Isolation and Molecular Characterization of Actinomycetes from Coastal Sediments of The Sundarbans

A Thesis Submitted by

Sharnali Das

Examination Roll – MS-180717

Session – 2017-2018

Submitted to Biotechnology and Genetic Engineering Discipline, Life Science School, Khulna University, Khulna-9208, Bangladesh in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology and Genetic Engineering.

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PREFACE

This research effort is hereby dedicated to my parents who have both made immeasurable sacrifices to support and inspire my passion for a career in biological sciences. May this be a small first step to their labor coming to fruition.

DECLARATION

This thesis paper is the original research work of the author Sharnali Das, except as otherwise appropriately cited. The materials presented has not been submitted either in whole or in part for a degree for this or any other university. The methods used were designed and implemented according to institutional and established international biosafety and bioethical guidelines. I also declare that the findings of the research have not been published or disseminated otherwise without the concurrence of the concerned supervisor.

Sharnali Das

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The Author

March,2022

ABSTRACT

Actinomycetes are unicellular organisms found in various environments across the world. It is a large order of bacteria and are potential source of a wide range of biologically active compounds. This study was conducted to isolate, identify and screen for potential antibacterial activity of Actinomycetes from coastal sediments of the Sundarbans. A total of nine isolates were screened which were subsequently subjected for 16s-rRNA gene subsequently. Eight isolates were found to be Actinomycetes (6 *Streptomyces* spp., 1 *Nocardia* spp., 1 *Cellulosimicrobium* spp.). *C.funkei* and *S.labedae* were selected for extraction of secondary metabolites to investigate the antibacterial and antioxidant potentials. Both isolates demonstrated moderate antioxidant potential in DPPH free radical scavenging assay compared to standard ascorbic acid. Extracts from both bacteria exhibited strong antibacterial activity against four pathogenic bacteria and their MIC value ranged from 0.25 to 2.0 mg/mL. *C.funkei* extract showed the highest antibacterial activity (MIC value of 0.0024mg/mL) against *Vibrio cholerae*. This study showed that the Sundarbans are rich in Actinomycetes specially *Streptomycetes* sp. Furthermore, Actinomycetes from coastal sediments of Sunderbans could be potential sources of antimicrobial agents.

Key words: Actinomycetes, 16s rRNA gene, antimicrobial activity, coastal sediments

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List of Abbreviations

Full form	Abbreviations
Milligram	mg
Microliter	µl
Minimum inhibitory concentration	MIC
Milliliter	ml
Water	H_2O
2,2-diphenyl-1-picrylhydrazyl	DPPH
50% Effective concentration	EC50
National Center for Biotechnology Information	NCBI
Polymerase Chain reaction	PCR
Tris base, acetic acid and EDTA	TAE
Degree celcius	°C
Rotation per minute	rpm
Ultraviolate	UV

1. Introduction:

1.1 Background

Actinomycetes are part of the microbial flora of most natural substrates (Nakeeb et al., 1963). Many are well known for their economic importance as producers of biologically active substances (Basilio et al., 2003). Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic, and enzymes like cellulase and xylanase used in waste treatment. Of these compounds, antibiotics predominate in therapeutic and commercial importance (Oskay et al., 2004). Over 5,000 antibiotics have been identified from the cultures of Gram-positive and Gram-negative organisms and filamentous fungi but only about 100 antibiotics have been commercially used to treat human, animal and plant diseases. The genus, *Streptomycetes*, is responsible for the formation of more than 60% of known antibiotics while a further 15% are made by a number of related *Actinomycetes* (Basavaraj et al., 2010). Though there is a large number of antibiotics produced from microorganisms, the demand for new antibiotics continues to grow due to the rapid emergence of antibiotic resistant pathogens causing life threatening infections in spite of considerable progress in the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds. This changing pattern of diseases and the emergence of resistant bacterial strains to currently used antibiotics continuously puts demand on the drug discovery scientists to search for novel antibiotics. Actinomycetes perform significant biogeochemical roles in terrestrial soils and are highly valued for their unparalleled ability to produce biologically-active secondary metabolites (Selvameenal et al., 2009) such as antibiotics.

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Most antibiotics in clinical use are direct natural products or semisynthetic derivatives from actinomycetes or fungi (Genolloud., 2017). Actinomycetes are attractive, bodacious and charming filamentous gram-positive bacteria, are the main source of clinically important antibiotics, most of which are too complex to be synthesized by combinatorial chemistry (Leng., 1998). Antibiotics has been used in many fields including agriculture, veterinary and pharmaceutical industry. Additional actinomycetes produced antibiotics likely could be discovered by subjecting soils and marine sediments to innovative enrichments and whole-cell screening methods. Combinatorial biosynthesis methods can generate derivatives of complex antibiotics and other secondary metabolites that would be difficult to generate using medicinal chemistry. Sequencing actinomycete genomes may provide insights useful in devising novel antimicrobial agents. Investigations can possibly reveal actinomycetes species that produce novel antibiotics. It is anticipated that the isolation, characterization and the study on actinomycetes can be useful in the discovery of antibiotics and novel species of actinomycetes (Oskay et al., 2004). Investigations can possibly reveal actinomycetes species that produce novel antibiotics. The isolation, characterization and the study on actinomycetes can be useful in the discovery of antibiotics and novel species of actinomycetes producing antibiotics (Tiwari and Gupta, 2012). So, this study will isolate, characterize and identify the antibiotic producing actinomycetes.

This research works hope to reveal the scientific basis of antibacterial compounds of actinomycetes under study and thus contribute to the consumers' confidence in low cost health care system. Clearly, bioactive compounds of the flora of the coastal sediments of

the Sunderbans has the potential to bring not only hope to human health in Bangladesh and beyond, but also to social justice and environmental conservation.

1.2 OBJECTIVES:

Hence, Actinomycetes are the major part of the microbial flora of most natural substrates. The secondary metabolites produced by bacteria work as antibacterial agent. Due to their nature as antibacterial agent these can be used to treat against many bacterial disease. These can be used as the alternative source of chemically synthesized antibiotics. The present research aims at to isolation and identification of the Actinomycetes can be used as antibacterial agent. The objectives of this study are

1. Isolation, Characterization and Identification of Actinomycetes from marine sediment.
2. Bioactivity analysis of these isolates.

2. Literature Review

Actinomycetes are microorganisms intermediate in form and function between bacteria and fungi. They are heterotrophic organisms that thrive on decomposable organic matter and proliferate in soils that are rich with plant and animal residues.

