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In-vitro antimicrobial assay of potential Streptomyces sp.NRI-20isolated from Mangrove soils of Machilipatnam, Andhra Pradesh, India

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ABSTRACT
Searching for novel antibiotics is the need of the hour to combat multidrug resistant pathogens. Hence, an attempt was made to isolate and identify promising antimicrobial metabolite producing actinomycetes from mangrove sediments of Machilipatnam, Andhra Pradesh,India along with the study of their antimicrobial potential. The samples collected from mangrove habitats were pretreated with calcium carbonate, diluted and plated on yeast extract malt extract dextrose (YMD), starch casein agar (SCA) and Actinomycetes isolation agar (AIA) media to isolate actinomycetes. Over all four strains designated as NRI-1, NRI-10, NRI-15 and NRI-20 were isolated and screened for their antimicrobial activity. Among them, one strain NRI-20 which exhibited high antimicrobial potential was identified as Streptomycessp. NRI-20 based on polyphasic taxonomy including micro-morphological, biochemical and molecular methods. The culture broth extracted with ethyl acetate was tested for antimicrobial activity and found to be active against pathogenic Gram-positive and Gram-negative bacteria as well as fungi. It was evident from the present study that mangrove habitats of Machilipatnam serve as a good source for potent actinomycetes possessing broad spectrum antimicrobial activity.

Keywords: Mangrove actinomycetes, Phylogenetic study, Streptomycessp. NRI-20, Antimicrobial activity.

I. INTRODUCTION

Actinomycetes are the strongest antagonists among microbes, producing effective and safe antibiotics. Actinomycetes are called filamentous bacteria due to their mycelium like thallus, belonging to the phylum actinobacteria, which represents one of the major lineages currently recognized within the domain of bacteria (Ventura et al., 2007). The filaments of actinomycetes are long and they may fragment into much smaller units and narrower than the fungal mycelium, usually 0.5 to 1.0 µm in few cases. They are gram positive bacteria, produce spores with DNA rich in G+C from 55-75%. They are characterized by complex life cycle. They reproduce by asexual spores called conidia. Soil actinomycetes produce sporangium which liberates spores. When spores are liberated by water drop mechanism from soil actinomycetes, they release a volatile compound geosmin with an earthy odour. They produce arthrospores during unfavourable conditions like dry heat and dessication. Actinomycetes produce aerial and substrate mycelium. They produce powdery mass of spores on the culture media. These are pigmented when aerial spores are produced.

Mangrove soil is a major source of actinomycetes (Vijayakumar et al., 2007). Members of actinomycetes which live in marine environment are moderately understood and only few reports are available pertaining to actinomycetes from mangroves (Sivakumar, 2007). The search of new and novel antibiotics and other bio active microbial metabolites is important for the fight against new emerging pathogens (Busti et al., 2006). There are many reports available on the research on antibiotics from marine actinomycetes, but only few reports are available on mangrove actinomycetes and their antibiotics. Therefore mangrove ecosystem was selected for the present study. Mangrove ecosystem is the most productive ecosystem diversified with variety of microbes (Kathiresan and Bingham, 2001).

Analysis of microbial bioactivity from these ecosystems will help in isolating and

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identifying new and potential microbes having high specificity for various applications. Isolation of actinomycetes from unique unexplored natural habitats is of interest to avoid re-isolation of strains that produce known bio active metabolites. Neglected habitats like mangroves are proving to be a good source of novel actinomycetes and bio active compounds. Mangrove forests are sometimes called "tidal forests", "coastal woodlands", or "oceanic rain forests" that grow at the interface between land and sea in tropical and sub tropical latitudes. Mangroves are distributed circumtropically, occurring in 112 countries and territories. Mangroves are largely restricted to latitudes between 30° north and 30°south. Global coverage has been variously estimated at 10 million hectares (Bunt, 1996), 14-15 million hectares (Schwamborn and Saint-Paul, 1996), and 24 million hectares (Twilley et al., 1992). Spalding (1997) gave a recent estimate of over 18 million hectares, with 41.4% in South and Southeast Asia and an additional 23.5% in Indonesia.

Mangroves provide unique ecological niche to different microbes which play various roles in nutrient recycling as well as various environmental activities. The fertility of mangrove water results from the decomposition of organic matter and recycling of nutrients by micro-organisms. Due to the presence of rich source of nutrients in mangrove soil, mangroves are called as homeland for microbes. Microbial counts are high during monsoon months, when nutrients are brought in by freshwater ingression (Kathiresan et al., 1996). These types of morphological adaptations in mangroves to live in fluctuated soil pH, temperature, soil nutrients and root exudates in the rhizosphere enhances to spread the microbial diversity including actinomycetes.

