CODEN [USA]: IAJPBB ISSN: 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

Available online at: http://www.iajps.com Research Article

"ISOLATION AND CHARACTERIZATION OF L-ASPARAGINASE-PRODUCING ACTINOMYCETES FROM MANGROVE SEDIMENTS: INSIGHTS INTO ANTI-MICROBIAL AND ENZYMATIC POTENTIAL"

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Article Received: March 2019 **Accepted:** April 2019 **Published:** May 2019

Abstract:

In recent decades, microorganisms have been shown to store bioactive chemicals. Actinobacteria manufacture secondary metabolites most efficiently. The rapid spread of epidemic illnesses, drug-resistant microorganisms, and the need for effective anti-cancer drugs need the development of effective antibiotics. Several actinobacterial species have been studied for bioactive chemical production from terrestrial environments in recent decades. Sediment samples from Nizampatnam (Station I) and Coringa (Station II) mangrove habitats were analysed using standard procedures. Parameters recorded included moisture content (15%), 17% (Station II), pH (7.6 and 7.2), organic carbon (7.2 and 6.8), and total nitrogen (4.54 and 4.92 µg/g). Air-dried sediment samples were pre-treated with four methods and plated on three selective medium employing soil dilution to extract and count actinomycete colonies (Yi., 2019). Calcium carbonate pre-treatments had higher actinobacterial counts than dry heat, phenol, and ringer's solution. Compared to asparagine glucose agar (AGA) (Smith, 1943) and glycerol-asparagine agar (GAA) (Waksman, 1961), starch-casein agar media (Wellington and Cross, 1961) promotes actinomycete growth. Nizampatnam mangroves yielded more actinobacterial strains than Coringa. Purified actinomycete strains were cultivated on yeast extract-malt extract-dextrose (ISP-2) agar.

Keywords: Enzymatic Potential, L-Asparaginase, Mangrove Sediments.

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*Please cite this article in press G. Madhavi and Vidyadhara S., **Isolation And Characterization Of L-Asparaginase-Producing Actinomycetes From Mangrove Sediments: Insights Into Anti-Microbial And Enzymatic Potential.,** Indo Am. J. P. Sci, 2019; 06[05].*

INTRODUCTION:

Enzymes, protein molecules composed of amino acids, are essential catalysts and regulators for various biological events. They exhibit more selectivity in their catalytic reactions compared to typical organic processes, making screening for novel enzymes necessary *Anand, P., et al. (2008)*. Screening a large number of microorganisms is an efficient and fruitful approach for uncovering novel enzymes due to their intrinsic variety and capacity to adapt. Enzymes have been used as therapeutics for various medical conditions, such as cancer, digestive difficulties, cutaneous ulcers, inflammation, heart problems, and cystic fibrosis. Calle, *E. E., & Kaaks, R. (2004).* Cancer is a medical term that describes abnormal growth of cells that can invade and spread to other tissues. Inadequate treatment of cancer cells could lead to organ dysfunction and mortality. The lymphatic and vascular systems facilitate the metastasis of tumor cells to distant body parts. All malignancies have six fundamental characteristics, known as "Hallmarks," which enable the conversion of normal cells into carcinogenic (malignant or tumor) cells. Cancer cells exhibit several characteristics that distinguish them from normal cells, including resistance to inhibitory signals, active promotion of growth, resistance to programmed cell death, angiogenesis, indefinite growth, and metastasis.

Environmental factors account for 90-95% of cancer cases, while genetic factors contribute to 5-10%. Factors such as poor nutrition, obesity, smoking, infections, environmental pollutants, strain, radiation exposure, and insufficient bodily activity increase the risk of cancer. Weight plays a role in 14-20% of all cancer fatalities and may increase the risk of developing certain cancers. Acute lymphoblastic leukemia (ALL), the most prevalent pediatric malignancy, affects six out of ten children and can manifest as either asymptomatic or acute, potentially leading to severe hemorrhage, infection, or respiratory distress. Acute leukaemia treatment includes steroids, radiation, chemotherapy, and a combination of therapies. L-asparaginase is a crucial enzyme in the management of acute lymphoblastic leukaemia (ALL) for diseases related to lymphoproliferative and lymphomas. It has been used in various cancer treatments, including pancreatic carcinoma, acute pancreatitis caused by bovine lymphosarcoma, and infantile ALL. L-asparaginase-II has been found to be essential in treating various forms of cancer, including reticulosarcoma, melanosarcoma, lymphosarcoma, acute myelocytic leukemia, chronic lymphocytic leukemia, and other forms of cancer Hanahan, D., & Weinberg, R. A. (2011).

L-asparaginase is catalyzed by the conversion of L-asparagine into L-aspartate and ammonia. It was first discovered in guinea pig serum in 1922 and has been shown to have anticancer properties. Kidd, J. G. (1953) The enzyme has also shown tumoricidal properties and has been used in both adult and pediatric ALL treatment. L-asparaginase is vital for the multiplication of immature lymphocytes and can inhibit the uptake of L-asparagine from the extracellular pool, leading to cell death in cancer cells Mashburn, L. T., & Wriston, J. C. (1964). However, it can also cause allergic reactions, inhibit blood coagulation, elevate blood sugar levels, trigger liver disease, and enhance liver enzyme blood tests. Fungal-derived L-asparaginase has shown non-toxic characteristics and demonstrated myelosuppressive and immunosuppressive effects. In recent decades, the identification rate of new molecules from earthly Actinomycetes has declined, making it essential to investigate fresh communities of Actinomycetes from underexplored environments as possible origins of unique therapeutic secondary metabolic products. Marine Actinomycetes from coastal environments are a significant source of novel antibiotics and may aid in recognizing new species. This study aims to classify the isolates of Actinomycetes that produce L-asparaginase by morphological, staining, and molecular characterization.

MATERIALS AND METHODS:

Isolation and Characterization of Actinomycetes from Mangrove Sediments: A Screening Approach.

Sediments from the Nizampatnam and Coringa mangrove habitats on the southern-east coast of Andhra Pradesh, India, were collected biweekly from April 2014 to February 2015. The first site is 15°54′0N, and the second is 16°44–16°53′N, 82°14′–82°22′E. *Das, S., Lyla, P. S., & Khan, S. A. (2008).* The samples were carefully gathered into sterile bags and transported to the lab after being removed from 6-10 cm. After transport to lab, samples air-dried at room temperature. The samples from each location were tested for hydration, pH, organic carbon, and total nitrogen Bull, A. T., & Stach, J. E. (2007).

Soil Characteristics

Moisture content

Ten grammes of sediment sample was heated to 1050 Celsius in a hot air oven until it attained a uniform weight to determine moisture content *Sharma, M. (2014).* After drying the sample, subtract its weight from its pre-drying weight to ascertain its soil moisture content. *Kathiresan, K., & Bingham, B. L. (2001)*

Soil pH
A DI-707 digital pH meter measured sediment sample pH (Jackson, 1973). A 100-millilitre beaker held 20 grammes of sediment sample and 40 millilitres of distilled water for this experiment. After complete mixing, the solution settled for 1 hour before pH was measured. The suspension's clay particles were eliminated during this period.