2.1 Classification of Actinomycetes:

Domain:	Bacteria
Phylum:	Actinobacteria
Class:	Actinobacteria
Order:	Actinomycetales
Family:	Actinomycetaceae
Genus:	***Actinomyces***

2.2 Characteristics of Actinomycetes:

The following characteristics of Actinomycetes are given below:

1. The size of Actinomycetes is 1-2 µm in diameter.

2. These are usually rod shaped with a filamentous and branched structure. The filaments contain mumaric acid.
3. Most of the species are aerobic, but few are anaerobic to facultative aerobes.
4. Cell wall and internal structures are similar to the bacteria. The cell wall of Actinomycetes consists of mycolic acid.
5. The growth or reproduction of Actinomycetes is slower than the bacteria and fungi and hence also refers as "Slow growers".
6. These are having 60-78% of G+C content.
7. Actinomycetes are most abundant in soil (10^6-10^8g) and marine habitat.
8. These are most usually non-motile, non-capsulated and non-acid fast.
9. The growth of actinomycetes is optimal at alkaline pH.

2.3 Habitat:

Terrestrial Actinomycetes:

According to Hayakawa (2008) a large population of actinomycetes live in the soil. In natural soil habitat *Streptomyces* spp. is the major population of actinomycetes. *Streptomyces* spp. was encountered to be the most abundant genus isolated, followed by *Micromonospora.* In a report of Ismet *et al,*. 2004 & Hong *et al,*. 2009 claimed that actinomycetes are abundant in mangrove soil. *Streptomyces* and *Micromonospora* which were isolated from mangrove soil, have the potential in producing bioactive secondary metabolites. Due to the tidal influence the actinomycetes isolated from mangrove rhizosphere was 1000to 10000 times smaller than arable lands. *Nocardia* which is a

common genus of actinomycetes isolated from mangrove soil produced new cytotoxic metabolites that strongly inhibited human cell lines such as gastric adenocarcinoma (Busi and Pattnaik, 2018). Tian *et al.*, 2014 isolated 274 strains of actinomycetes from rice's sterilized root and leaves. This proves that actinomycetes can be found on the surface of plant, especially on leaves, stem and root. Most of the strains belong to the genus *Streptomyces* spp. and few are in *Streptoverticillium.* Isolation of endophytic actinomycetes from roots of healthy wheat plants was done by Coombs & Franco, 2003. *Streptomyces, Micromonospora, Saccharomonospora, Streptoverticillium, Actinoplanes* are the main groupsof thus isolates.

Aquatic Actinomycetes:

Waksman (1959) said that Actinomycetes are abundant in fresh water. It is also reported on an old report that some thermophilic actinomycetes have found in river water. At about 60 degree C they have seen growing well in sewage water. That was proved when Cross isolated some members of the genera *Actinoplanes, Micromonospora, rhodococcus, Streptomyces*, and other spore forming *Thermoactinomycetes* from fresh water.

Waskman (1959) also said that members of the genus *Micromonospora* is a group of microbes that can live both in water and bottom deposits of inland lakes. So it can be isolated easily from the lake sediment. Moreover it has been observed that various complex organic compounds like chitin, cellulose lignin etc. accumulated in the lake mud can be decomposed by this genus of actinomucetes. Rowbotham & Cross (1977) experimented that spores from *Micromonospora* can survive as dormant propagules as they washed into stream, riverand lakes. Jiang & Xu (1996) showed that *Micromonospora* , *Streptomyces*

can also be isolated from the sediments of lakes. The presence of *Streptomyces* in freshwater was because of their spores being continuously washed into rivers and lakes. This enables *Streptomyces* spores could be found in foam of rapids at the water-air interface. From the experiment of Terkina *et al.*, 2002 it can be said that *Streptomyces* can be found dominantly in water sample while great members of *Micromonospora* were found in sediments.

Marine actinomycetes:

Mainly marine water contains low concentration of organic matter and high concentration of inorganic salts and posses a high hydrostatic pressure. That's why it is very difficult to survive in marine environment. Thus, microorganisms surviving both in marine and terrestrial environment are expected to be totally different. Waksman (1959) enlisted that there were very few reports available concerning the occurrence of actinomycetes in the sea and sea bottom. Early studies showed that the existence of actinomycetes in that environment was believed because of soil contamination, or to their presence on algal material floating on the surface of the sea, or to the fact that the samples of water were obtainsd near the docks. Grein & Meyers (1958) first isolate the actinomycetes from inshore marine sediments. Based on the absence of apparent morphological and biochemical difference between both marine and terrestrial isolates, they elucidated that the actinomycetes might be originated from terrestrial but adapted to salinity level of sea water. According to Okami & Okazaki (1974) the spores of actinomycetes transports to the shallow sea mud. They have showed that the spores of actinomycetes could be transferred from the land to the sea by rain or river and survived. Spores were precipitated by the NaCl acceleration after reaching the sea level into sediment. By formulating media with sea

water they showed terrestrial strain could grow well besides showed a wide range of halo-tolerance, whereas halo-tolerance of NaCl-sensitve strains could be induced by stepwise exposure to increasingly NaCl concentrations.

However, Ganesan., 2018 isolated atinomycetes from the deep sea sediments and proved the existence of indigenous marine actinomycetes. Jensen *at al.* (1991) also experimentally showed that the maximum number of actinomycetes can be isolated from near-shore sediments in both shallow and deep sampling sites showing a bimodal distribution in relation to depth. A report showed that about 98% of streptomycetes are obtained from the depth less than 3 meters. However, actinoplanetes number was increased with up to 33m of depth increment. These evidences indicated that the theory that marine-derived actinomycetes are originated from territorial could be argued. They also proved that near-shore marine sediments actinomycetes are well-adapted and functional mambers of the marine microbial community. Jensen *at al.* (1991) claimed that marine actinomycetes are metabolically active and adapted to life in the sea. However Hakvag *et al* (2008) isolated a sum of 217marine actinomycetes. From the sea surface micro layer. Almost all of them isolates are in the genus *Streptomyces* spp. It is also said that they have the ability to survive under marine water due to their salt tolerance ability. Ghanem *et al* (2000) said that marine actinomycetes isolated from sediments, counts far exceeded those found in the sea water. Thus, sediments are the best source of actinomycetes. A sum of about 192 marine actinomycetes were isolated from sediments and those are mainly in the family *Micromonosporaceae* and a few *Streptomyces* spp. (Magarvey *et al.*2004). All these prove the value of marine sediment as the source of marine actinomycetes.

