

IDENTIFICATION OF ANTIFUNGAL COMPOUND FROM MARINE ACTINOMYCETES

Submitted in partial fulfilment of the requirements for the award of Bachelor of
Technology degree in Biotechnology

by

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INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
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APRIL – 2021**



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of D HARI HARAN (37230023) and ABHISHEK SAHU (37230100) who carried out the project entitled "Identification of antifungal compound from marine actinomycetes" under our supervision from December 2020 to April 2021.

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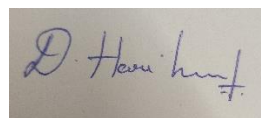
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DECLARATION

I, D Hari haran (37230023) and Abhishek sahu (37230100) hereby declare that the Project Report entitled “**Identification of antifungal compound from marine actinomyces**” done by us under the guidance of **Dr. C Karthikeyan** at KIRND Institute of research and developments PVT. LT is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

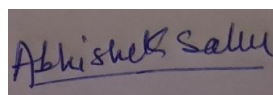
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ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to Board of Management of SATHYABAMA for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to Dr. V Ramesh Kumar, Head of the Department, Dept. of Biotechnology for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Project Guide Dr. Y Swarnalatha and Dr. C Karthikeyan for their valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work. I also thank them for taking me under their wing, for mentoring me and providing access for utilization of resources and facilities at the KIRND Institute of research and developments despite their academic and professional commitments.

I express my sincere thanks to research scholar Ms. S. Seema for providing feedbacks and suggestions that helped in troubleshooting of problems.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology and KIRND Institute of research and developments who were helpful in many ways for the successful completion of the project

ABSTRACT

Candida biofilm cause variety of infections such as urinary tract infection, skin infection, catheter mediated infection and forms of dental plaques. The major risk is due to the colonization of the Candida on matrix surfaces. Formation of candidal biofilm showing resistance to conventional antibiotics. Therefore, there is an urgent need to identify novel drugs to overcome resistant in Candida sp. Anti-fungal agents targeting the virulence genes can act as the potential drug candidate to prevent resistance developed by the biofilm producing fungal pathogens. The objective of the present study is to identify the antifungal compounds from marine actinomycetes against *C. albicans* biofilm which is of clinical importance. The bio active fractions from actinomycetes extract showed tremendous activity which was confirmed using bio assays and characterization techniques such as FTIR, and GCMS.

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LIST OF ABBREVIATIONS

DAP	Diaminopimelic acid
GC-MS	Gas chromatography–mass spectrometry
TLC	Thin layer chromatography
FTIR	Fourier Transform Infrared Spectroscopy
NIST	National Institute of Standards and Technology
ECR	East Coast Road
SCA	Starch casein agar
PBS	Phosphate-buffered saline
SD	Sabouraud Dextrose
BIC	Biofilm inhibitory concentration

IDENTIFICATION OF ANTIFUNGAL COMPOUND FROM MARINE ACTINOMYCETES

CHAPTER 1

INTRODUCTION

1.1 ACTINOMYCETES:

Actinomycetes generally resemble filamentous, Gram-positive bacteria that form branching filaments at some stage of their development. In 1875, Ferdinand Cohn was initially described as the actinomycetes then he observed a filamentous organism during a concretion from a person's lachrymal duct and named it *Streptothrix Foerster*. Later in 1878, C.O. Harz named an organism that he isolated from a case of bovine cervicofacial actinomycosis, as *Actinomyces bovis*. In Greek, the term actinomycete was called "ray fungus" and it is still mentioned as "ray fungi". Actinomycetes can be present in a variety of ecosystems, including soil, aquatic environments, plant litter, composts, and food. Plants, animals, and humans are all connected to them. Actinomycetes mediate degradation of complex polymers like chitin, lignin, and cellulose, help within the biocontrol of other bacteria and fungi by the assembly of antimicrobial agents and cause deterioration of stored food products. They are a crucial group of prokaryotes that are ready to synthesize metabolic products like antibiotics, pigments, enzyme inhibitors, and biotechnologically relevant enzymes and they are also pathogenic to plants, animals, and man. Plant diseases like rot of sweet potato and human diseases like tuberculosis, paratuberculosis, mycetomas, actinomycosis, allergic pneumonia, and various sorts of abscesses are caused by actinomycetes.

