Comparative Study on Isolation and Characterization of Actinomycetes from Terrestrial and Marine Environments

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Abstract: Actinomycetes are diversely present in both terrestrial and marine environment. Most of the actinomycetes are known to produce secondary metabolites, their production varies with the species level and the culture condition. This study is aimed to explore the diversity and bioactive potential of actinomycetes isolated from distinct environments, specifically marine and terrestrial samples collected from the Bellary's mine and estuary sand in Mangaluru, India. The isolation process involved pre-treatment of samples followed by cultivation on selective media, resulting in the successful identification of nine actinomycete strains. Morphological characterization revealed variations in colony colour and mycelial structure, while Gram staining confirmed their Gram-positive nature. Biochemical tests further elucidated their metabolic capabilities, indicating potential for antibiotic production. The results of this study not only contribute to the understanding of actinomycete diversity across different ecosystems but also emphasize their promising applications in developing novel antimicrobial agents, justifying further research into their biotechnological potential.

Keywords: Actinomycetes, Serial dilution, Morphological characterization, Mycelia, Gram's staining, Biochemical Tests.

I. INTRODUCTION

Actinomycetes are the 'ray fungi' with both the characteristics of bacteria and fungi. These organisms are present in various environment like deserts (Kumar *et al*., 2021), Himalayan region (Salaria and Furhan, 2021), marine (sediments, water, mangrove, seaweed, corals, sponges) (Ribeiro *et al*., 2020; Wang *et al*., 2020), terrestrial regions and plant root exudates. Microorganisms isolated from the marine environment has gained much attention due to the production of natural compounds with great applications. The deep sea has distinctive characteristics like low temperature, less availability of light, high pressure, high salinity and lack of oxygen and variation in oxygen concentration (Bull *et al*., 2000). Actinomycetes interact with various marine organisms like sponges (Abdelmohsen *et al*., 2010), corals, seaweeds, seagrasses, sea vertebrates (Wu *et al*., 2005) etc., and this may influence the chemical structure of metabolites (Ghanem *et al*., 2000). Most of the actinomycetes are isolated from the marine sediments, deep sea sediments than from sea water while actinomycetes isolated from soil samples include rhizosphere samples, surface soil samples, cave sediment samples etc. Isolating actinomycetes requires culturable conditions, specific parameters and specific media (Adam *et al.*, 2018). According to Bredy *et al*., 2005, about one fourth of the secondary metabolites are produced by actinomycetes. Among the actinomycetes species, *Streptomyces* spp. has produced about 7600 secondary metabolites and the discoveries of new bioactive compounds from actinomycetes are increasing. Actinomycetes are also known for their medical uses, they are the primary produces of various antibiotics. About 75% of the antibiotics present are obtained from *Streptomyces* spp. and rest are from non-*Streptomyces* spp., also other organisms like bacteria, fungi (Bredy, 2005). *Streptomyces* sp. are known to be the principle producers of antibiotics (Hasani *et al*., 2014). These biocatalysts (enzymes) help in degrading complex organic matters. Various enzymes such as amylase, protease, lipase, tyrosinase, cellulose, esterase and L- asparaginase are produced by marine actinomycetes (Mobeen *et al*.,

2018). Discovering novel bioactive chemicals with promising applications in the biotechnology and pharmaceutical industries requires investigating the habitats and comprehending the adaptations of actinomycetes in various maritime and terrestrial settings.

Recent studies are focusing on the advantages obtained from the marine ecosystem and mainly on medical and pharmaceutical compounds. The present study was planned to isolate actinomycetes from terrestrial and marine samples and check physiological characterization of the actinomycetes isolates.

II. MATERIALS AND METHODS

A. Sample collection

Samples were collected from Bellary mines and Sasihithulu beach, Mangaluru region. Marine sand samples were collected from estuaries and the terrestrial soil sample was collected from mine area. Terrestrial sample was collected from soil which was dug 5 -10cm beneath the soil surface (Jensen *et al*., 1991; El-Nakeeb and Lechevalier, 1993). Both the sample was collected in sterile polyethylene zipper bags and brought to the laboratory for further isolation process.