Extreme environment:

Many actinomycetes have been isolated from unusual environment. For example members from *Streptomyces* and *Nocardiopsis* genus are seen growing in the extreme environment like alkaline soil (pH 10-12) surrounding minerals springs (Jiang *et al.*, 1993). Groth et al. (1997) showed that a new genus and species of alkalophilic actinomycetes from a soda lake soil (pH 10) was also been described as Bogoriella caseilytica

Meves *et al.*, 2000 isolated actinomycetes from mountain soil and observed the optimum growth at about 11-13 degree C temperature. Some actinomycetes, for example, *Cryobacterium psychilum*, are seen growing at about 9-12 degree C temperature but they can't survive temperature more than18 degree C which was isolated from Antarctica soil (Suzuki *et al.* 1997). On the other hand Zakalyukina et al., 2004 showed that actinomycetes can survive in acidic condition. Streptomyces and Micromonospora have shownthe ability to survive in acidic soil and peat soil. However actinomycetes are also seen to survive at extreme temperature. Streptomyces spp. isolated from silt and sample of meteoritic crater are seen growing on agar at 55 degree C, with 18hours sporulation time. At pH 7.5-12 and temperature 55 degree C, they have seen producing protease enzyme which helps to survive this microorganism up to 85 degree C (Dey & Chaphalkar 1998). Takahashi et al. (1996) had reported that this microorganisms can be isolated from desert soil. Saccharothrix, Actinomadura, Amycolaptosis are isolated from desert of Califotnia, Mojava etc. which can grow at temperature up to 55 degree C.

2.4 Importance:

Actinomycetes play significant role in various purpose. They are consider as the major component in most soils. They function as the main soil organic matter decomposer (Arai,

1997). According to Goodfellow & Williams, 1983, actinomycetes can degrade plant litter, particularly in the recalcitrant lignocelluloses components and thus the play a major role in ecosystem. Actinomycetes will automatically germinate in the occasional presence of exogenous nutrients as their spores, sporangia or resting cocci are present in the soil and it is proved by Mayfield *et al.* in 1972. Along with available nutrients soil temperature, moisture, pH etc. factors play a vital role in growing this actinomycetes (Goodfellow & Williams, 1983).

Actinomycetes are able to produce various secondary metabolites specially antibiotics. In fact they are the major antibiotic producing group of microorganisms. The actinomycetes growing in the rhizosphere soil are capable of producing antibiotic and other metabolites. Getha & Vikineswary (2002) reported that *Streptomyces* spp. have the ability to control the fungal pathogen which are harmful to crops. This is proved when Vercesi *et al.*, 1992 experimentally showed that *Streptomyces* spp. which is isolated from grapes, have the antifungal activity against pathogenic yeast and fungi from the same habitat. However, Miyajima *et al.*, 1998 isolated a species called S. turgidiscabies which cause erumpent scub lesions on potatoes in Hokkaido, Japan.

2.5 Antioxidant Assay:

DPPH, an antioxidant assay characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole (Fig. 1), so that the molecules do not dimerise, like most other free radicals. The delocalisation also gives rise to the deep violet colour, with an absorption in ethanol solution at around 520 nm. On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet colour. Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is

$$Z^{*} + AH = ZH + A^{*}$$

Where ZH is the reduced form and A• is free radical produced in the first step. The latter radical will then undergo further reactions which control the overall stoichiometry The reaction (1) is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the autoxidation of a lipid or other unsaturated substance; the DPPH molecule Z• is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH (Kedare and Singh, 2011).

2.6 Antimicrobial activities of actinomycetes:

Actinomycetes is the leading antibiotic producing microorganism. According to Berdy, 2005 over 10,000 bioactive compound is produced by filamentous actinomycetes and it is about 45% of all microbial metabolites. Among them 75% of valuable production of bioactive compound is found from *Streptomyces* spp. and 25% are from other rare actinomycetes (*Micromonospora, Actinomadura, Streptoverticillium*). Actinomycetes play an important drug producing role. In an article of Okami & Hotta (1988) it was reported that some significant drugs are produced by actinomycetes like aminoglycosides,

anthracyclines, chloramphenicol, Bita-lactams, tetracycline, macrolides. *Streptomyces* spp. is the most potent genus in showing a wide range of antimicrobial activities (Ogunmwonyi *et al.*,2010). The ethyl acetate extraction of *Streptomyces* spp. exhibited activities against at least 6 and up to 26 of the 32 test bacteria screened. *Streptomyces* spp. also play as an antifungal agent (Getha & Vikineswary, 2002). Baharlouei *et al.*, 2010 experimentally showed that *Streptomyces plicatus* strain 101 have the chitinolytic activity and have antifungal effect on mycelial growth of *Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium graminearum, F. solani, Rhizoctonia necatrix, & Pythium aphanidermatum.* And this can be happened due to the extracellular chitinase production.

2.7 Biomedical Use:

Members of Actinomycetes produce many of the best-known antibiotics like amphotericin, neomycin, novobiocin, chloramphenicol, tetracycline etc.

- Tetracycline and erythromycin etc. target bacterial ribosomes and used to cure respiratory infections.
- Vancomycin mainly attacks the bacterial cell wall of pathogenic bacteria (Streptococcus aureus).
- Rifampicin targets bacterial RNAP (RNA-Polymerase) and used to cure tuberculosis and leprosy.
- Adriamycin use in the treatment of cancer.
- Amphotericin attacks fungal membranes and shows few side effects.
- Rapamycin enables organ transplant.

2.8 Molecular technique for identifying diversity

Methods used for detection of Actinomycetes are identification of colony morphology, fermentation patterns, serotyping or some combination of these. Although these traditional methods have limitations they are used for identification. With the developing technology about the molecular typing it is getting more reliable to identify and differentiate bacterial strains. Classical microbiological techniques are really important for selection, enumeration and biochemical characterization (fermentation profiles, salt-pH-temperature tolerances) but it is not efficient to classify a culture taxonomically. Molecular characterization methods are powerful, even between closely related species. There are a number of alternative taxonomic classification methods well known, including hybridization with species-specific probes and generation of profile PCR applicants by species-specific primers (Klaenhammer and Kullen, 1999). Polymerase chain reaction based methods (PCR-RFLP, REP-PCR, PCR ribotyping and RAPD) are mainly used as molecular tools.

2.9 Molecular Markers

The molecular markers are one of the versatile tools in molecular biology and genetic engineering. With the advent of molecular markers, new generation of markers have been introduced over the last two decades, which has revolutionized the entire scenario of biological sciences. Ever since their development, they are constantly being modified to enhance their utility and to bring about automation in the process of genome analysis. The discovery of polymerase chain reaction was a landmark in this effort and proved to be a unique process that brought a new class of DNA markers. Molecular markers discriminate organisms at the level of DNA and are inherited in simple Mendelian fashion (Waltson, 1993). There are different types of DNA markers and many more being discovered. There are two important types of markers viz., hybridization based markers (Restriction fragment length polymorphism RFLP) and PCR based markers (RAPD, AFLP, SSR, ISSR, SCAR, SNP, CAPS, etc.).

2.10 Random Amplified Polymorphic DNA (RAPD)

Randomly amplified polymorphic DNA (RAPD) involved the use of random primers in PCR reactions (Welsh and McClelland, 1990; Williams et al., 1990). It has been used increasingly to distinguish closely related organisms (Bassom et al., 1992) based on polymorphism among RAPD products.

RAPD technology is a useful, simple, fast and informative. Further, the technique gives an opportunity to get information about the biodiversity in a group of isolates to distinguish them from one another.