1.1.1 Classification of actinomycetes:

The actinomycetes area unit is classified into quite one hundred fifty genera. Members of this cluster area unit cosmopolitan in nature and might be found during a style of habitats across the planet. This classification is predicated on morphological and chemical criteria. 9 different cell wall chemotypes area unit distinguished on the thought of the kind of the diaminopimelic acid (DAP) chemical compound and thus the presence of characteristic amino acids and sugars in their cell walls. The order includes phylogenetically numerous however morphologically similar aerobic and anaerobic bacterium that exhibit thin branching structures that fragment into bacillary or round forms. Eubacteria, Arachnia,

Rothia, and Bifidobacterium are some of the most important anaerobic genera in medicine. Aerobic actinomycetes are a large, diverse group of gram-positive bacteria that includes Gordonia, Nocardia, and Rhodococcus, among other genera. Actinomycetes happiness to the genera Gordonia, Nocardia, and Rhodococcus area unit cosmopolitan in natural environments. However, they are acknowledged to cause infections of the skin and soft tissues in upset human patients. they need been shown to share a variety of characteristics with fungi (e.g. mycelial growth) and cause constant unwellness as fungi within the eye. These organism's area unit expedient human pathogens and infections could usually be post-operational and hospital noninheritable.

1.1.2 Distribution and ecology:

Actinomycetes, which belong to the phylum Actinobacteria, are found all over the world. Since they can survive in a variety of habitats and eat a variety of foods, they have been able to spread and flourish in many different parts of the world, competing with other species in their habitat. Whereas such diseases/infections as zoonosis (caused by actinomyces) conjointly occur worldwide, studies have shown prevalence to be influenced by such factors as socioeconomic standing and hygiene, etc. While actinomycetes have the ability to occur in different kinds of habitat, they occur within the soil in significant numbers in several varieties of soil (about one million cells per gram of soil). Some of the opposite factors which will influence the sort of species in soil include temperature and oxygen. Actinomycetes are especially useful in soil because they break down a wide range of tough compounds/polymers, from lignocelluloses to pectin, and thus fungi cell membranes, among other things. In doing so, they play a crucial role in recycling organic matter within the soil while also controlling the expansion of other microbial organisms that tend to be pathogenic to plant. Actinomycetes also are involved in the organic process in soil. The activities of actinomycetes in soil induce the earthy scent of freshly turned soil. A variety of species can be found in marine habitats in addition to the soil. In comparison to their prevalence in terrestrial and freshwater environments, they were found to have a lower population in marine habitats. Actinomycetes are active in the breakdown of various compounds, much as they are in terrestrial/soil environments. Here, they need been shown to assist in the decomposition of cellulose, alginates, and various hydrocarbons. Thermophilic Actinomycetes are often found in compost/manure

particularly during the first stages of decomposition. The warmth level in manure/compost is increased as a result of their participation in decomposition, providing a favourable living climate. When looking into the characteristics of actinomycetes, it was discovered that they are found in freshwater (lakes, rivers, etc.) and that the majority of these organisms wash in from the land.

1.2 MARINE ACTINOMYCETES:

Actinomycetes are a rich source of lead compounds, which are critical in the development of microbial drugs. Owing to their unusual living environment, marine actinomycetes have unique metabolic pathways compared to terrestrial microorganisms, which has the ability to supply a wide range of bioactive substances. Actinomycetes in general, and marine actinomycetes in particular, have a wide range of applications, including the synthesis of biologically active products, the development of drugs to treat diseases, the production of enzymes, the development of antibacterial, antifungal, and anticancer products, and their function in biofouling. In today's environment, both of these areas are critical.