Organic carbon:
Walkey and Black method was used to measure sediment sample organic carbon (Walkley, 1934). A 500 ml Erlenmeyer flask held 10 g of sample. The flask was gently shaken to include the sediment after adding 10 millilitres of 1 N K2Cr2O7. After slowly adding 20 ml of concentrated H2SO4, strong agitation lasted 1 minute. The suspension was filtered after adding 200 ml distilled water to the flask. After that, a few drops of O-phenanthroline indicator were added to the filtered solution and titrated against 0.5 N FeSO4. 7H2O. Control sample was soil-free. We calculated the soil sample's organic carbon using this formula.

Organic carbon (%) = (milliequivalents $K_2Cr_2O_7$ – milliequivalents $FeSO_4$) x 0.003 x 100) / Soil weight (g) x *f*

f = correlation factor = 1.33

Total nitrogen:
Cappuccino and Sherman (2004) used the Micro-Kjeldahl method to find the total nitrogen in the soil sample.

Reagents
The required reagents include concentrated hydrochloric acid, potassium bichromate, sodium hydroxide-sodium thiosulate solution (60% NaOH and 5% Na2SO¬3. 5H2O in distilled water), 0.02 N standard hydrochloric acid, 4% boric acid solution, and a mixed indicator solution (10 ml methyl red and 50 ml bromocresol green in ethanol).

Procedure
Procedure: A flask was used to digest 100 g of finely sieved material. After adding 2 grammes of K2SO4, 90 milligrammes of mercuric oxide, and 2 millilitres of concentrated H2SO4, the mixture was carefully put in the flask and heated to high temperatures for digestion. Finally, the flasks were chilled before carefully adding the minimal water. The funnel was rinsed with water after adding NaOH-Na2SO3 to the digest. Following distillation, ammonia was dissolved in boric acid and collected. We got 20 ml distillate. Rinsing the condenser tip with water, the distilled sample was added to a standard solution of HCl (0.02 N) and titrated until a violet tint was seen, denoting the end point. The same volume of water was used as a control for the distilled sample. After using the technique, we calculated sediment nitrogen.

Total Nitrogen = HCl (ml) in sample – HCl (ml) in blank x normality of acid x 14.01x100/ Weight of sample (mg)

Isolation:
Several soil pre-treatments increased actinobacterial population and reduced contaminants in air-dried samples from 6-10 cm depth. Methodologies include

a) Dry heat treatment at 100°C for one hour [P-1]

b) Pre-treatment with 1.5% phenol [P-2] Seong et al. (2001)

c) Pre-treatment with calcium carbonate [P-3] (El-Nakeeb and Lechevalier, 1963) and

d) Pre-treatment utilizing an osmoprotectant, such as quarter-strength Ringer's solution [P-4]

Pre-treated soil samples were homogenised, serially diluted (10^-4 dilution), and inoculated onto media such as starch-casein, asparagine-glucose, and glycerol-asparagine agar, supplemented with nalidixic acid (25 µg/ml) and secnidazole (25 µg/ml). The inoculation plates were incubated at 30°C for 10 days. Actinomycete strains with robust, leathery colonies partially embedded in the agar (Jensen et al., 2005) were selected, isolated, and subcultured on YMD (ISP-2 medium) slants and stored at 4°C for further study.

Screening of morphologically distinct actinomycete strains for bioactive metabolites
Actinomycete strain secondary metabolites were extracted using Ellaiah et al. (2004). The strains' pure cultures were aseptically transferred to seed media (ISP-2 broth). After 24 hours, 10% of the seed culture was put into the production medium. The fermentation lasted one week at 35° C with 250 rpm agitation. At 24-hour intervals, flasks were retrieved and soup biomass was removed. Biomass dry weight was measured in mg/100 ml. A crude residue was obtained by filtering cultures, extracting them twice with ethyl acetate, and evaporating the solvent extracts under vacuum. After dissolving the remains in 0.2 ml of DMSO, 0.8 ml of distilled water was added. Antibacterial activity was assessed on extracted

samples. Antimicrobial tests employed Staphylococcus aureus (MTCC 3160), Escherichia coli (ATCC 35218), and Candida albicans (ATCC 10231).

Screening of L-asparaginase producers using a rapid plate assay

A modified M-9 medium with phenol red, a food pH indicator, was used to measure each strain's L-asparaginase activity. After that, the medium was incubated at 30°C for 14 days. Gulati and colleagues found a pink zone in colonies of L-asparaginase-positive strains in 1997. M-9 liquid medium was used for quantitative enzyme activity estimation. The actinomycetes with the greatest enzyme activity and widest zone were selected for further research. The powerful actinomycete strain Pseudonocardia endophytica RM-5 was identified utilizing colony morphology, spore organization, physiological and biochemical properties, and other factors. The strain's identification was further validated by molecular analysis of the 16S rRNA gene sequence, which was submitted in GenBank under the accession number JN087501.

Determination of L-asparaginase activity

L-asparaginase was quantified using an assay technique according to Imada et al. (1973). After adding 0.5 ml of culture filtrate and 0.04 M L-asparagine as substrate, 0.5 ml of 0.05 M Tris-Hcl buffer at pH 7.2 started the reaction. The mixture was incubated in a water bath shaker at 30°C for 20 minutes. The process ended with 1.5 M tricholoroacetic acid as the catalyst. After adding 0.2 milliliters of Nessler's reagent, the precipitated protein was centrifuged off and the ammonia measured at 450 nanometers. IU measured enzyme activity. One international unit (IU) of L-asparaginase is defined as 1 µmol of ammonia discharged per millilitre per minute (µmole ml-1 min-1).

Construction of the standard graph

A standard graph was constructed by treating 1ml of ammonium sulphate solutions with volumes of 0.02, 0.04, 0.08, 0.16 and 0.32 ml using Nessler's reagent. The optical density (OD) was determined at a wavelength of 450 nm after a 20-minute incubation of the containers at a temperature of 20 °C.

Stock solution of 10 mM

The solution was prepared by mixing 100 ml of distilled water with 132 mg of ammonium sulphate in a volumetric beaker.

Working solution of 1mM

It was created by adding 50 ml of distilled water to 5 ml of stock solution.

Taxonomic studies of the potent actinomycete strain RM-5

To identify the strains, normal protocols were followed, including morphological, cultural, physiological, and biochemical characterisation, as well as genomic (16S rRNA gene sequence) analysis.

Cultural characterization

The strain RM-5's cultural characteristics were examined on tryptone-yeast extract agar (ISP-1), YMD agar (ISP-2), oat-meal agar (ISP-3), starch inorganic salts agar (ISP-4), asparagine-glycerol agar (ISP-5), tyrosine agar (ISP-7), and non-ISP media like starch-casein, maltose-tryptone, malt-extract, and Czapek Do. Cultural parameters included growth type, aerial and substrate mycelia colour, and pigment synthesis.