A research team carried out different PCR based DNA fingerprinting techniques for identification and discrimination of bacterial strains.In Randomly Amplified Polymorphic DNA fourteen strains of *Salmonella* divided into 9 groups out of which maximum number of strains i.e., 5 presented in one group. In box PCR method primer with highly conserved repeated sequences which was longer than the used in RAPD produced six different groups which showed the presence of a common band. Therefore, serotype *typhimurium* could be placed in the same group by Box PCR. Results show that RAPD had a highly discriminating power for *Salmonella* isolates compared to the other two methods

Gupta et al., 2001 isolated the 16 isolates of *Xanthomonas oryzae* representing different geographical locations in India along with 2 isolates from the Philippines were analyzed using polymorphic RAPD with seven primers viz., OPA-03, OPA-04, OPA-10, OPA-11, OPK-7, OPK12 and OPK-17 IS-1112 based PCR with primers PJEL1 and PJEL2. Results showed that the RAPD primers generated simples, specific and reproducible fingerprinting patterns, indicating the usefulness of RAPD markers in differentiating *Xanthomonas oryzae*isolates. The IS-1112 based PCR also generated specific and reproducible fingerprints patterns for the same set of *Xanthomonas oryzae* isolates based on RAPD-PCR (seven primers) and IS 1112 PCR (two primers) data at a similarity of 0.57, 16 out of 18 isolates were grouped into five different clusters and two isolates from the Philippines were loosely grouped with them. Indicating that the usefulness of RAPD-PCR and IS1112-PCR in assessing genetic variation among the isolates.

Bradic et al., 2003 isolated the nine strains of indigenous *Sinorhiziobiummeliloti* from alfalfa nodules collected from different field sites in Croatia and three reference strains were analyzed for genetic characterization by PCR-RFLP of the 16s rDNA, rep-PCR and

RAPD-PCR. The results of PCR- RFLP of the 16s rDNA revealed that most isolates (77%) had two reference strains 469,528 and indigenous strain C16 differ from other a separate group.

Susana et al., 2004 determined genetic diversity of 323 strains of *Lactobacilli* isolated from an Almagro eggplant fermentation was analyzed by using random amplified polymorphic DNA (RAPD). Thirty-four distinct RAPD patterns were obtained with 95% of isolates grouped into 18 main clusters. Genetic diversity was higher in brines from season II, *Lactobacillus plantarum* and *Lactobacillus fermentum/ cellobiosus* being the species with the greatest number of genotypes. A single *L. fermentum/cellobiosus* genotype comprised isolates from both seasons and could be considered endemic to that factory.

Weiss et al., 2005 isolated and identified forty potentially probiotic *Lactobacillus* strains. For identification, all strains were subjected to genus-specific polymerase chain reaction (PCR), Using two species-specific primer-pairs for *Lactobacillus*reuteri, specific amplicons observed for of the forty investigated strains. For differentiation, these eight strains as well as the reference strains of the species *L. reuteri*and closely related species were subjected to Randomly Amplified Polymorphic DNA (RAPD)-PCR using fourteen arbitrary primers.

Ashmaig et al., 2009 isolated and characterized *Lactobacillus* spp. from camel's fermented milk. They found two major groups among the isolates and identified as *Lactobacillus plantarum* (66.6% of the gariss isolates) and the second was composed of 6 isolates recognized as *Lactococcus raffinolactis* (33.3% of the gariss isolates). The remained minor groups were classified as *L. animalis, L. brevis, L. divergens, L. rhamnosus, L. gasseri, L. paracasei, L. fermentum, L. alimentarium, Lactobacillus* sp. The genetic relationships of the isolates were determined by the Random Amplified Polymorphic DNA (RAPD)-Polymerase Chain Reaction (PCR), the results demonstrated a distinction comparative genetic clusters and their pattern was greatly related to the clustering obtained with the API 38 CHL group identification.

Random Amplified Polymorphic DNA profiling revealed 28 different genetic profiles among the *Lactobacilli* isolates. Their probiotic potential was evaluated through different assays, including production of antimicrobial compounds, adherence to intestinal mucin,

degradation of mucin and pattern of antibiotic sensitivity. Some strains showed potential for future applications as canine probiotics (Martin et al., 2010).

The diversity of 72 isolates of *Lactobacillus plantarum* identified from different raw vegetables and fruits, was studied based on phenotypic (Biolog System) and genotypic (Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction, RAPD-PCR, and Amplified Fragment Length Polymorphism, (AFLP) approaches. A marked phenotypic and genotypic variability was found. Eight clusters were formed at the similarity level of 92% based on Biolog System analysis. At high values of linkage distance, two main clusters were obtained from both UPGMA (Unweighted Pair Group With Arithmetic Average) dendrograms of RAPD-PCR and AFLP analyses (Cagno et al., 2011). The two clusters mainly separated the isolates of tomatoes and carrots from the isolates of pineapples.

Banwo et al., 2012 were isolated a total of 104 lactic acid bacteriafrom ogi, wara, fermenting cassava mash for local food product gari and raw milk for nono. They were characterized phenotypically and divided into six main groups. They were further characterized employing genotypic fingerprinting techniques, such as RAPD-PCR with primer M13, rep-PCR with primer (GTG)5 and sequencing of the 16S rDNA genes for starter culture development. RAPD-PCR did not give accurate delineation of the strains, but with the combination of rep-PCR fingerprinting and 16S rDNA sequencing, representative strains from rep-PCR clusters at 80% Pearson's correlation coefficient (r) grouped the organisms to nine (9) clusters. These were identified as *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus pentosus, Pediococcuspentosaceus, Pediococcusacidilactici, Enterococcus faecium,* and the unidentified *Lactobacillus* species were assigned into *Lactobacillus* fermentum group.

Sharma et al., 2016 characterized 13 strains of *Lactobacillus brevis*, isolated from different vegetable products of South Korea. Two primers i.e. 239 and KAY3 were used. The primer 239 produced bands ranged from 500-4,000 bp and KAY3 primer produced bands with sizes from 600-4,000bp. Both primers produced thirteen different RAPD profiles. Phylogenetic dendrogram showed that all the isolates could be divided into six major clusters both the primers.

Abdollahniya et al., (2018) conducted study on 26 specimens collected from traditional dairy products in Bukan. *Lactobacillus* species were separated and purified employing biochemical tests. Then, the intra/inter-species diversity was investigated using RAPD-PCR technique. According to the dendrogram result, the highest level of genetic similarity was observed between B.L7 and B.L9, postulating that they were 2 different strains from thesame genotype, because they were taken from the source. The G.L15, G.L25, and G.l23 isolates, which were located in the same group, were all in the samples taken from the cow.

