig 11).

16S rRNA gene sequencing

The taxonomic identification of the ACT 1F was based on 16s rRNA analysis to elucidated the taxonomic position. The 16s rRNA sequence was compared with the sequences in GenBank using BLAST and aligned with the sequences retrieved from NCBI GenBank data using Clustal W method (Fig 11). The phylogenetic tree was constructed based on neighbor joining tree method and illustrated in (Fig 12). The database was deposited in NCBI GenBank with an accession number. Based on the cultural, morphological, physiological and molecular analysis, the ACT1F was identified as *Streptomyces longisporoflavus*.

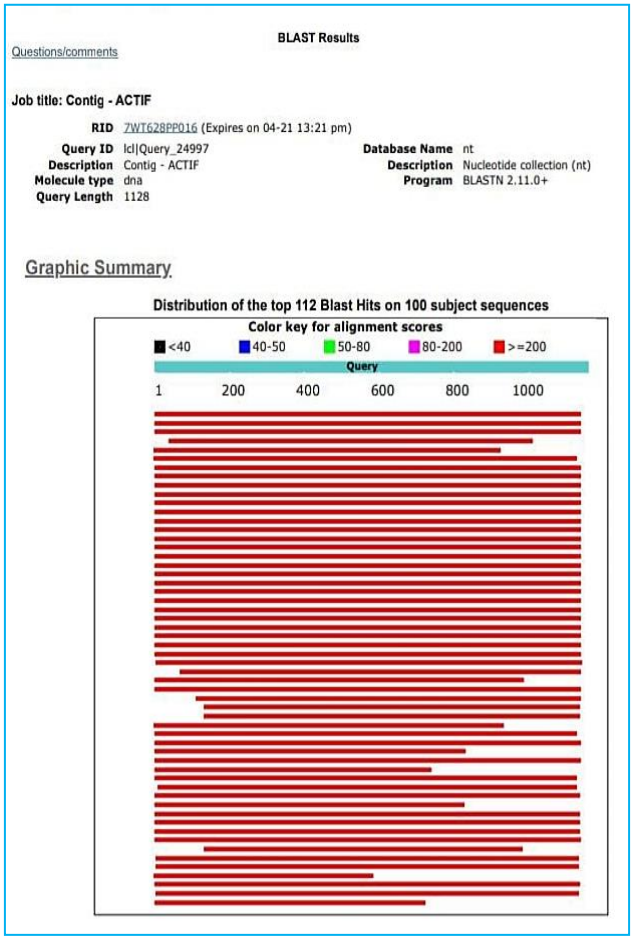

Fig 12 Blast data: Alignment view using combination of NCBI

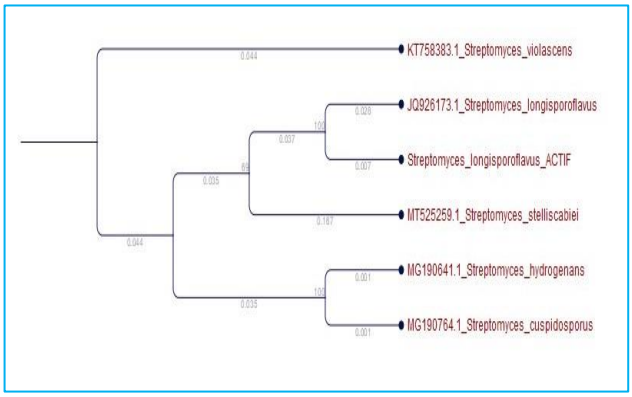

Fig 13 Phylogenetic tree

CONCLUSION

In conclusion, the isolation and characterization of the Actinomycete strain ACT1F from soil sample led to the identification of *Streptomyces longisporoflavus*. The strain demonstrated distinctive morphological features, such as rough colonies with branched or aerial mycelia and long chains of spores. Biochemical tests confirmed the strain's ability to hydrolyze starch, casein, gelatin, urea, and nitrate, as well as its positive results in catalase and other enzyme activity tests. The ACT1F strain exhibited significant antimicrobial activity, particularly against *Escherichia coli* and *Proteus*, indicating its potential as a source of bioactive compounds. The purification of these compounds, along with the strain's antioxidant properties, further highlights its potential for pharmaceutical applications. Molecular analysis using 16S rRNA sequencing reinforced the taxonomic classification of ACT1F as *Streptomyces longisporoflavus*, paving the way for future research into its bioactive metabolites and their potential therapeutic uses.

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