Table 1. Composition of ISP and Non-ISP media

Medium code	Name of the medium	Composition (%)
	ISP media	
ISP-1	Tryptone-yeast extract agar	Tryptone-1.0; yeast extract-0.3; agar-2.0; pH-7
ISP-1	Yeast extract malt extract dextrose agar	Yeast extract-0.4; malt extract-1.0; Dextrose-0.4; $CaCO_3$-0.2; agar-2.0; pH-7
ISP-3	Oat meal agar	Oat meal-2.0; trace salt solution ($FeSO_4.7H_2O$-0.01, $MnCl_2.4H_2O$-0.01, $ZnSO_4.7H_2O$-0.01); agar-2.0; pH-7
ISP-4	Starch inorganic salts agar	Soluble starch-1.0; K_2HPO_4-0.1; $MgSO_4.7H_2O$-0.1; NaCl-0.1; $(NH_4)_2SO_4$-0.2; $CaCO_3$-0.2;Trace salt Solution ($FeSO_4.7H_2O$-0.01, $MnCl_2.4H_2O$-0.01, $ZnSO_4.7H_2O$-0.01); agar-2.0; pH-7.
ISP-5	Asparagine glycerol agar	L-asparagine-0.1; glycerol-1.0; K_2HPO_4-0.1; trace salt solution ($FeSO_4.7H_2O$-0.01, $MnCl_2.4H_2O$-0.01, $ZnSO_4.7H_2O$-0.01); agar-2.0; pH-7
ISP-6	Peptone yeast extract iron agar medium	Peptone-1.0; yeast extract-0.4; $FeSO_4.7H_2O$-1.0; agar-2.0; pH-7
ISP-7	Tyrosine agar	L-tyrosine-0.1; agar-2.0; pH-7
Non-ISP media		
NA	Nutrient agar	Peptone 0.5; Beef extract-0.3; NaCl-0.5; agar-2.0; pH-7
CD agar	Czapek Dox agar	Sucrose-3.0; sodium nitrate-0.2; K_2HPO_4-0.1; $MgSO_4.7H_2O$-0.05; KCl-0.05; $FeSO_4.7H_2O$-0.001; agar-2.0; pH-7
ME agar	Malt extract agar	Malt extract-2; pH-6.5
MT agar	Maltose tryptone agar	Maltose-1.0;tryptone-0.5; K_2HPO_4-0.1 ; $FeSO_4.7H_2O$-0.01; agar-2.0; pH-7
SC agar	Starch casein agar	Starch-0.1;casein-0.2; K_2HPO_4-0.1; $MgSO_4.7H_2O$-0.1; $FeSO_4.7H_2O$-0.01; agar-2.0; pH-7.2

Morphological studies

Slide culture was used to analyse the strain's micromorphology using ISP-2 medium. The strain's sporophore morphology was photographed using SEM according to Bozzola and Russell (1999). The culture was fixed for 24 hours at 4 ºC with 0.249 mol L-1 glutaraldehyde in 0.1 mol L-1 phosphate buffer (pH 7.2). It was then post-fixed for 4 hours in 0.078 mol L-1 aqueous osmium tetroxide in the same buffer. Ruska Labs, Acharya NG Ranga Agricultural University, Hyderabad, India, dehydrated the sample in ethanol and dried it to the critical point using the Electron Microscopy Science CPD unit. Desiccated specimen was taped on aluminium stub using double-sided carbon tape. An automated sputter coater (JEOL JFC-1600) applied a thin gold coating to the sample for three minutes. Sample analysis was conducted using a JEOL-JSM 5600 scanning electron microscope at ×5500 and ×6500 magnifications.

Physiological and Biochemical tests

Utilization of carbon compounds

The strain consumed carbon sources according to Gottlieb (1961). The carbon utilisation assay minimal medium contains KH2PO4 (0.2%), K2HPO4 (0.5%), MgSO4 (0.1%), CuSO4 (0.1%), FeSO (0.1%), MnCl2 (0.1%), ZnSO (0.02%), (NH4)2SO (0.2%), and agar (2%), pH adjusted to 7.0. Arabinose, glucose, fructose, galactose, lactose, maltose, mannitol, starch, sucrose, and xylose were added to molten carbohydrate-free minimum medium at 1% and poured onto sterile Petri plates. The plates were infected with the strain and cultivated at 30°C for 10 days after solidification. The organism grew well, moderately, and poorly on different carbon sources.

Indole test

The Indole test measures the strain's tryptophan-to-indole capability. Make tryptone broth, aliquot it into test tubes, and sterilise at 15 pressure for 15 minutes. A loopful strain culture was injected and cultured at 30°C for 48 hours. After incubation, 10 drops of Kovac's reagent were added and the test tubes were left alone for several minutes. The tubes were checked for cherry red (Holding and Collee, 1971).

Methyl red and Voges-Proskauer (MR-VP) test

In MR-VP broth include glucose, peptone, and phosphate buffer. Mixed-acid fermentation organisms create acid that exceeds the broth's buffering capacity, lowering pH. Other fermentation microbes can not overcome the broth's buffering. The soup received pH indicator methyl red after incubation. Methyl Red was present at pH < 4.4 (positive) and yellow at pH > 6.0. The strain was inoculated in MR-VP broth, incubated at 30 ºC for 48-72 hours, and monitored for colour changes.

Citrate utilization test

The citrate utilization test assessed the strain's ability to use sodium citrate without fermentable glucose or lactose. A loopful bacterial culture was inoculated onto sterilised Simmons citrate agar slants. The medium pH was calibrated at 7. A loopful strain culture was seeded onto an agar slope and incubated at 30°C for 48 hours. Uninoculated slant was control. Slants were inspected for colour changes after incubation. A positive test changes the medium's hue from green to blue, whereas a negative test does not (Holding and Collee, 1971).

Hydrogen sulphide test

The strain's H2S generation was assessed using SIM agar. The medium was prepared and the strain was stab-inoculated. Without strain inoculation, the control condition held. The tubes were kept at 30°C for 48–72 hours to check for colour changes in the stabbed area (Kuster and Williams, 1964).

Melanin Test

The strain's melanin synthesis was tested on ISP-7 medium. Tyrosine agar plates were cultured at 30°C for 5–7 days with the strain. After incubation, the plates were checked for brown or black colour around the colonies, indicating a positive melanin test (Baumann et al., 1976).

Sodium chloride tolerance test

The strain's NaCl tolerance was tested using Tresner et al.'s (1968) technique. After adding NaCl (0-15%) to the ISP-2 medium, it was autoclaved at 15 lbs of pressure (121°C) for 15 minutes. Sterilised material was poured onto Petri plates to solidify. The strain was inoculated onto plates and incubated at 30°C for 7 days to determine its growth at different NaCl concentrations.

Gelatin liquefaction

Nutrient gelatin medium detected gelatin hydrolysis. This medium contains peptone, beef extract, and gelatin (Schaad et al., 1988). The culture was stabbed into the medium under aseptic conditions and incubated at 20°C for 4 days. The test is positive when secreted gelatinases hydrolyse gelatin and liquefy the media. Gelatin digests and loses its gelling property, therefore refrigeration or ice baths keep the medium liquid. Negative results occur if the medium stays solid following cold treatment.

Enzymatic profile of the strain

Amylase

The strain was tested for amylase production on starch agar. At 30°C for 48 hours, the culture-inoculated media was incubated. Gram's iodine solution, a 2:1 combination of potassium iodide and iodine in 300 ml distilled water, was applied to the plate after incubation. A positive zone around the colony on the blue backdrop (Holding and Collee, 1971).