2.11 Dendrograms

A dendrogram is a visual representation. Specifically, it is a tree or branch diagram where there are many elements at one end, and few, or one, at the other. The branches represent categories or classes and the diagram implies an order or relationship between these categories or classes. The members of these categories are similar in some fashion or have a number characteristics in common. Another name for these categories or classes is cluster. And the process of placing the items into a specific cluster is known as clustering. Dendrograms are often used with clusters (CaliNski, 2014).

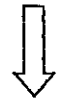
3. Materials and Methods:

3.1 Collection of soil sample:

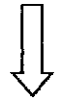
Soil samples was collected from marshy wet land of marine ecosystem especially from at least four different locations of the Sundarbans at a depth of 10–15 cm. The samples will be placed in sterile plastic bag, which was tightly sealed and transported to the laboratory.

3.2 Isolation of the Actinomycetes:

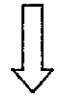
Sample drying at room temperature



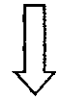
Heat treatment for 2 hours at 60° C



25ml sample into 100ml distilled water and lefty with shaker for 30 minutes

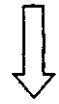


Serial dilution



Transferred into starch casein agar (SCA) media (supplemented with fluconazole and streptomycin) at 30° C for 10 days

Subculture



Identification by morphologically, color of aerial spores and pigmentation

3.3 Actinomycetes DNA Extraction protocol:

Culture into 100ml conical flask containing nutrient broth

⇩

Incubated in rotary shaker at 37°C for 3days at 180 rpm

⇩

Centrifuged at 9000rpm for 10 minutes

⇩

Discard supernatant and retain pellet in 1.5 ml Eppendorf tube

⇩

Keep the tube in liquid nitrogen for 4-5minutrs

⇩

Ground with micro pestles with 500 µl of TE buffer (10mm Tris and 1mm EDTA)

⇩

Grinding is repeated till the pellet is dissolved

⇩

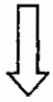
Incubated at 37°C for 45 minutes

⇩

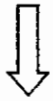
Added 20 µl of 10% SDS (w/v) and 20 µl of proteinase K

⇩

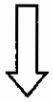
Incubated at 60°C for 20 minutes



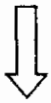
Cool lysate and extract with equal volume of tris saturated phenol and Chloroform solution (1:1) at 10000 rpm for 10 minutes at 16°C



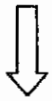
Transferred into fresh tube



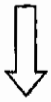
5-8 µl of RNase is added and inoculated at 37°C for 40 minutes



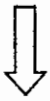
Extraction with equal volume of phenol and chloroform solution 9 1:1) at 10000 rpm for 10 minutes at 16°C



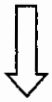
Transferred into fresh tube



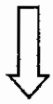
DNA precipitated by adding 0.6 volumes of isopropanol



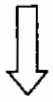
Centrifuged at 10000 rpm for 8minute at 4°C



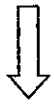
Washed pellet with 70% ethanol



Dissolved the pellet in 80 µl TE buffer



Analysis of purity and concentration by UV-spectrophotometer, wave length, A260/A280



Agarose gel electrophoresis (0.8%)

3.4. Gel electrophoresis

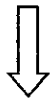
Gel electrophoresis separates DNA fragments by size in a solid support medium (an agarose gel). Application of an electric current at the top (negative) end causes the negatively-charged DNA to migrate towards the bottom (positive) end. The rate of migration is proportional to size: smaller fragments move more quickly, and wind up at the bottom of the gel. Non PCR Gel electrophoresis was performed for detecting the presence of DNA. In this gel electrophoresis process 1% Agarose gel, Ethidium bromide, 50X TAE, 1X TAE were needed to prepare before starting this process.

3.4.1. Preparation of 1% Agarose Gel

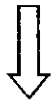
Requirement: Agarose, 50X TAE buffer, Distilled water

Procedure

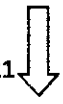
At first, 90 ml distilled water was placed into the conical flask



Then, 1 g of Agarose was added to the mix



The whole mixture was placed in oven until melted of Agarose



After melting, 700 µl of 50X TAE was added to the mix

The mix was then subjected into gel running tray and cooled until concentrated.

3.4.2 Gel electrophoresis Procedure

DNA sample was taken and 6X dye was added

Vortexed the DNA sample and then centrifused for proper mixing

DNA was then loaded into the pore of the gel

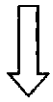
DNA was allowed to run into the gel about 120 volts for 80 mins

After running, the gel was placed in shaking incubator staining into Et. Bromide.

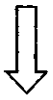
3.4.3 Documentation of the DNA samples

Procedure

After electrophoresis, gel was taken out carefully from the gel chamber



The gel was then placed in the dark chamber of UV transilluminator



The image was visualized on the monitor and the photo was taken for further analysis.

3.5 Amplification of the 16s rDNA:

3.5.1 Master mixer preparation:

Table 3. Master mixer

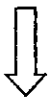
No.	Reagent	Concentration
1	5X PCR mixer	5l
2	Forward primer	10.2
3	Reverse primer	10.2
4	Water	173.4
5	Template	1µl

3.5.2 Amplification:

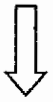
Initial denaturation at 94°C for 5 minutes

Denaturation for 1minute at 4°C

Annealing at 58°C for 1 minute



Extension at 72° C for 1 minute



Total 35 Cycle

Final extension at 72° C for 5 minutes

Hold at 8/10° C

3.6 Sequencing: Gene sequencing was done by JUST. Then the data was submitted to gene bank. Then the data was analysed in MEGA software for phylogenetic tree construction.

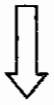
3.7 Fermentation of isolates:

3.7.1 Fermentation media preparation:

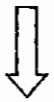
No.	Name of the chemical	Quantity
1	Soluble starch	2g
2	Soyabean meal	1.5g
3	KH_2PO_4	0.3g
4	$MgSO_4.7H_2o$	0.05g
5	$CoCl_2.6H_2O$	0.0002g
6	$MnCl_2.4H_2O$	0.0002g
7	Na_2HPO_4	0.2g
8	Sea water	100ml
9	pH	7.0

3.7.2 Fermentation:

Culture the isolates into NA media



Inoculate the isolates into fermentation media

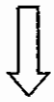


Keep it for 25-30 days at room temperature

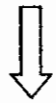
3.8 Extraction of secondary metabolite:

Performed a single extraction using approximately 25mL of dichloromethane (CH_2Cl_2, an exact amount is not necessary), as described previously, with some differences.

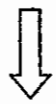
- (As CH_2Cl_2 is prone to emulsions, invert the funnel and shake *gently* for one minute with venting.
- After allowing the layers to separate in the funnel, drain the bottom organic layer into a clean Erlenmeyer flask (and label the flask, e.g. "bottom organic layer"). Do not drain the top aqueous layer from the funnel).