The secondary metabolites produced by the mangrove oriented actinomycetes are entirely different from terrestrial actinomycetes. Even the extracts from mangroves and mangrove-dependent species have proven activity against human, animal and plant pathogens. (Abeysinghe and Pathirrana, 2006; Bobbarala et al., 2009 and Shelar et al., 2012). In general, the microbial population is about seven-fold higher in mangrove water than in adjoining Bay of Bengal.

II. MATERIALS AND METHODS

OVERVIEW:

The Godavari-Krishna Mangroves are an integral part of the intertidal coastal ecosystems along much of the eastern coast of the Indian Peninsula. These form larger mangrove forest tracts around the deltas of the major east-flowing river systems bridged by thin slivers of mangrove belts along mudflats and coasts. They form dense interlocked stands of specialised trees adapted to recurring inundation, high salinity, and the dynamic coast – ocean interface. It provides habitat for nursing young fish and larvae and are highly productive ecosystems with unique estuarine vertebrates and invertebrates. The mangroves crucially shield the structural integrity of the eastern shoreline against extreme climate events and large-scale erosion. For all their ecological and social value, mangrove ecosystems are little known and are threatened by anthropogenic pressures like urbanisation, industrial intrusion, farming, and pollution.

Collection of soil samples and sample pretreatment

The mangrove soil samples were collected at a depth of 20 cm by using an aseptic metal trowel from different locations of mangrove region in Machilipatnam, Andhra Pradesh, India (Fig. 1). The samples were randomly collected, labeled then brought to the laboratory in sterile zip log bags and stored them in refrigerator at 4^0 C. Air dried samples were ground with mortar and pestle. All the samples were subjected to dry heating (50^0 C/30min) to control unwanted microbial contamination, pre-treated with calcium carbonate (10:1 w/v) and phenol (1.5%) to enrichment of actinobacteria (Manimaran et al., 2017).

Fig. 1 Mangrove Sample collection area Machilipatnam mangrove habitats, A.P.

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Isolation and purification of mangrove actinobacteria

Actinobacteria were isolated by employing dilution plate technique using selective media ISP-2 (YMD), starch casein agar (SCA) and Actinomycetes isolation agar (AIA) media supplemented with 3% NaCl and at pH 7.0. The three selective media were amended with rifampicin (25µg/mL) and nystatin (25µg/mL) to minimize the bacterial and fungal contamination**.** The pretreated samples were dissolved in 1ml of sterile water and diluted up to 10^{-5} dilutions. Aliquots of 0.1 ml of each dilution were spread evenly on plates of above media in triplicates. The plates were incubated at 30±2^{o} C up to 3 weeks. After incubation, the plates were examined for actinobacteria colonies based on color, dryness, rough, convex colony**.** The fifteen prominent colonies were selected, purified and maintained on YMD slants at 4^0 C for further study (Mangamuriet al., 2012).

Preliminary screening or perpendicular screening of isolated actinobacteria:

Preliminary screening was performed to all isolated actinobacterial strains. The culture medium (YMD medium) was prepared and the actinobacterial strains were streaked in a line at the middle of the each plate. The test bacterial pathogens were also streaked perpendicularly to the each actinobacterial streaked line. All the plates were allowed for incubation for 24 to 48hrs. Based on the screening results, the strain was selected for further studies.

Characterisation studies of the Actinobacteria

Morphological characterisation by Slide culture Technique: The potent actinobacterial strain was characterized by morphological, biochemical and molecular methods.This technique also called as "inclined coverslip technique". For the study of morphological characteristics with reference to aerial mycelium, substrate mycelium and sporulation this technique were used. The isolate was cultivated on YMD agar, sterile coverslip was inserted at angle of about 45^0C into a solidified medium in a Petri dish so that the half the coverslip is in the medium. The inoculum was spread along the line, where the upper surface of the coverslip meets the agar, with a fine wire needle and plates were incubated at ambient temperature for 3-7 days. After incubation period the isolate was grown on both, the medium and coverslip. The coverslip was carefully withdrawn from the microscope to differentiate between substrate and aerial mycelium. Slides were prepared by using Lacto-phenol Cotton Blue (LPCB) reagent. The observation was also recorded about the presence of colour of spore mass (grey, blue, red, violet, yellow or white), spore chain morphology (rectiflexibiles, spirals or retinaculiaperti) and presence of sclerotia.