Cellulase
Modified ISP-4 medium with 1% cellulose was used to test the strain's cellulolytic activity. Inoculated plates incubated at 30°C for 7 days. After hydrolysing cellulose with 1 ml of 0.1 N HCl and 5 ml of 2% KI, a yellow zone appeared against a reddish-brown background (Yeoh et al., 1985).

L-asparaginase
The strain was tested for its ability to produce L-asparaginase, which hydrolyses L-asparagine to L-aspartic acid and ammonium ions. The strain was grown on modified M-9 medium with 1% L-asparagine and 0.03% phenol red at 30 ºC for 5 days at pH 7.0. Petri plates were filled with sterilised media to harden. The strain was inoculated on plates and incubated at 30°C for 48–72 hours. Changing the dye colour surrounding the colony from yellow to pink suggests a favorable response, whereas no change indicates a negative reaction (Gulati et al., 1997).

Catalase
Sterilized trypticase soy agar slants were incubated at 30°C for 48–72 hours with the strain. The culture received 3-4 hydrogen peroxide droplets after incubation. The tubes were checked for air pockets (Holding and Collee, 1971).

Chitinase
Colloidal chitin was made using Rojas-Avelizapa et al.'s (1999) technique. Chitin powder (5 mg) was vigorously stirred with 90 cc of strong HCl for 2 hours. Then, 1L distilled water was added. The fine white precipitate was collected by centrifugation at 6,000 rpm for 10 minutes at 4ºC. Regularly rinsing with distilled water brought the colloidal chitin to pH 7. After drying at 50°C, it was used for study. Colloidal chitin (0.5%) was added to the other CYS bouillon components. Inoculating the plates with the strain after 7 days at 30°C revealed a zone of clearing surrounding the actinomycete colony.

Deoxyribonuclease (DNase)
The strain was inoculated on DNAase agar plates to test its DNA-degrading enzyme. After 3-5 days of incubation, plates were flooded with 1 N HCl, and a clear zone surrounding the actinomycete colony shows DNA degradation (Hankin and Anagnostakis, 1975).

Nitrate reductase
Sterilised trypticase nitrate broth was incubated at 30°C for six days with the strain. To detect the colour change, add five drops of reagent A (8 g sulphanilic acid in 1000 ml 5 N acetic acid) and five drops of reagent B (10 g α-naphthalamine in 1000 ml 5 N acetic acid) to the culture vessels after incubation. Cherry red indicates the strain's nitrate reductase production (Holding and Collee, 1971).

Protease
Caseinolysis assessed the strain's proteolytic activity. Starch casein agar media was incubated at 30°C for three days with the strain. A clean zone around the colony passes the test (Holding and Collee, 1971).

Ribonuclease (RNase)
The culture media reported by Jeffries et al. (1957) was used to evaluate the strain's ability to digest ribonucleic acid. A 7.0 pH test medium was created. After carefully dissolving 2 mg/ml RNA in 1 N NaOH, it was added to the liquefied basal medium. Pouring plates began soon after the medium cooled to 50°C. The strain was inoculated onto solidified dishes and cultured at 30°C for 5 days. After saturating plates with 1 N HCl, the actinomycete colony had a clear zone, indicating medium RNA destruction (Hankin and Anagnostakis, 1975).

Urease
Sterilised Christensen agar medium was placed on sterile Petri plates to test the strain's urease activity. The strain was injected at the middle of the agar medium, incubated at 30°C for 48 hours, and checked for a yellow-to-pink colour change around the colonies, indicating a positive response (Holding and Collee, 1971).

Antibiotic sensitivity of the strain by paper disc method
Formulated ISP-2 medium was autoclaved at 15 psi and 121°C for 15 minutes. Aseptically, sterilised media was distributed onto petri dishes and injected with the strain using spread plates. Commercial antibiotic discs were placed on inoculation plates with sterile forceps. The strain's antibiotic sensitivity was tested on 48-hour plates at 30°C (Cappuccino and Sherman, 2004).

RESULTS AND DISCUSSION:

Soil characters

The properties of soil
The sediment samples from the mangrove habitats of Nizampatnam (Station I) and Coringa (Station II) on the southeast coast of Andhra Pradesh, India, were analysed using soil characteristics techniques. The

study found that Station I had 15% moisture, Station II 17%, pH 7.6 and 7.2, organic carbon 7.2 mg/g and 6.8 mg/g, and total nitrogen 4.54 µg/g and 4.92 µg/g, respectively. Actinomycetes thrive in moist (ten to fifteen percent), slightly acidic or neutral pH, organic carbon-rich soils.

This study compared actinobacterial diversity in two mangrove ecosystems. Compared to the Coringa environment, the Nizampatnam mangrove habitat has the most actinobacterial strains, with 31 from station I and 24 from station II. This may be because Nizampatnam mangroves have more nutrients than Coringa mangroves.

The season-actinobacteria distribution study is shown in Fig.1. In two sites, actinobacteria counts peaked in February and dropped in December. Li-Hua xu et al. (1996) observed reduced actinobacteria in cooler climates, supporting our findings. Like Ravi Kumar and Sugandhi (2011), Thondi's actinobacteria count peaked in July and January and dropped in September then October. Actinobacterial populations in mangrove habitats increased during dry and warm seasons.

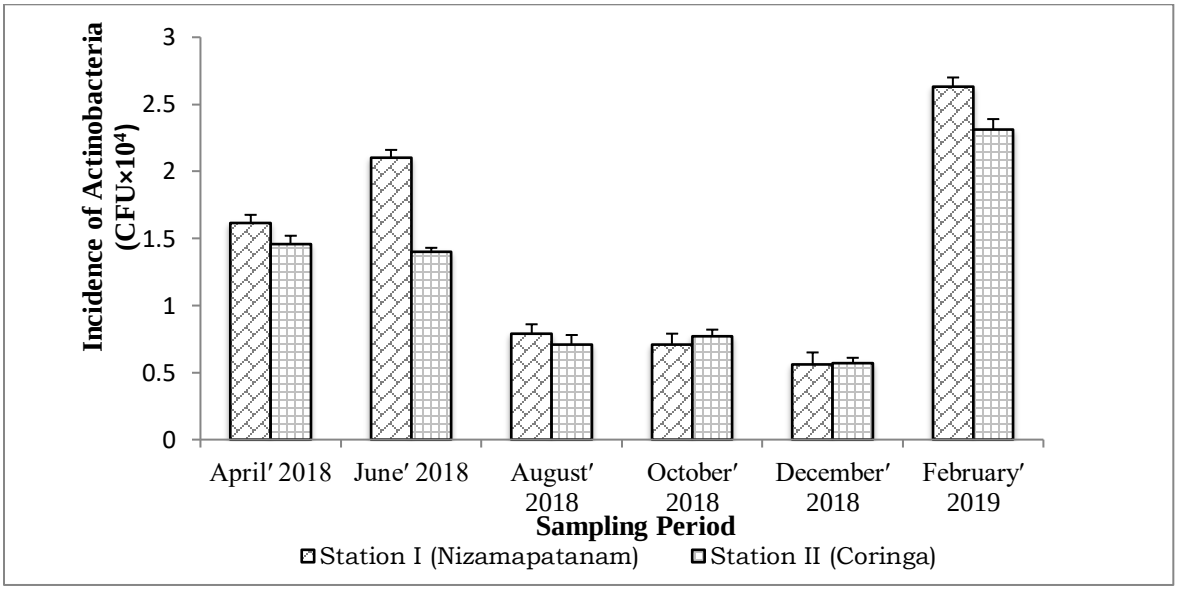
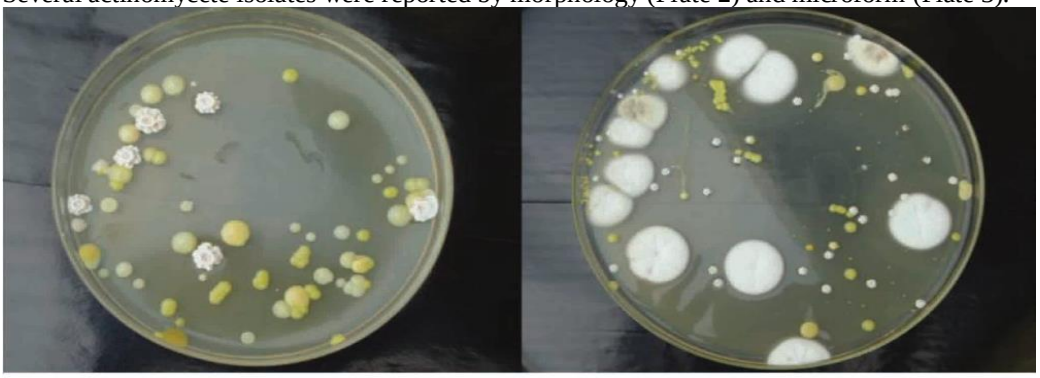