B. Pre-treatment of the samples

The collected samples were pre-treated before isolation process. Collected sediment and soil samples were air dried in laboratory of 24hrs before inoculation (Gebreselema *et al*., 2013) and they were also treated with calcium carbonate (El-Nakeeb and Lechevalier, 1993), inoculated on actinomycetes selective media.

C. Isolation of marine and terrestrial actinomycetes

Three different methods of isolation included direct plate method, spread plate method and pour plate method. Starch casein agar was used for the isolation of actinomycetes from sand and starch nitrate agar medium was used for isolating actinomycetes from soil samples (Williams *et al*., 1993). Serial dilution was done by mixing 1gm of sand sample in 9ml of distilled water and dilution up to 10^{-7} was prepared. Dilution 10^{-3} and 10^{-4} were used for spread plate and pour plate method whereas for direct plate method a pinch of fine sand sample was sprinkled on the media plate, incubated at room temperature for 5-7 days. Powdery colonies are sub-cultured in triplicates and stored in refrigerator for further work.

D. Phenotypic characterization of marine actinomycetes

Morphology of the isolated marine actinomycetes was observed using selective media like Starch casein agar and Starch nitrate agar. Morphological, cultural characteristics of the isolates were compared with Bergey's Manual of Systematic Bacteriology Volume 5 (Goodfellow *et al*., 2012) and biochemical tests was performed using standard protocols (Cappuccino and Sherman, 2010). Gram's staining was done and observed in 100x magnification light microscope to identify the isolates.

E. Gram's staining:

Gram's staining of actinomycetes isolates was carried out as mentioned in Benson (2002) manual. A thin smear of the isolate was prepared on the clean glass slide and heat fixed. The smear was stained with primary stain, crystal violet and was kept for 60 seconds. The primary stain was washed with distilled water and stained with mordant stain, Gram's iodine and was kept for 30 seconds. Stain was washed with distilled water and decolorizing agent, 2-3 drops of 95% ethanol was used to wash the smear. The smear was counter stained with safranin, was kept for 30 seconds and rinsed with distilled water. The slide was observed under microscope at various magnification.

F. Starch hydrolysis test:

Starch hydrolysis test was performed to check the ability of actinomycetes isolates to breakdown starch. Starch agar media (0.2% starch was added to the Nutrient agar media) was for this test. The isolate was inoculated into the medium and incubated for 4 days at 37^0C. After incubation, the culture plates was flooded with iodine crystals and was shook. The halo zone formed around the colony showed the hydrolysis of starch by the isolate (Benson, 2002).

G. Citrate utilization test:

This citrate test was used to screen the ability of the actinomycetes isolate to utilize citrate as sole source of carbon. Simmon's citrate agar medium was used in this test and the isolates were inoculated on the medium slants and incubated for 4 days at 37^0C. After incubation, if it resulted in colour change from green to blue then indicates citrate was utilized by the isolates (Collee *et al*., 1996).

H. Triple Sugar Iron (TSI) Test:

For this test, Triple sugar iron agar medium is used and it is also a differential media. TSI test is used to determine the carbohydrate fermentation (glucose, sucrose, lactose) and hydrogen sulphide production by the isolates. Isolates were inoculated to TSI slants and incubated for 4 days at 37^0C. After incubation, the colour change from orange-yellow to pink or red colour indicates the carbohydrate fermentation and black colour at the butt or slant region shows hydrogen sulphide production (Cappuccino and Sherman, 2010).