Scanning electron microscopy:

The conidia morphology of strain NRI-20 was studied under the scanning electron microscope (SEM). Samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C and postfixed 2% in aqueous osmium tetroxide for 4 h. Dehydrated in a series of graded alcohols and dried to critical point drying with CPD (EMS 850) unit. The processed samples were mounted over the stubs with double-sided carbon conductivity tape, and a thin layer of gold coat over the samples was done by using an automated sputter coater (Model - JEOL JFC-1600) for 3 min and scanned under Scanning Electron Microscope (SEM - Model: JOEL-JSM 5600) at required magnifications as per the standard procedures (Antony-Babu, and Goodfellow 2008 and Arijit Das et al., 2014).

Biochemical Characterisation:

Staining (Gram's Staining): In this staining procedure, a thin smear was made on a clean glass slide which was air dried and fixed by flaming. Then the thin smear was subjected to gram's staining. The smear was flooded with crystal violet solution for 1 min, decolorized crystal violet with 95% ethanol for a few seconds and then counter stained with saffranine solution. Then, the slide was blot dried and observes under microscope under oil immersion (Benson, 1994).

Indole production test: This test was conducted to test the ability of bacteria to decompose the amino acid tryptophane to indole, which accumulates in the medium. Tryptone broth inoculated with 24-48 hours old culture and incubated for 48 hours at 37^0C. Indole production was then tested by the coloured reaction with the addition of Kovac's reagent. The red colour in the alcohol layer indicates a positive reaction (Collee et al., 1996).

Methyl red test: Using YMD (Yeast Extract Malt extract dextrose (ISP-2)) broth, the actinobacteria culture was inoculated and incubated at 28^0 C for 5-7 days. Addition of 5 drops of methyl red was done after incubation expecting the development of red colour. Appearance of red colour indicates the positive for methyl red test.

International Journal of Pharmaceutical Research and Applications
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V-P (VOGES-PROSKAUER) test: Using YMD (Yeast Extract Malt extract dextrose (ISP-2)) broth, loopful of test culture was inoculated followed by incubation at 28°C for 5-7 days. Post incubation, 0.6 ml α-napthol& 0.2 ml Potassium Hydroxide were added per ml of broth recording the result after 15-60 mins expecting the development of Cherry red color. If appeared cherry red colour indicates the VP test positive.

Citrate utilisation test: This is a test the ability of an organism to utilize citrate as the sole carbon and nitrogen source for growth and an ammonium salt as the sole source of nitrogen. Simmon's citrate agar medium was used. The medium was inoculated with 24-48 hours old culture and incubate at 37^0C for 3-5days. An observation made for the colour change from green to blue was due to change of pH in the medium (Collee et al., 1996).

Catalase production test: An enzyme that catalyzes the oxygen from hydrogen peroxide. One or two drops of 3% hydrogen peroxide was taken on a clean glass slide and one loopful of 24-48 hours old culture was added or mixed with hydrogen peroxide. Observation was made for the emergence of bubbles due to release of oxygen (Collee et al., 1996).

In-vitro screening for antibiotic susceptibility

The antibiotic sensitivity test was conducted to assess the susceptibility of the strain to different antibiotics by following the Kirby-Bauer disc diffusion method. Few antibiotic discs viz., azithromycin (15µg), chloramphenicol (10µg), kanamycin (30µg) etc were used and procured from Hi-Media Pvt. Ltd., India. The plates were inoculated with the potent actinobacteria, specified antibiotic disc was placed and incubated them at $30\pm2^{\circ}$ C for 48 h. After incubation, the inhibition zones around the antibiotic discs were observed. Based on observation, the strain was considered as either sensitive (S) or resistant (R) to an antibiotic (Ragalatha, 2020).

Production and screening of secondary metabolitesand Extraction of Metabolites:

The homogenous culture suspension of the actinobacteria is prepared by suspending 7-day-old culture in sterile saline was used to inoculate YMD broth (seed medium) and incubated at 30°C for seven days on a rotator shaker at 180 rpm. Seed culture at the rate of 10% was transferred to YMD broth (fermentation medium). The fermentation was carried out at 30 ± 2°C for twenty days under agitation at 180 rpm. The antimicrobial compound was recovered from the filtrate by the solvent extraction method. Ethyl acetate was added to the filtrate (1:1) and shaken vigorously. The ethyl acetate extract was evaporated to dryness on a water bath and the residue thus obtained was used to determine antimicrobial activity.