Fig. 1: Incidence of actinobacteria in Nizampatnam and Coringa mangrove ecosystems

Four pre-treatment procedures and three selective media were used to study actinomycetes in Nizampatnam and Coringa mangrove ecosystem. Calcium carbonate pre-treatments had higher actinobacterial counts than dry heat, phenol, and ringer's solution. Starch-casein agar (Plate 1) had the most actinomycete count, followed by asparagine glucose and glycerol-asparagine agar (Table 2). These findings are congruent with Williams and Davies (1965), Mackey (1977), and Anand et al. (2008), who developed starch casein agar medium for actinomycetes isolation and counting. Several actinomycete isolates were reported by morphology (Plate 2) and microform (Plate 3).



(a) (b)

Plate-1: Colonies of Actinomycetes on starch casein agar plates

Table 2: Enumeration of actinobacteria by four different methods of pre-treatment of soil by plating on three different growth media

Station 1 Nizampatnam							
Counts of actinobacteria							
Pre-treatment	Media	April	June	August	October	December	February
P-1 (Dry Heat)	M1	$2x10^4$	$3 x10^4$	$1 x10^4$	0	$1 x10^4$	$2 x10^4$
	M2	$1 x10^4$	$1 x10^4$	$1 x10^4$	$1x10^4$	0	$1 x10^4$
	M3	$1 x10^4$	0	$1 x10^4$	$1x10^4$	0	$1 x10^4$
P-2 (Phenol)	M1	$1 x10^4$	$2 x10^4$	$2 x10^4$	$2x10^4$	$1 x10^4$	$3 x10^4$
	M2	$1 x10^4$	$2 x10^4$	0	0	$1 x10^4$	$3 x10^4$
	M3	$2 x10^4$	$1 x10^4$	0	0	0	$1 x10^4$
P-3 ($CaCo_3$)	M1	$1 x10^4$	$2 x10^4$	$1 x10^4$	$1 x10^4$	$1 x10^4$	$4 x10^4$
	M2	$1 x10^4$	$3 x10^4$	$2 x10^4$	$1 x10^4$	0	$3 x10^4$
	M3	$2 x10^4$	$2 x10^4$	0	0	$1 x10^4$	$2 x10^4$
P-4 (Ringers Solution)	M1	$2 x10^4$	$2 x10^4$	$1 x10^4$	$1 x10^4$	0	$4 x10^4$
	M2	$1 x10^4$	$2 x10^4$	0	0	0	$1 x10^4$
	M3	0	$1 x10^4$	$1 x10^4$	0	0	$2 x10^4$
Station 2 Coringa							
Counts of actinobacteria							
Pre-treatment	Media	April	June	August	October	December	February
P-1 (Dry Heat)	M1	$2 x 10^4$	$3 x10^4$	$1 x10^4$	$1 x10^4$	$1x10^4$	$3 x10^4$
	M2	$2 x10^4$	$1 x10^4$	0	$1 x10^4$	$1 x10^4$	$2x10^4$
	M3	$1 x10^4$	$1 x10^4$	$1 x10^4$	0	0	$2 x10^4$
P-2 (Phenol)	M1	$2 x10^4$	$1 x10^4$	0	$1 x10^4$	$1 x10^4$	$2 x10^4$
	M2	0	$1 X10^4$	$1 X10^4$	$1 X10^4$	0	$3 X10^4$
	M3	$1 x10^4$	$1 x10^4$	$1 x10^4$	0	$1 x10^4$	$1 x10^4$
P-3 ($CaCo_3$)	M1	$2 x10^4$	$2 x10^4$	$1 x10^4$	$1 x10^4$	0	$4 x10^4$
	M2	$2 x10^4$	$2 x10^4$	$2 x10^4$	0	$1 x10^4$	$3 x10^4$
	M3	$1 x10^4$	$1 x10^4$	0	$1 x10^4$	$1 x10^4$	$2x10^4$
P-4 (Ringers Solution)	M1	$1 x10^4$	$2 x10^4$	$1 x10^4$	$2 x10^4$	0	$2 x10^4$
	M2	$2x10^4$	$1 x10^4$	0	$1 x10^4$	0	$2x10^4$
	M3	0	$2 x10^4$	$1 x10^4$	0	$1 x10^4$	$1x10^4$

M1- Starch casein agar; M2- Asparagine glucose agar; M3- Glycerol asparagine agar