To the aqueous layer remaining in the funnel, add a fresh 25mL25mL portion of dichloromethane. Stopper the funnel, invert and shake *gently* for 1 minute with venting, then allow the layers to separate.



Since it is most common to combine the organic layers in multiple extractions, the bottom organic layer can be drained from the separatory funnel into the same flask that was used for the organic layer in the first extraction (that may have been labeled "bottom organic layer"). In this flask, there should be roughly 50mL of dichloromethane from the two extractions.



Repeat the extraction by adding another fresh 25mL portion of dichloromethane to the aqueous layer in the separatory funnel. Stopper the funnel, invert and shake gently with venting, then allow the layers to separate.

⇩

Drain the bottom organic layer into the flask used previously, where there should be roughly 75mL of dichloromethane from the three extractions.

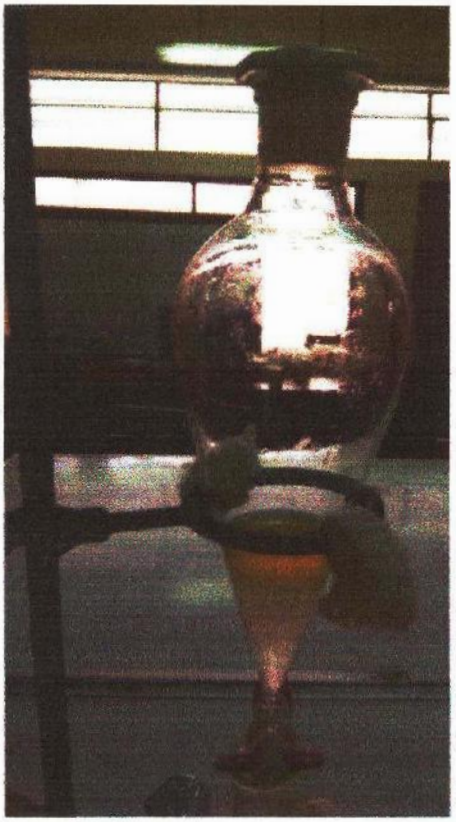


Figure: 3.1 Extraction of Secondary metabolites from isolates

3. 9 Antioxidant activity test:

3.9.1 DPPH assay materials:

- Ethanol
- Volumetric flask
- DPPH

3.9.2 DPPH Scavenging Assay:

Added 50 mL of absolute ethanol to the beaker to dissolve the salt

⇩

Transfer your solution to a 100 mL volumetric flask

⇩

Using absolute ethanol, complete the solution's volume to reach 100 mL

⇩

Read the absorbance of the solution at 517 nm in a microplate reader

⇩

If the absorbance of the solution is above 1.12, dilute the solution by adding absolute ethanol
If the absorbance of the solution is lower than 1.08, add DPPH;

⇩

Incubation of the samples

⇩

Reading of samples

⇩

Calculated data and determined results

⇩

Determined the percentage of inhibition of the DPPH radical

3.9.3 Calculation and Interpretation

Based on the standard curves, concentrations of samples (reconstituted with PBS) were taken and the experiments were repeated accordingly for each sample (x3) and blank (PBS). For both assays, % of scavenging activity was calculated as:

$$\%SA = \frac{Absorbakce_{Blank} - Absorbakce_{Treatment}}{Absorbakce_{Blank}} \times 100$$

Effective concentration (EC_{50}) for DPPH and H_2O_2 radical scavenging were derived from concentration vs %Scav plots and calculated as:

$$EC_{50} = \frac{50 - Iktersept_{Conc.\ vs\ \%SÆ}}{Slope_{Conc.\ vs\ \%SÆ}}$$

$Abs_{control}$ = The absorbance of DPPH
Abs_{sample} = The absorbance of your sample
Abs_{blank} = The absorbance of methanol as negative control

3.10 Antibacterial Activity Assay:

3.10.1 Preparation of resazurin solution:

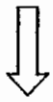
Resazurin dye (13.4 mg) was dissolved in 2 mL of sterile water and mixed well using a vortex mixer

3.10.2 Resazurin based MIC (minimum inhibitory concentration) assay:

Prepared 96-well micro titer plate at step-03 was incubated for 18 to 24 h.

Then 5 µl resazurin solution was added to each well.

Again 96-well microtiter plate was incubated for 10 min.



Finally, the absorbance was taken by microtiter plate reader at 600 nm wavelength.

(Pavithra et al., 2010)

4. Result:

4.1 Isolation of the Actinomycetes:

Soil samples was collected from marshy wet land of marine ecosystem especially from at least four different locations of the Sundarbans. I have isolated 9 isolates, among them 8 were Actinomycetes but two were studied for bioactive analysis.

Table 4.1 Identification of bacteria from coastal sediment of Sundarban

Internal code	Name of bacteria	Accession Number	BLAST match sequence		
			Reference number	Query length	Similarity (%)
T-2R	*Streptomyces olivoverticillatus*	OL714360	*Streptomyces olivoverticillatus* NR_115786.1	362	100
T-4	*Streptomyces cyaneus*	OL714362	*Streptomyces cyaneus* NR_112525.1	625	100
T-5	*Cytobacillus oceanisediminis*	OL716090	*Cytobacillus oceanisediminis* NR_117285.1	598	99.83
T-6	*Streptomyces albogriseolus*	OL716091	*Streptomyces albogriseolus* NR_112489.1	644	99.69
T-7	*Nocardiopsis synnemataformans*	OL716094	*Nocardiopsis synnemataformans* NR_029343.1	651	100
T-8	*Streptomyces albogriseolus*	OL714346	*Streptomyces albogriseolus* NR_112489.1	662	100
T-9	*Streptomyces atrovirens*	OL714352	*Streptomyces atrovirens* NR_112449.1	711	100
T-10	*Cellulosimicrobium funkei*	OL714357	*Cellulosimicrobium funkei* NR_042937.1	564	100
T-11	*Streptomyces labedae*	OL714360	*Streptomyces labedae* NR_115446.1	669	100