In-vitro Antimicrobial activity:

The antimicrobial activity of the strain was determined by agar well diffusion assay. YMD broth was used as a production medium for the extraction of crude secondary metabolites. The potent actinobacterium NRI-20 was inoculated and the fermentation was carried out, Bioactive compounds were recovered from the filtrate by solvent extraction method. Ethyl acetate was added to the filtrate (1:1 v/v) and shaken vigorously. The ethyl acetate phase that contains bioactive secondary metabolites was separated from the aqueous phase using separating funnel.

For antimicrobial assay, to culturing test bacterial samples, Nutrient agar medium (NAM) was prepared. The medium was mixed with the test bacteria and poured into the petri plates then allowed for solidification. Similarly, for culturing the fungal samples, Czapek dox agar medium (CDA) was prepared. The medium was mixed with the test fungi and poured into the petri plates then allowed for solidification. After solidification of all plates, wells were made by using 6mm cork borer. A volume of 80µL of crude extract was carefully dispensed into separate well and allowed to diffuse followed by incubation at 37°C for 24h. Standard antibiotic (streptomycin 80µL) was used as a positive control. After incubation, the zone of inhibition (mm) was measured and recorded.

Microbial pathogens used for antimicrobial activity: Thetested bacterial pathogens wereStaphylococcus aureus (MTCC 3160), Klebsiella pneumoniae (MTCC 109),Xanthomonas campestris, Bacillus megaterium (NCIM 2187), Pseudomonas aeruginosa and Escherichia coli (ATCC 9027). The fungal pathogens used for this bioactivity wereCandida albicans, Aspergillus flavus and Penicillium citrinum.

III. RESULTS AND DISCUSSION

Sample collection and isolation of actinobacteria

Soil samples were collected(fig. 2A), they were subjected for pre-treatment processes. After pre-treatment, isolation procedure was done by using three different selective media (fig.2B,C). A total of 4actinobacterial strains were isolated from the mangrove habitats of Machilipatnam and designated as NRI-1, NRI-10, NRI-15 and NRI-20.

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 6 Nov - Dec 2025, pp: 146-154 www.ijprajournal.com ISSN: 2456-4494

Among them, one actinobacterial strain NRI-20 was found to be potent and exhibited strong antimicrobial activity against test bacteria and fungi.

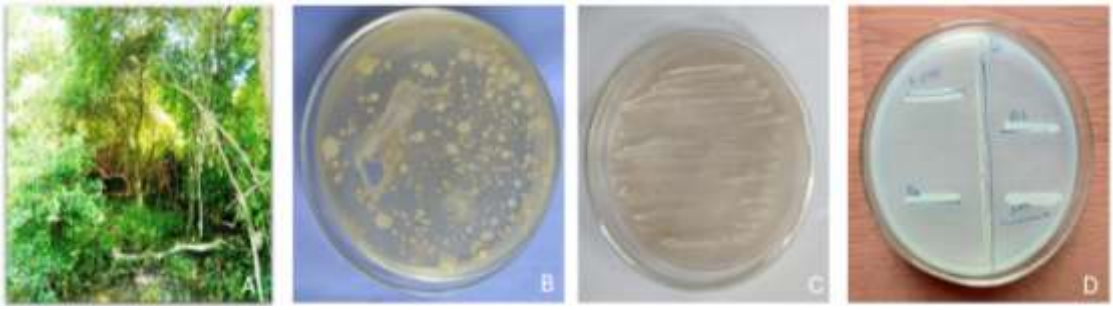

Fig. 2A. Sample collection in Machilipatnam mangroves; B & C. Isolation and Subculturing of actinomycete strains; D. Preliminary screening of isolated actinobacteria

Preliminary screening of isolated actinobacteria and Morphological characteristics of the strain NRI-20:

In preliminary screening, the actinobacteria NRI-20 was exhibited significant potential antimicrobial property against the E.coli, Pseudomonas aeruginosa, Bacillus subtilis and Streptococus mutans(Fig. 2D).Based on the screening results, the strain NRI-20 was selected for further studies.The culture colony exhibits white colour mycelia. Micro-morphological view of the strain was examined under Scanning electron microscopy. The strain displayed massive sporulation with branched mycelium and abundant aerial hyphae. The Spores of the strain was spherical, globose with smooth-surface. Paired spores on hyphae were observed (fig. 3A,B).