1.2.1 Actinomycetes in marine environment:

Oceans cover 71 percent of the Earth's surface, contain 97 percent of the planet's water, and nearly 87 percent of life with mostly unchanged fauna and flora, and are a rich source of undiscovered species such as microorganisms and novel natural products. Rare actinomycetes obtained from the sea are thought to contain a large number of chemicals and structurally distinct secondary metabolites. The existence of several novel actinomycetes that were not previously observed in cultivation studies has been revealed thanks to high-throughput metagenome sequencing methods. In most cultivation studies, the retrieval of rare actinomycetes falls short of that of streptomyces strains. However, a better understanding of the physiological, chemical, and structural options available to marine actinomycetes has led to the development of selective isolation media. Up until 2010, actinomycetes produced a total of 13,700 bioactive metabolites, with ten,400 derived from streptomyces and 3300 from uncommon eubacterium strains. In 1974, solely one hundred twenty-five active metabolites had been isolated from rare actinomycetes, increasing to 361, 745, 1276, 2250, 2500 bioactive metabolites by 1980, 1984, 1988, 2005, and 2010, severally. In our previous review, we tend to summarize the novel families, novel genera, and new species of rare actinomycetes from marine habitats

together with bioactive compounds according from 2007 to mid-2013. The aim of this review is to summarise new species of marine rare actinomycetes and, as a result, bioactive compounds discovered between mid-2013 and mid-2017, as well as to address their chemical diversity and biotechnological potential.

1.2.2 Role of marine actinomycetes:

Actinomycetes play an important role within the marine environment aside from antibiotic production (Das et al. 2006). The degradation and turnover of varied materials are endless processes mediated by the action of a spread of microorganisms (Jensen et al., 2005a, Lam, 2006). There's speculation that the rise or decrease of a selected enzyme-producing being might indicate the concentration of natural substrate and conditions of the setting (Ramesh and Mathivanan 2009). Chandramohan et al. (1972) reported on the cellulolytic behaviour of marine actinomycetes, Pisano et al. (1992) reported on chitinolytic actinomycetes, and a variety of industrially important protein manufacturing actinomycetes were reported (Ramesh and Mathivanan 2009). Actinomycetes also are reported to contribute to the breakdown and recycling of organic compounds (Goodfellow and Haynes 1984). They also help with organic matter mineralization, mineral nutrient immobilisation, nitrogen fixation, physical parameter development, and environmental protection (Goodfellow and Williams 1983).

1.2.3 Secondary metabolites from marine actinomycetes:

Almost millions of years before all these organisms were created and evolution happened. The breakdown of substances including fats, proteins, or nucleic acid is called primary metabolites. Secondary metabolites directly do not give to the basal metabolites but rather act as critical factors to kill or attract other organisms and thus increases their survival. Secondary metabolites are both prokaryotic and eukaryotic micro-organisms with unicellular bacteria. They can also banish predator's organism because of their toxic type nature.

1.2.4 Characterization of streptomyces species using FTIR and GC-MS analysis:

The identification of the antifungal compound present in the *Streptomyces* sp. The extract was performed with the aid of GC-MS analysis. From FTIR we can analyze chemical bond functional groups by absorption of infrared radiation which provokes vibration modes in bond, the especially chemical bond of organic material. Detection limits vary great but are

sometimes. The gas chromatography and mass spectrometry (GC-MS) approach was used to identify the active compounds from TLC plates. The mass spectrum was captured using an AGILENT GC-MS at 70 degrees Celsius. After obtaining the peaks from gas chromatography, the compounds were identified using the NIST library's database.

1.3 INTRODUCTION AND CLINICAL EFFECT OF *C. ALBICANS*:

Candida albicans are fungal pathogens of humans. It can populate nearly every tissue & organ and cause serious invasive infection. It is a common fungal pathogen of humans, causing mucosal and systemic infection in immune patients. *C. albicans* have the ability to survive in every slit of a mammalian host. It is a member of the healthy microbiota.

CHAPTER 2

LITERATURE SURVEY

Zhongihu Zheng, Wei Zeng, *et al.*, (2000) determined that marine actinomycetes showed antitumor activity. 20.6% of marine actinomycetes showed cytotoxicity activity on P388 cells and 18.6% on KB cells. The genus *Micromonospora* revealed the highest positive rate of inducing activity. They concluded that the actinomycetes could be a promising source for antitumor bioactive bioagents.