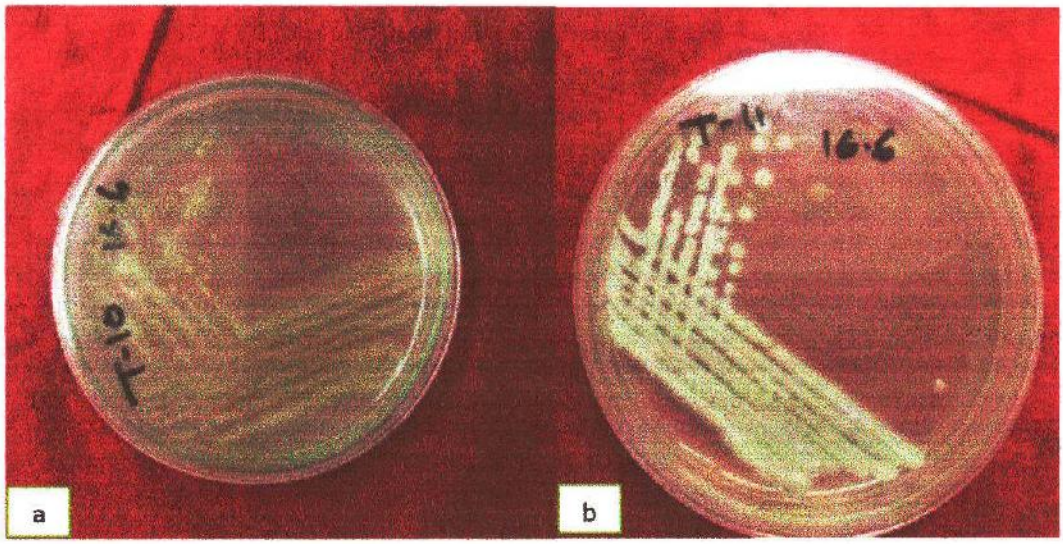


Figure 1. Pure colony development of two Actinomycetes isolates a. *Cellulosimicrobium funkei* b. *Streptomyces labedae*

4.2 Actinomycetes DNA Extraction:

In the present study extraction was done and gel electrophoresis was done. The gel electrophoresis was performed and the gel was visualized in the gel documentation system at 254nm absorbance. The data recorded from the extracted DNA band were well enough for molecular characterization of the DNA samples.

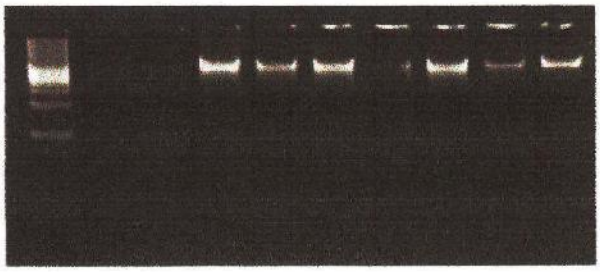


Figure 4.2 Extracted Actinomycetes DNA band after gel electrophoresis.

4.3 Amplification of the 16s rDNA:

In the present study Polymerase Chain Reaction (PCR) based 16s rDNA marker was used to amplify 2 sample of Actinomycetes. Post Polymerase Chain Reaction (PCR), the gel electrophoresis was performed and the gel was visualized in the gel documentation system at 254nm absorbance. Data obtained from the band by binomial calculation. The data recorded from the amplification band were well enough for molecular characterization of 2 DNA samples.

Figure 4.3. 16s rDNA profile generated by forward primer C27: AGAGTTTGATCCTGGCTCAG and reverse primer RC1492: TACGGCTACCTTGTTACGACTT

4.4 Identified Actinomycetes: Two of the isolated Actinomycetes were selected for further analysis. They are *Cellulosimicrobium funkei* (OL714357) and *Streptomyces labedae* (OL714360) mentioned in table 4.1

Table 4.2 Actinomycetes isolated from Sundarbans with NCBI accession numbers

Internal code	Name of the Actinomycetes	Gene bank accession number	Reference accession number	Max. identification
T 10	*Cellulosimicrobium funkei*	OL714357	*Cellulosimicrobium funkei* (ref no. 042937.1)	100%
T 11	*Streptomyces labedae*	OL714360	*Streptomyces labedae* (115446.1)	100%

4.5 Dendogram:

Dendogram was made for the two isolates for showing the similarity with others of same species.

Figure 4.4 Phylogenetic tree of *Cellulosimicrobium funkei* isolated from Sundarban, was constructed based on rDNA sequence by using MEGA.

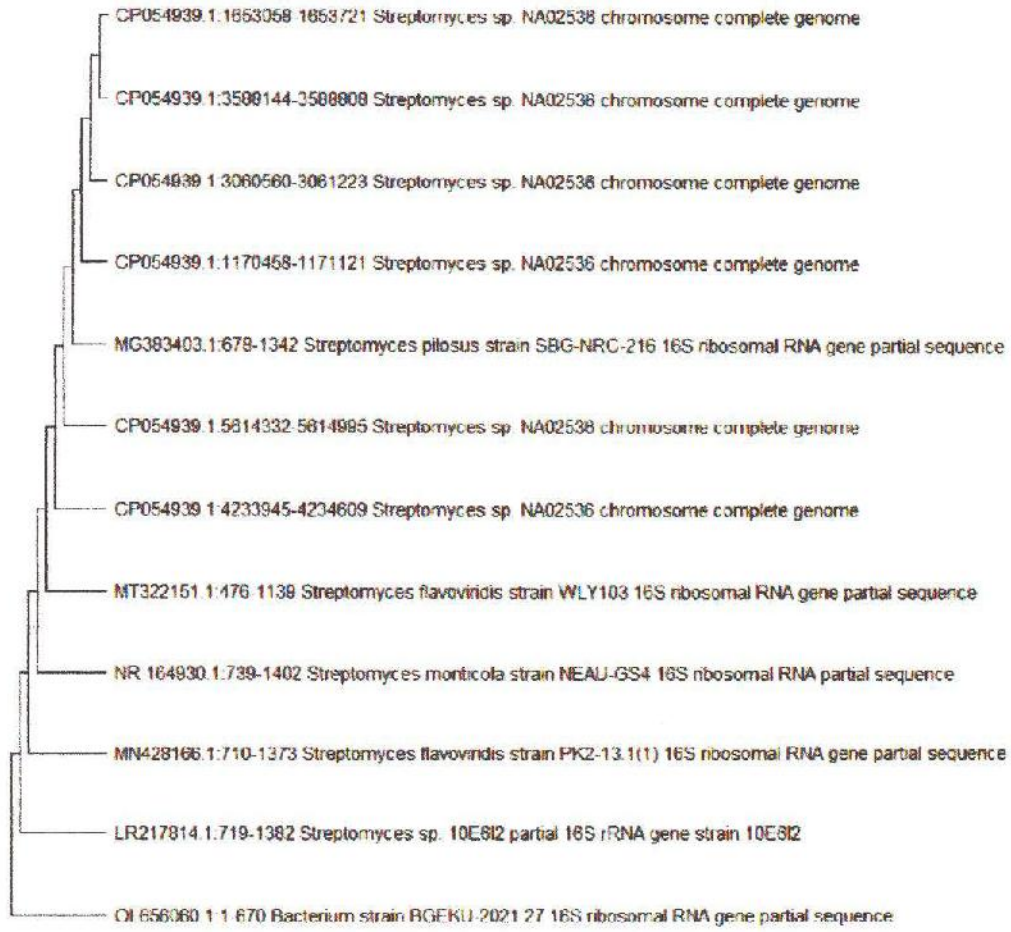


Figure 4.5 Phylogenetic tree of *Streptomyces labedae* isolated from Sundarban, was constructed based on rDNA sequence by using MEGA.