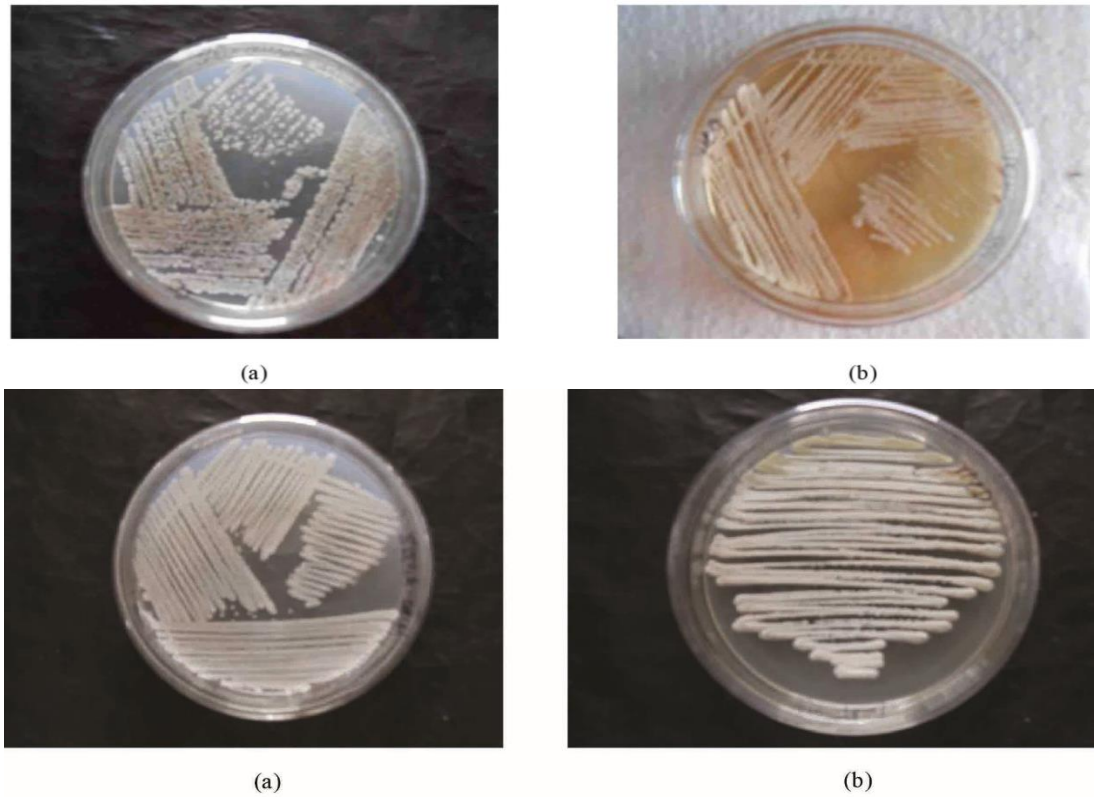


Plate-2: Colony morphology of isolated actinomycetes

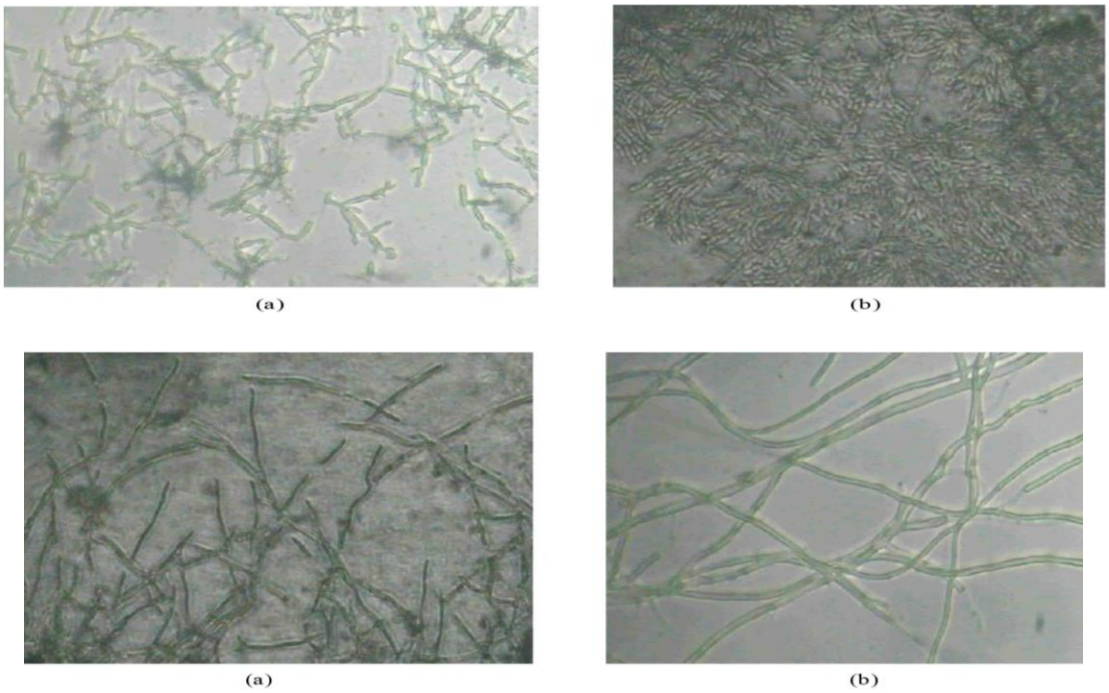


Plate-3: Micro morphology of isolated actinomycetes

Bioactive chemical production was assessed in actinomycete isolates. 28 of 55 strains had antimicrobial activity; 19 were effective against all pathogens, including S. aureus, E. coli, and C. albicans; four were active against both, and five against both (Table 3). Antibacterial activity was absent in 27 isolates. Many studies have demonstrated that actinobacteria fight many human illnesses. Rameshe (2006) found 111 antibacterial compounds in 208 Bay of Bengal actinobacterial species. Mitra et al. (2008) identified several actinobacteria from the Sunderban mangrove ecosystem, with 50.84% being antibacterial. According to Remya and Vijaykumar (2008), 32.8% of 64 actinobacterial strains from marine and mangrove sediments on India's West coast were antibacterial.

Table 3: Screening of mangrove actinomycetes for antimicrobial activity (Inhibition zone represented in mm)

S.NO	Isolate	*Staphylococcus aureus*	*Escherichia coli*	*Candida albicans*
1	RM-4	12	12	11
2	RM-7	14	11	10
3	RMK-8	-	10	14
4	RM-10	19	18	17
5	RM-12	15	16	12
6	RM-15	17	14	13
7	RM-17	15	12	12
8	RM-18	15	13	-
9	RM-19	-	15	13
10	RM-21	18	11	15
11	RM-24	19	17	12
12	RM-27	14	12	10
13	RM-28	20	12	-
14	RM-29	-	12	11
15	RM-31	18	15	-
16	RM-A	19	17	15
17	RM-B	15	17	15
18	RM-C	17	15	15
19	RM-D	17	16	16
20	RM-F	17	18	15
21	RM-H	17	14	11
22	RM-I	15	10	10
23	RM-K	16	12	12
24	RM-N	18	15	13
25	RM-O	-	13	12
26	RM-R	16	14	-
27	RM-S	15	12	10
28	RM-T	12	12	-

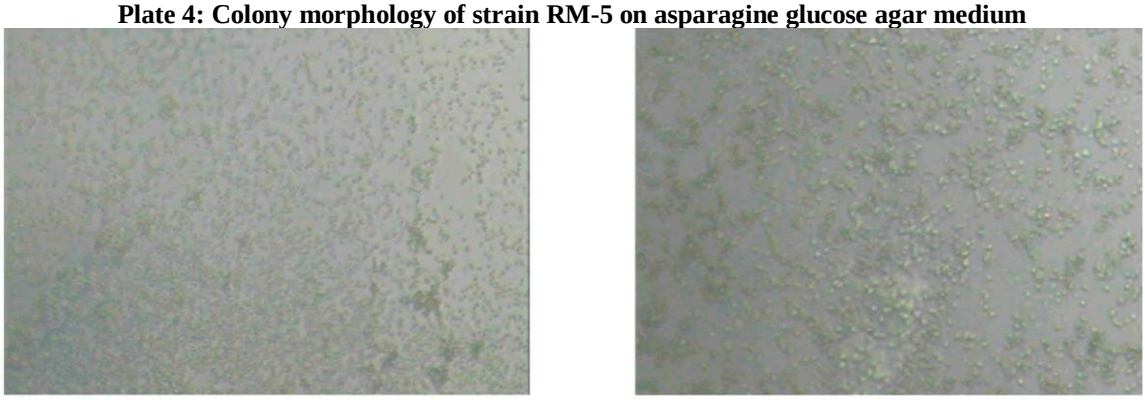
**Isolates from Nizampatnam represented in numbers while from Coringa in alphabets*