N.K. Asha Devi, M. Jeyarani, *et al.*, (2006) indicated that *Streptomyces sp.* showed a good level of antibacterial and antifungal effect against particular human pathogens like *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Vibrio cholerae*, *Klebsiella sp.* And *Aspergillus niger*. Therefore, it is identified that streptomyces of actinomycetes can offer the potential to develop treatments for disease based on the physiological role of secondary metabolites and also be used in the discovery of antibiotics and novel species of marine actinomycetes.

N S Basha, R Rekha, *et al.*, (2007) explained that the marine actinomycetes may act as a potential source of high substrate specificity L-asparaginase (an anti-leukemia agent). Isolates showed the highest productivity of protein content and had an optimum activity at pH 7.5.

Subramani Ramesh and Narayanasamy Mathivanan (2009) explained that among 208 marine actinomycetes 111 isolates showed antimicrobial activity against human pathogens and also against two plant pathogens. Some of the isolates produced lipase, gelatinase, and cellulase which show that there is a rapid increase in finding industrially marine actinomycetes because they act as the vital source for the discovery of useful enzymes.

E. Esin HAMEŞ-KOCABAŞ (2012) was discovered the new species of actinomycetes and novel metabolites from the marine environment namely, sponge and soil sediments. Many factors such as the use of enrichment and pre-treatment techniques, and selection of antibiotic supplements and growth media are considered for the successful isolation of marine actinomycetes. Important considerations for the cultivation of marine

actinomycetes are stopping the growth of irrelevant fast-growing species, providing favorable nutrients to enrich the growth, and increasing the time of incubation for slow growing strains.

Vaibhav Ambavane, Pradipta Tokdar, *et al.*, (2014) identified Caerulomycin from marine actinomycete which is a novel and superior antifungal compound used for treating infectious diseases.

S. Usha Nandhini, P. Jaya Bharathy, *et al.*, (2014) discovered a strain as *Streptomyces rubralavendulae* strain SU1 (HQ234765) which could be used as an alternative to the host for medicinal activity against fungal infections.

Mohamed Saleh Abdelfattah *et al.*, (2016) evaluated the cytotoxic activity of different actinomycetes species isolated from the Red Sea coast in Sharm el-Sheikh, Egypt. He found the result that five ethyl acetate extracts exhibited cytotoxicity towards breast cancer cell lines MDA-MB-231. The isolate EGY8(crude extract) exhibits slightly high antimicrobial activity against different test microorganism, he finally concluded that the marine sediment has the potent source of novel species of actinomycetes and the extracted isolates plays important role in the development of microbial natural product research.

Olabisi Flora Davies-Bolorunduro *et al.*, (2019) reveal the anticancer potential of a metabolic compound from marine actinomycetes isolated from Lagos Lagoon, which could be exploited for therapeutic purposes. They purified the two novel anticancer compounds: ULDF4 and ULDF5, which were isolated from the metabolic extracts. These compounds evaluated for cytotoxicity activity and justified that these compounds have the anticancer therapeutic potential of actinomycetes, which could immensely benefit the development of useful chemotherapeutics. Finally, they concluded that these studies could improve our understanding of the distribution of actinobacteria in underexplored tropical marine habitats and help develop high-throughput screening programs for anticancer drugs and chemotherapeutics.

Mary Sulakshana Palla *et al.*, (2018) isolated the actinomycetes from Koringa mangrove soil samples and assessed for antagonistic activity against various bacteria and fungi.

Based on physiological, morphological characteristics, and 16s rRNA gene sequencing the isolated strain KMFA-1 was identified as *Streptomyces hydrogenates*. The bioactive metabolite was extracted from the crude supernatant and it was found to stable at 80 °C. He concluded that that the *Streptomyces hydrogenates* produced an interesting antifungal metabolite and further investigations are necessary for isolation and chemical characterization of the compound by chromatographic and other spectral analysis.

CHAPTER 3

AIM AND OBJECTIVES

3.1 AIM

The present study aims to identify and isolate high antifungal activity producing actinomycetes strains from marine habitat, to study antifungal production to determine theirs for the inhibition of the growth of the common human pathogens.