I. Urease Test:

Urea broth (Stuart's Medium) which contain 2% urea and phenol red as a pH indicator, is used for urease test. This test is performed to check the capability of the isolates to hydrolyse urea to produce ammonia and carbon dioxide. Isolates were inoculated into urea broth and incubated for 4 days at 37^0C. After incubation, if results in colour change from orange-yellow to pink is considered a positive for urea hydrolysis (Cappuccino and Sherman, 2010).

J. Catalase test:

This is enzyme- based test used to identify catalase enzyme producing isolates. Here catalase test, hydrogen peroxide breakdown into oxygen and water and forms bubbles when organism is inoculated. The formation of bubbles on the slide of cultural plate on addition of hydrogen peroxide is a positive result for catalase enzyme production (Collee *et al*., 1996).

K. Carbohydrate fermentation test:

Carbohydrate fermentation test is performed to detect the ability of the isolates to ferment different sugars. Fermentation broth with addition of 5-10% of different sugars and phenol red as pH indicator along with Durham's tube which is used for the gas production. Isolates were inoculated to the fermentation broth and incubated for 4 days at 37^0C. After incubation, it broth changed its colour from orange-yellow to yellow shows the carbohydrate fermentation has taken place and bubble formation inside Durham's tube indicates gas production (Cappuccino and Sherman, 2010).

III. RESULT AND DISCUSSION

Isolation of actinomycetes from different environment has its own advantages. The ecosystem in which actinomycetes are found places a very important role in the secretion and production of various bioactive compounds. Characterization of the microorganisms also play an important role in studying their ecological role (Sharma *et al*., 2020). In the present study, actinomycetes colonies was isolated from marine estuary sand sample collected at Sasihithulu beach and mines soil sample collected at Bellary mines. Total nine actinomycetes was isolated on starch casein agar and starch nitrate agar (Fig 1). Isolates was morphologically characterized by observing the aerial mycelial colour and substrate mycelial colour (Table 1). Most of them was grey colonies and had yellow substrate mycelia. Two of the isolates, RB10 and RB11 produced diffusible pigment. Gram's stained isolates was observed under microscope at 100x magnification and the isolates were Gram positive, purple thread like structure was observed (Fig 2). They were further physiologically characterizes by biochemical tests which included citrate utilization, triple sugar iron test, urease, catalase, starch hydrolysis (Fig 3) and carbohydrate fermentation for different sugars like sucrose, maltose, xylose, raffinose and rhamnose. Sucrose was most fermented by the isolates and rhamnose was the least fermented by the isolates. RLNBT1 showed negative for all the five sugar fermentations and the biochemical analysis results are shown in Table 2. These test results are the major keys to identify the microorganisms and each isolates showed its own characteristics and how different they are from each actinomycetes that are isolated.

A similar study by Sapkota *et al*., (2020) reported forty one actinomycetes isolates were isolated from the soil sample collected from different regions of Nepal. The isolates were characterized and identified by morphological, physiological, sugar utilization, protein utilization and hydrolysis tests study. Further characterization study included the optimization, where the growth of isolates were observed at different temperature, pH. Isolates were identified as they belong to the species of *Streptomyces* (70.7%), *Nocardia* (19.5%) and *Micromonospora* spp (9.5%). Total 11 isolates produced diffusible pigment and among them yellow pigment was observed to be more prominent. In the previous study, seawater samples was collected from Gujarat marine region and isolated twenty haloalkaliphillic actinomycetes. On the basis of morphological characterization, physiological characterization and phylogenetic

characterization by 16S rRNA sequencing the twenty isolates was identified and belonged to *Nocardiopsis* sp (Sharma *et al*., 2021). This *Nocardiopsis* sp. was isolated for the first time in the Arabian Sea coast and which is very rare, as this particular species is highly found in Pondicherry coast, Bay of Bengal (Suthindhiran and Kannabiran, 2010).