Taxonomic characterization of strain RM-5

In our search for new actinomycetes-derived antimicrobial metabolites and therapeutic enzymes in the Nizampatnam and Coringa mangrove area, we isolated the dominant and morphologically unique isolate RM-5 on asparagine glucose agar medium using the soil dilution plate method (Plate 4). The strain RM-5 has Pseudonocardia-like morphology (Warwick et al., 1994; Huang et al., 2002). Slide culture was utilised to analyse the strain's micro-morphology on ISP-2 medium by light microscopy (Plate 5). Morphological and micro-morphological study of the strain showed abundant, well-developed, fragmented aerial and vegetative hyphae with rod-shaped, smooth-surfaced spores. The strain produced no soluble pigments in the culture medium, except for melanin on tyrosine (ISP-7) agar. Plate 6 shows the strain's sporophore morphology (SEM images at x 4,500 and x 6,500).

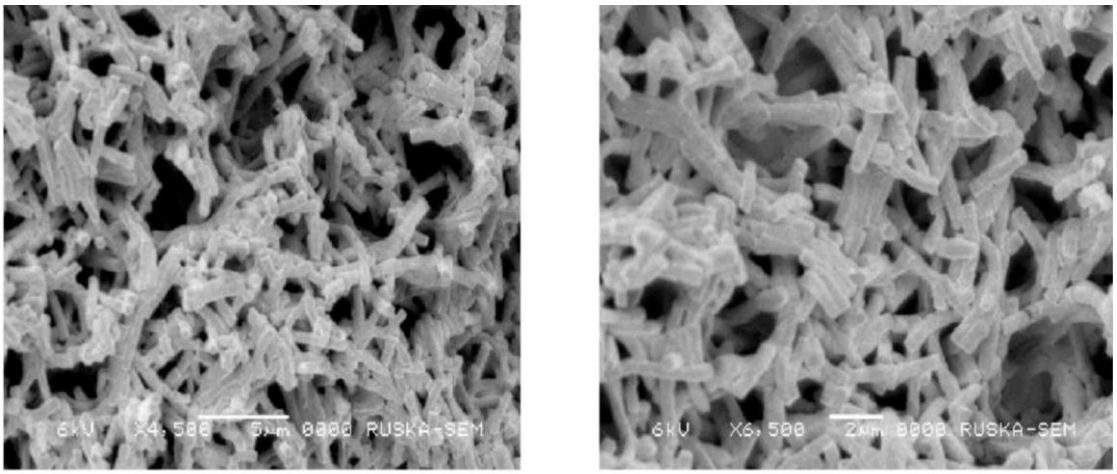
(a) (b)

Plate 4: Colony morphology of strain RM-5 on asparagine glucose agar medium



(a) (b)

Plate 5: Micro morphology of the strain RM-5 by Slide culture technique



(a) (b)

Plate 6: Scanning electronic microscopic images of the actinomycete strain RM-5 at different magnifications (a) x 4,500 (b) x 6,500

Table 4 shows strain RM-5's cultural features. Strain RM-5 grew well on ISP-1, ISP-2, ISP-3, ISP-5, Czapek-Dox, and maltose-tryptone. On ISP-4, ISP-7, and starch casein agar, it grew effectively but not on nutrition. The aerial mycelium on ISP-1, ISP-2, ISP-7, maltose tryptone, and starch casein agar was creamy white. It was creamy orange on Czapek-Dox agar and cream-coloured on ISP-4 and ISP-5. Nutrient agar did not support aerial mycelium. ISP-1 and ISP-2 substrate mycelium is orange-red. ISP-3,-4,-5, and-7 are brown. It is light orange on Czapek-Dox, maltose tryptone, and nutrient agar. Siva Kumar (2001) suggested using culture features to identify strains. Classifying and identifying actinomycetes and altering their development rate need physiological testing. The bacteria grew from pH 6 to 9, with best growth at pH 7. The ideal development temperature was 35°C, while 20–45°C was possible. As per Tresner et al. (1968), the strain showed intermediate salt tolerance up to 10%, with optimum growth at 3% NaCl (Table 5).

Biochemical Characterization

RM-5 tested positive for catalase and citrate expenditure but negative for urease, hydrogen sulphide generation, nitrate reduction, starch hydrolysis, gelatin liquefaction, indole, methyl red, and Voges-Proskauer. Morphological, physiological, and biochemical characteristics of the isolate are reported in Table 5.

Table 4: Cultural characteristics of strain RM-5

S.No	Medium	Growth	Aerial	Substrate	Pigmentation
1	Tryptone yeast-extract agar (ISP-1)	Good	Creamy white	Brownish orange	Nil
2	Yeast extract malt extract dextrose agar (ISP-2)	Good	Creamy white	Brownish orange	Nil
3	Oat-meal agar(ISP-3)	Good	White	Brown	Nil
4	Inorganic salts starch agar (ISP-4)	Good	White	Brown	Nil
5	Glycerol asparagine agar (ISP-5)	Good	Cream	Brown	Nil
6	Tyrosine agar(ISP-7)	Moderate	Creamy white	Brown	Melanin
7	Starch-casein agar	Moderate	Creamy white	Light brown	Nil
8	Czapek - Dox agar	Good	Creamy white	Light range	Nil
9	Maltose tryptone agar	Good	Creamy white	Light range	Nil
10	Nutrient agar	Good	Creamy white	Orange	Nil

Table 5: Morphological, Physiological, and biochemical characteristics of strain RM-5

Character		Response
S. No	**Morphological characters**	
1	Mycelial form	
2	Sporophore morphology	Fragmentation
3	Color of aerial mycelium	Creamy white
4	Color of substrate mycelium	Brownish orange
Physiological characters		
5	Gram reaction	+
6	Acid-fast reaction	-
7	Production of melanin pigment	+
8	Range of temperature for growth	25-50°C
9	Optimum temperature for growth	30 °C
10	Range of pH for growth	5-9
11	Optimum pH for growth	7
12	NaCl tolerance	10%
Biochemical characters		
13	Catalase production	+
14	Urease production	-
15	Hydrogen sulphide production	-
16	Nitrate reduction	-
17	Starch hydrolysis	-
18	Gelatin liquefaction	-
19	Methyl red test	-
20	Voges proskauer test	-
21	Indole production	-
22	Citrate utilization	+
23	Casein hydrolysis	-

Carbon source use by strains may assist identify species (Pridham and Gottlieb, 1948). D-glucose, lactose, maltose, sucrose, galactose, fructose, and starch were utilised by strain RM-5, but not xylose, arabinose, or mannitol (Table 6). The strain generated commercially significant enzymes including amylase, cellulase, chitinase, and L-asparaginase but not protease. Plate 7 and Table 7 show DNase and RNase. Goodfellow and Orchard (1980) suggested using antibiotic susceptibility to classify certain nocardioform bacteria. Susceptibility tests showed that the isolate was susceptible to streptomycin, gentamicin, rifampicin, chloramphenicol, and oxytetracycline but resistant to penicillin and nalidixic acid (Table 8).