3.2 OBJECTIVES

- To isolate and characterize the marine actinomycetes from marine soil sediments near ECR beach.
- To analyze the indigenous marine isolates with high potential to produce bioactive secondary metabolites.
- To evaluate the antibiofilm activity and Fourier Transform Infrared (FTIR) spectral analysis of the bioactive metabolite.
- To determine the antifungal bioactive compound with the help of GC-MS analysis

CHAPTER 4

MATERIALS AND METHODS

4.1 SAMPLE COLLECTION AND ISOLATION OF ACTINOMYCETES:

With reference to the modified methodology of Rajivgandhi *et al.* (2016), the isolation of actinomycetes was performed. For this study, the marine soil sediment was collected from the eastern coastal region (ECR), Akkarai beach, Chennai (Tamil Nadu, India). Samples are packed in the polythene bag and were immediately transported to the laboratory. The soil samples were air-dried and kept at 45°C in a hot air oven for 10 mins to prevent fungal and bacterial contamination. About 1g of soil sample was transferred to a flask containing 99ml of sterile 50% seawater. The soil sediment suspension was further diluted to a 10^{-5} dilution level. 1ml of the diluted suspension was spread over the surface of the Starch casein agar (SCA) medium. Starch casein agar (SCA) medium was prepared with amikacin (2.5 mg mL^{-1}) and fu-conazole (7.5 mg mL^{-1}) antibiotics. The Petri plates were then incubated for 21 days at 28 °C. After the 5th day, the colonies were periodically monitored with diverse morphologies were purified in SCA by frequent sub-culturing via streak plate technique. In SCA slants, the isolated pure cultures were preserved at 4 °C for further experiments.

4.2 MORPHOLOGICAL MANIFESTATION OF PURE ISOLATE:

The morphological method consists of micro-morphological and macro-morphological characterization of the pure isolate of marine actinomycetes was studied (Srivastava and Dubey 2016). The isolated actinomycetes were observed for macroscopic characterization such as size, shape, aerial mycelium, and substrate mycelium. Then, the microscopic observation was performed by standard coverslip technique and Gram staining method. The coverslip technique was performed by inserting the coverslip into the solidified starch casein agar medium plate at the angle of 45° and the plates were incubated at room temperature. Using sterile forceps, the coverslip was removed and placed on a clean glass slide and processed for microscopic observation.

4.3 TEST PATHOGENIC FUNGI:

A Clinical isolate of *C. albicans* (CA1) was obtained from KIRND Institute of Research and Developments PVT. LT, Tiruchirappalli, Tamil Nadu, India. *C.albicans* was maintained on suborned dextrose agar (SDA) at 4 °C

4.4 FERMENTATION AND EXTRACTION OF SECONDARY METABOLITES:

In a 250-mL culture medium, the selected fully-grown *Streptomyces sp.* was used as an inoculum. The culture medium contains 0.5% dextrose, 0.2% peptone, 0.25% sodium chloride (NaCl), 0.4% Calcium Carbonate (CaCO₃), and 0.2% Potassium nitrate in a 500-mL Erlenmeyer flask. The culture flask was incubated for 7 to 10 days in a shaker incubator at 28 °C. After incubation, the culture broth was centrifuged at 5000 rpm for 20mins at room temperature. From the separated biomass, the cell-free culture supernatant was collected and mixed with the equal volume of ethyl acetate and placed in a rotary shaker at 200 rpm overnight. The extracted supernatant was then concentrated using a rotary vacuum evaporator and air-dried. The air-dried extract was dissolved in 5% DMSO and processed for further investigation of antifungal activity.

4.5 ANTIBIOFILM ACTIVITY ASSAY AGAINST C. ALBICANS:

The Crystal Violet assay is based on staining cells that are attached to cell culture plates which were performed with 24-well polystyrene microtiter plates. With the help of 200 µl PBS, the microtiter plates were washed twice and air-dried for 45mins. The reaction mixture for the antibiofilm assay (1 mL) contains 100 µL of *C. Albicans* suspension (10⁶ cell mL⁻¹) and various concentrations (1–10 µl mL⁻¹) of *Streptomyces sp.* metabolite extract. The wells with the absence of *Streptomyces sp.* extract served as the control and the microtiter plates were placed under incubation for 2 days at 37 °C. After the incubation, the wells were stained with 100 µl of 0.4% aqueous crystal violet solution for 5-6 mins. Afterward, washing was done twice with MilliQ water to remove excess stain and then air-dried. Subsequently, destaining was done with the 1mL of 95% ethanol, and using a multi-label reader, the optical density was measured at 570 nm.