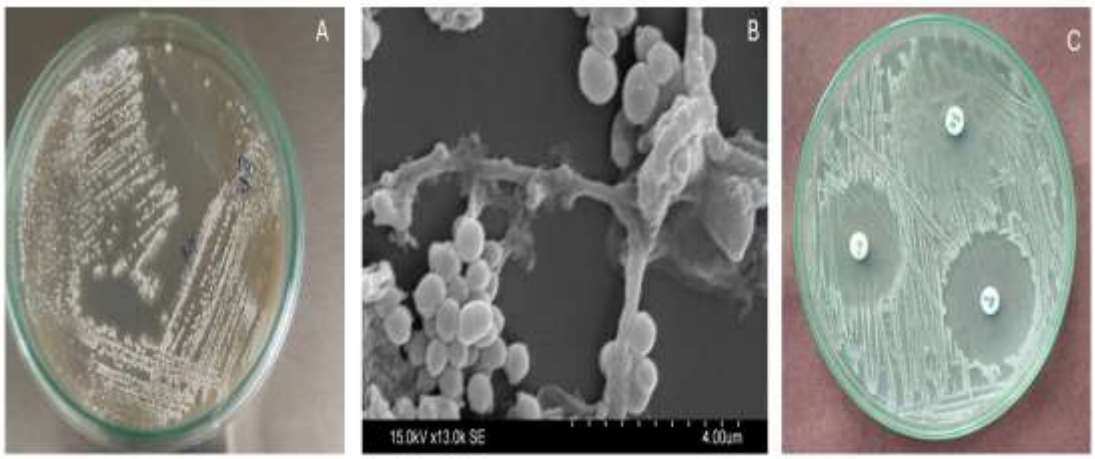

Fig. 3A. Actinobacterium grown on YMD medium the strain NRI-20; B.SEM Photograph of Streptomyces sp. NRI-20; C. Antibiotic sensitivity test

Biochemical and Physiological characteristics of the strain NRI-20

The physiological and biochemical characteristics were significant tools for identification of actinobacteria. The strain NRI-20 exhibited a positive response to indole production, Voges-Proskauer, and catalase activity, citrate utilization but negative to H_2S production and methyl red test (figure 4; table 1).The strain showed good growth at pH range between 5 and 9 with the optimum being 7 and the range of temperature for growth was 30-40°C with the optimum being 35°C. The strain could grow well in the medium supplemented with 3% sodium chloride and showed tolerance up to 7%. The strain showed sensitivity to imipenem, ampicillin, chloramphenicol and tetracycline. It showed resistance to Penicillin, clotrimazole, cefixime and amikacin (table 2).

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 6 Nov - Dec 2025, pp: 146-154 www.ijprajournal.com ISSN: 2456-4494

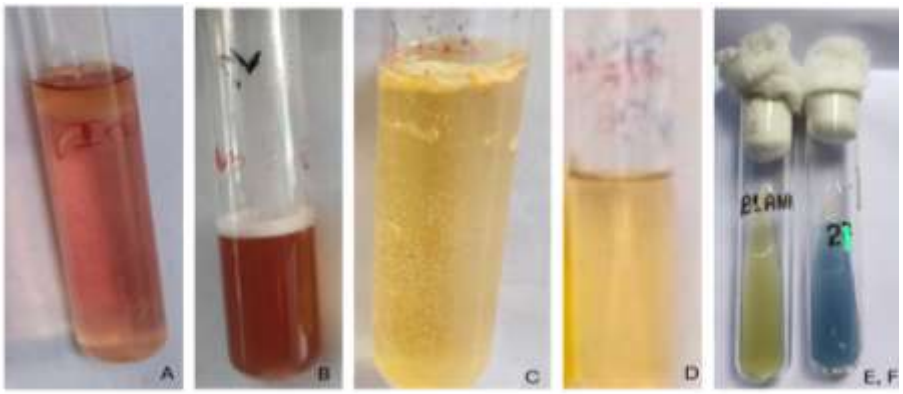

Fig. 4 Different biochemical tests A: Indole test, B: VP test, C: Catalase test, D: MR Test, E: Citrate utilisation test (Blank and Test)

Table 1: Morphological and Biochemical characteristics of NRI-20

Character	Response
Morphological characters	
Sporophore morphology	Recti flexible
Color of aerial mycelium	White
Color of substrate mycelium	White
Physiological characters	
Gram's reaction	+
Biochemical characters	
Catalase production	+
Methyl red test	-
Voges proskauer test	+
Indole production	+
Citrate utilization	+
H_2S test	-