Table 6: Utilization of the carbon sources by the strain RM-5

S. No	Carbon source	Response
1	Lactose	++
2	Maltose	++
3	Xylose	-
4	Sucrose	++
5	Arabinose	-
6	D-Glucose	+++
7	Galactose	++
8	Fructose	++
9	Starch	+
10	Mannitol	-

Excellent (+++); Moderate (++); Poor (-).

Table 7: Enzyme activity of the actinomycetes strain RM-5

Enzymatic activity	Result
Amylase	+
Cellulase	+
Chitinase	+
L-Asparginase	+
Protease	-
RNase	-
DNase	-

+ = Positive; - = Negative

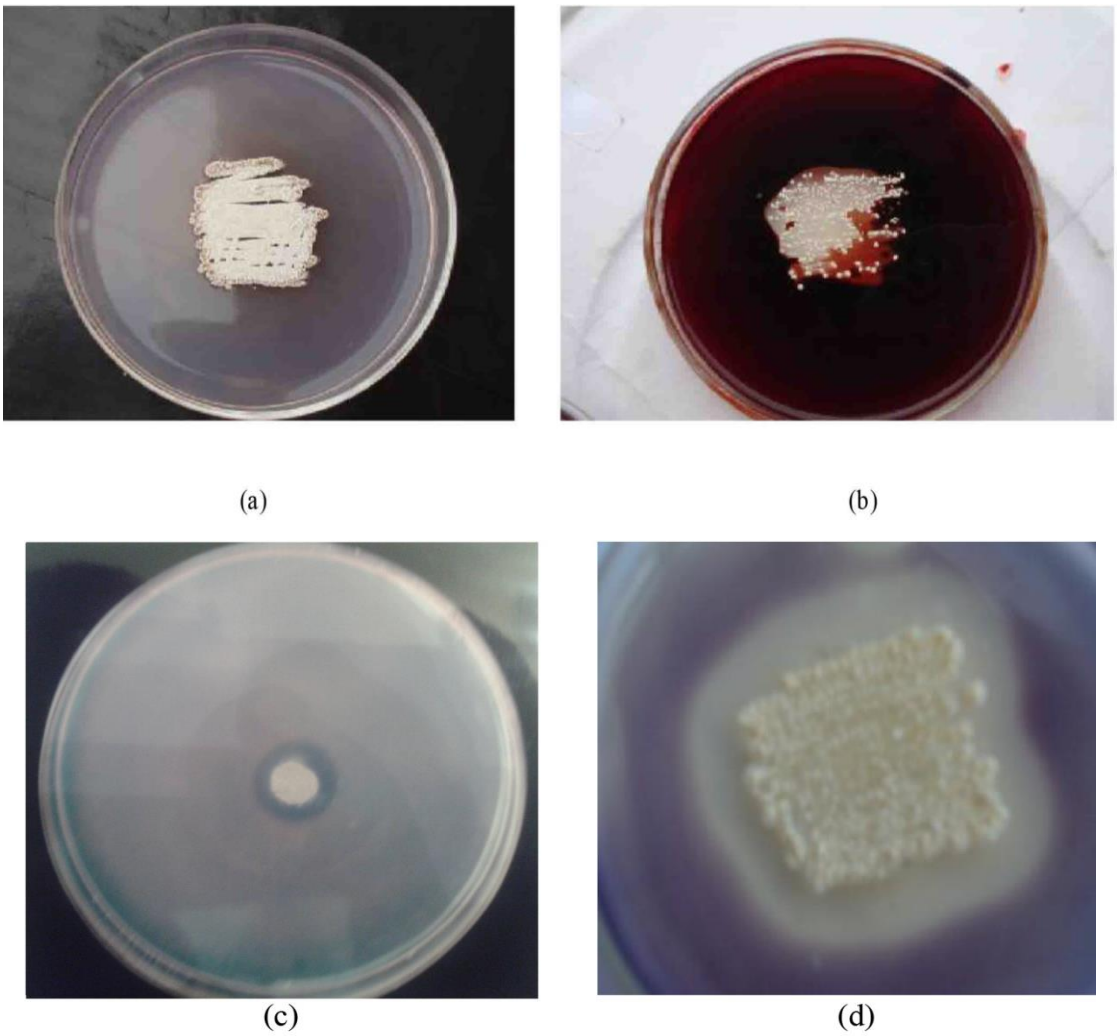


Plate 7: Screening of the actinomycete strain RM-5 for the production of enzymes.

(a) L-asparaginase; (b) Cellulase; (c) Chitinase; (d) Amylase

Table 8: Antibiotic susceptibility testing of RM-5 (µg/disc)

Susceptibility to Antibiotics: (µg/disc)		
1	Nalidixic acid (30)	R
2	Streptomycin (10)	S
3	Penicillin (10)	R
4	Chloramphenicol (30)	S
5	Rifampicin (30)	S
6	Oxytetracycline (10)	S

R-Resistant; S-Sensitive

CONCLUSION:

Most medications nowadays are natural secondary metabolites. In recent decades, microorganisms have been shown to store bioactive chemicals. Actinobacteria manufacture secondary metabolites most efficiently. The rapid spread of epidemic illnesses, drug-resistant microorganisms, and the need for effective anti-cancer drugs need the development of effective antibiotics. Several actinobacterial species have been studied for bioactive chemical production from terrestrial environments in recent decades. Thus, scientists must explore unexplored marine habitats to isolate novel actinobacteria that produce novel carbon skeletons with new biological activities. This has shifted biotechnologists' emphasis from discovery to new secondary metabolite habitats. Actinobacteria in mangroves are diverse and new. This research investigates how a unique actinomycete bacterium in Southeast Coastal Andhra Pradesh mangroves manufactures L-asparaginase.

Sediment samples from Nizampatnam (Station I) and Coringa (Station II) mangrove habitats were analysed using standard procedures. Parameters recorded included moisture content (15%), 17% (Station II), pH (7.6 and 7.2), organic carbon (7.2 and 6.8), and total nitrogen (4.54 and 4.92 µg/g). Air-dried sediment samples were pre-treated with four methods and plated on three selective medium employing soil dilution to extract and count actinomycete colonies (Yi., 2019). Calcium carbonate pre-treatments had higher actinobacterial counts than dry heat, phenol, and ringer's solution. Compared to asparagine glucose agar (AGA) (Smith, 1943) and glycerol-asparagine agar (GAA) (Waksman, 1961), starch-casein agar media (Wellington and Cross, 1961) promotes actinomycete growth. Nizampatnam mangroves yielded more actinobacterial strains than Coringa. Purified actinomycete strains were cultivated on yeast extract-malt extract-dextrose (ISP-2) agar.

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