IV. CONCLUSION

The study involved a systematic isolation process that included pre-treatment of samples and cultivation on selective media, resulting in the identification of nine distinct actinomycete strains. Morphological characterization revealed variations in colony colour and mycelial structure, while Gram staining confirmed their Gram-positive nature. Biochemical tests further elucidated their metabolic capabilities, indicating a potential for antibiotic production. The results contribute to the understanding of actinomycete diversity across different ecosystems and emphasize their promising applications in developing novel antimicrobial agents. The study highlights the need for further research into the biotechnological potential of these microorganisms, particularly in the context of natural compound production for various applications.

V. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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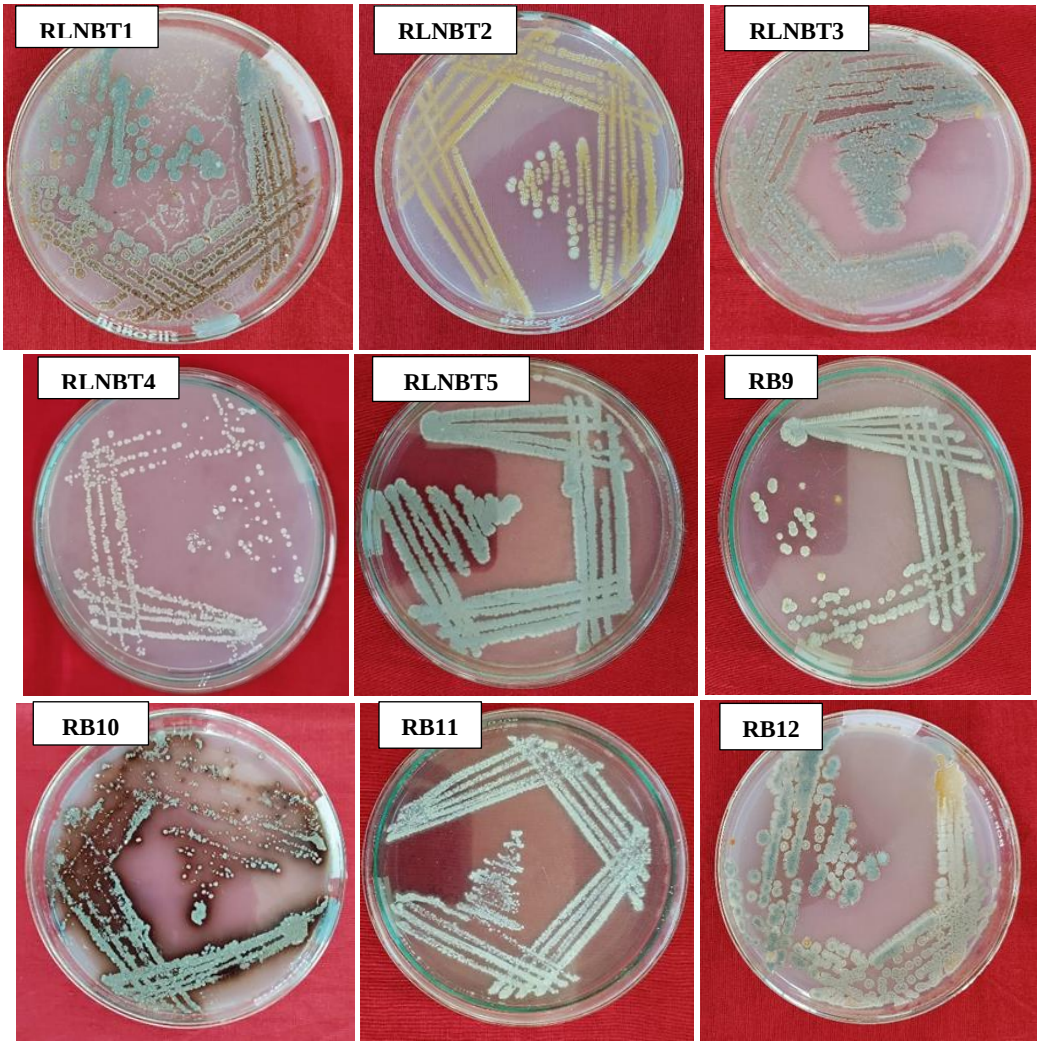

Fig 1: Actinomycetes isolates of terrestrial and estuary sample.