Table 2: Antibiotic susceptibility/resistance of NRI-20

Antibiotics (µg/disc)	Susceptibility/Resistance
Tetracycline (30 µg)	S
Penicillin (10 µg)	R
Chloramphenicol (50 µg)	S
Clotrimazole (25 µg)	R
Novobiocin (10 µg)	S
Cefixime (30 µg)	R
Ampicillin (10 µg)	S
Amikacin (10µg)	R

***S – Sensitive; *R – Resistant**

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 6 Nov - Dec 2025, pp: 146-154 www.ijprajournal.com ISSN: 2456-4494

Antimicrobial profile of the strain Streptomyces sp. NRI-20

The antimicrobial profile of Streptomyces sp. NRI-20was studied at regular intervals checking up to 10 days in batch culture. The stationary phase of the strain extended from 144 h to 168 h of incubation**.** The bioactive secondary metabolites obtained from 8-day-old culture showed high antimicrobial activity against the test microbes. The secondary metabolites obtained from 5 day-old culture extracts of S. albogriseolous VJMS-7, S. violaceoruber VLK-4 and S. cellulosae VJDS-7 exhibited high antagonistic activity. Similarly, the antimicrobial metabolites extracted from 6 day-old culture ina extracts VJMS-4, of S. rectiverticillatus VJMS-8 and 4-day-old culture extracts of S. cheonanensis VUK – A, S. tendae TK-VL 333 showed high antimicrobial activity against the test microorganisms. The antimicrobial spectrum of the strain cultured on YMD broth for 8 days was tabulated (table 3). The metabolites extracted from 6-day-old culture showed maximum activity against B. megaterium, E. coli and K. pneumoniae. In case of fungi, C. albicans showed high sensitivity.

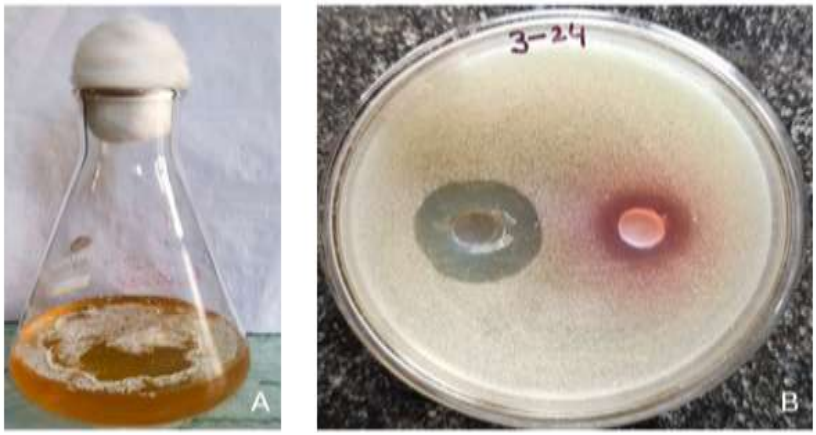

Fig.5A. The strain NRI-20 growing on YMD broth for antimicrobial activity; B.Antimicrobial activity of secondary metabolites produced by the strain against Escherichia coli

Table 3: Antimicrobial activity of Streptomyces sp.NRI-20:

Test organism name	**Zone of Inhibition (mm)**
Bacteria	**NRI-20**
Staphylococcus aureus	12
Escherichia coli	22
Xanthomonas campestris	18
Pseudomonas aeruginosa	17
Bacillus megaterium	20
Klebsiella pneumoniae	20
Fungi	
Candida albicans	18
Aspergillus flavus	12
Penicillium citrinum	14

International Journal of Pharmaceutical Research and Applications
Volume 10, Issue 6 Nov - Dec 2025, pp: 146-154 www.ijprajournal.com ISSN: 2456-4494

Statistical Analysis: Readings were taken as the mean ± standard deviation of the mean of two replicates calculated using Microsoft Excel XP 2007.

IV. CONCLUSION

The present investigation highlights the antimicrobial potential of Streptomyces sp.NRI-20. It is evident from the present study that mangrove habitats of Machilipatnam, serve as a good source for the isolation of potent actinomycetes with broad spectrum antimicrobial activity. Further study on chemical characterization of bioactive compounds of the isolate is in progress.

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CONFLICT OF INTERESTS: Declared none

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