4.6 Antioxidant activity test (DPPH Scavenging Assay):

Free radical scavenging-based assays used stable radicals DPPH that predict ability of treatments to inhibit active oxidation. The assay results inferred from % scavenging and subsequent calculation of EC_{50} values predict a set of secondary metabolites from Actinomycetes to be strong antioxidants. Extracted sample from *Cellulosimicrobium funkei* exhibited the lowest EC_{50} values than *Streptomyces labedae*

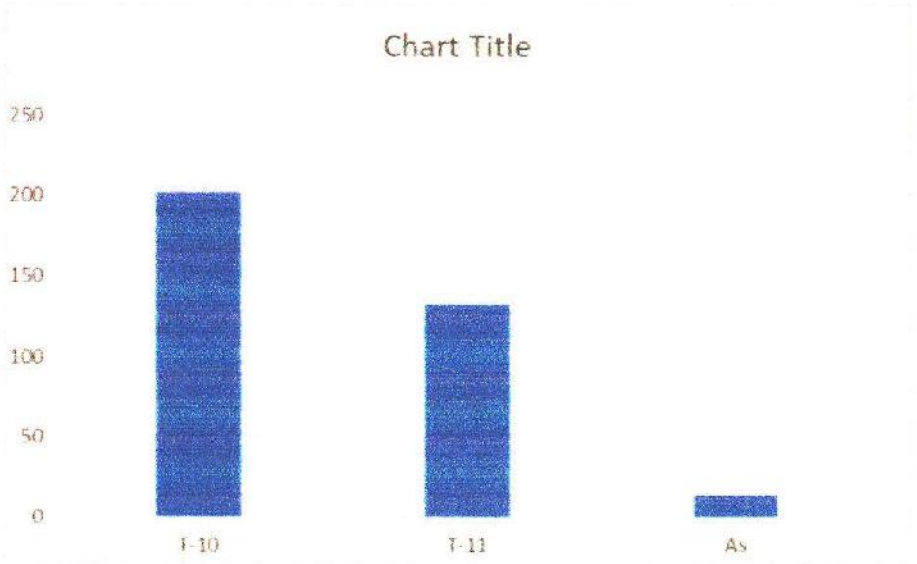
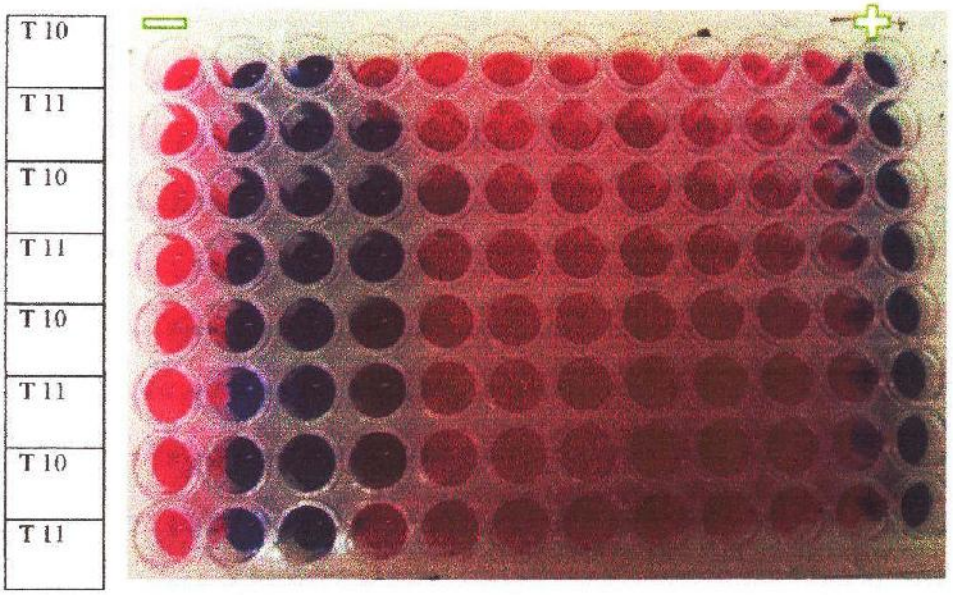


Figure 4.6 Antioxidant Potentials of secondary metabolites from Actinomycetes Evaluated using in-vitro Assays. EC_{50} values express the required concetration of treatment for effective (50%) scavenging of free radicals in solution – lower EC_{50} values correspond to higher antioxidant activity of Actinomycetes, ascorbic acid was used as a positive control for comparison of antioxidant potentials.



4.7 Resazurin based MIC for checking antimicrobial activity against 4 pathogenic bacteria. The 1st row is for negative control and the last one is for positive control (Streptomycin).

5. Discussion:

Actinomycetes have been reported from a variety soil samples that contribute to the diversity of microorganisms in the ecosystems. Actinomycetes produce various biologically active compounds which play versatile roles in their inherent surroundings. Sundarbal coastal area can thrive in a unique habitat with high microbial and fungal competitions and brackish tidal environment (Dilip et al., 2013). In an extensive review, Sengupta et al. (2015) documented 54 species of mangrove Actinomycetes. In another study, *Cellulosimicrobium* spp. was found to be one of the most abundant Actinomycetes species in mangrove sediment (Tanveer et al., 2021). Our study is also in good agreement with these previous studies as we have isolated and identified two Actinomycetes species of two different genera. However, in our case, *Cellulosimicrobium* spp. was the most dominant species followed by *Streptomyces* spp.

Although we didn't identified the Actinomycetes based on morphological characteristics, however, this type of identification is presumptive and requires fair bit of experience. Therefore, we chose nucleotide sequence analysis for more accurate identification, due to its simplicity and straightforward nature of operation. It was done by 16s rDNA sequencing and offer a better identification of closely related species. By sequence determination and comparison with the sequences of Gene bank database, all the strains were identified to the species level 99% homology to the best-matching reference sequence. Monciardini et al., 2002 also used this technique for identifying their Actinomycetes spp. In a research they found the EC 50 value for *Cellulosimicrobium fuenki* is close to us (Tanveer et al., 2021). We estimated the value 201.9302. Gozari et al., 2018 found satisfactory result in case of

Streptomyces labedae and in our study we found EC 50 value 132.8517. Utami and Juliasih 2021 found MIC value 0.25mg/ml in case of their Actinomycetes isolates which is same to our result against *V.cholerae*.

6. Conclusion:

In conclusion, the current findings show that Actinomycetes are highly promising sources to find medically important natural metabolites. We have isolated 8 Actinomycetes out of 9 isolates. We then introduced *Cellulosimicrobium fuenki Streptomyces labedae* as sources of bacterial growth inhibitors and is promising sources free radical scavenger. So Actinomycetes from coastal sediment of Sundarbans could be potential sources of antimicrobial agents. Here two activities were analyzed but there are so many activities, which are needed to be analyzed further.

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