Table 1: Colony morphology of the actinomycetes isolates.

Isolates	Aerial mycelia	Vegetative mycelia
RLNBT1	Grey	Pale yellow
RLNBT2	White	Cream
RLNBT3	Grey	Pale yellow
RLNBT4	White	Cream
RLNBT5	Grey	Cream
RB9	Greyish-white	Yellow
RB10	Grey	Black
RB11	White	Brown
RB12	Greyish- white	Yellow

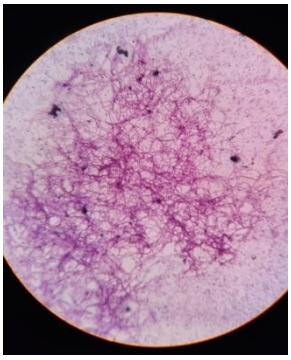

Fig 2: Gram's staining of the isolate RLNBT3 observed at 100x magnification

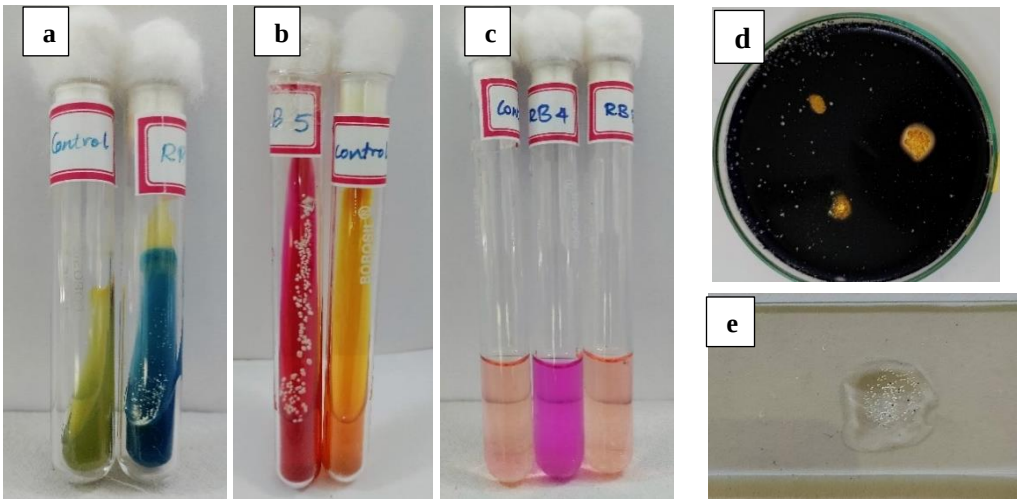

Fig 3: Biochemical tests- a) Citrate utilization; b) Triple Sugar Iron (TSI); c) Urease test; d) Starch hydrolysis; e) Catalase test.

Table 2: Physiological characterization of actinomycetes isolates.

Biochemical tests	RLNBT 1	RLNBT 2	RLNBT 3	RLNBT 4	RLNBT 5	RB 9	RB 10	RB 11	RB 12
Starch hydrolysis	+	+	+	+	+	+	+	+	+
Citrate utilization	+	+	+	-	+	-	+	+	-
Triple sugar iron test	+	+	-	-	+	+	-	+	+
Urease test	+	-	+	+	-	+	-	-	+
Catalase test	-	+	-	+	-	+	+	-	+
Carbohydrate fermentation									
Sucrose	-	+	+	+	-	+	+	+	+
Maltose	-	-	+	-	+	+	-	+	-
Xylose	-	+	+	+	+	-	+	+	+
Raffinose	-	-	-	+	+	+	+	-	+
Rhamnose	-	-	-	+	-	+	+	-	+

+: Positive; -: Negative