4.6 LIGHT MICROSCOPIC OBSERVATION OF C. ALBICANS BIOFILM:

In sterile glass slides (1×1 cm), the *C. albicans* biofilms were allowed to grow on the slides and placed inside the wells of the microtiter plates. These microtiter plates contain SD broth supplemented with a concentration of *Streptomyces sp.* extract, which inhibits the activity of biofilm (BIC). Without shaking the plates were kept in the incubator for 2 days at 37 °C. Once the incubation period over, the slides were introduced for crystal violet

staining, followed by ethyl alcohol destaining. Then, the slides were kept under the light microscope for observation at a total magnification of 400× (Nikon Eclipse Ti 100, Japan)

4.7 CHARACTERIZATION OF *STREPTOMYCES SP.* EXTRACT:

4.7.1 Fourier Transform Infrared (FTIR) spectral analysis

FTIR spectrum of each bioactive extract was identified using Shimadzu IR-470 plus. The scanning range of the spectra ranges from 400 to 4000 cm^{-1} and the graph was plotted between intensity and wave number. FTIR spectroscopic analysis was used to identify the major functional groups and structural present in the *Streptomyces sp.* ethyl acetate extract. Bioactive sample (5mg) and KBr pelleting were mixed properly and a thin KBr disk was prepared for infrared radiation analysis. Scans were obtained with a resolution of 4 cm^{-1} . The symmetrical, asymmetrical, and stretching frequencies were examined and studied from infrared radiation bands. Then, the functional group in the *Streptomyces sp.* ethyl acetate extract was identified. (Perkin Elmer, Inc., USA).

4.7.2 Identification of bioactive compound by GC-MS analysis

The identification of the bioactive compound present in the *Streptomyces sp.* ethyl acetate extract was performed with the aid of GC-MS analysis. GC-MS analysis was carried out on Agilent GC-MS technologies. The extract was examined for bioactive metabolites with an AOC-20i/s autosampler. The capillary column was directly coupled to the MS. Analysis conditions were 20mins at 100 °C, then 280 °C at a rate of 5 °C/min and the injector port temperature was maintained at 250 °C. Helium was used as the carrier gas. Once the peaks are obtained from the gas chromatography, the compounds were reported by analyzing with the database in the NIST library (Ezhilan and Neelamegam 2012).

CHAPTER 5

RESULTS AND DISCUSSION

5.1 SAMPLE COLLECTION AND ISOLATION OF PURE CULTURE:

The collected soil sediment sample was air-dried and kept in a hot air oven for 10mins. Then, serially diluted to a 10^{-5} dilution level and cultured in starch casein agar (SCA) medium.



Fig 5.1. Soil sediment sample



Fig 5.1.1. Serial dilution

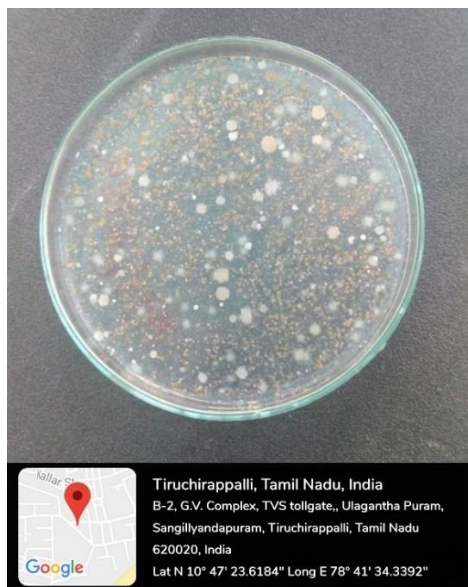


Fig 5.1.2. Microbial consortium from sediment

A total of 9 marine actinomycetes were isolated from the soil sediment sample collected from ECR beach. Only *Streptomyces* sp. colonies were isolated and sub-cultured in the isolation Petri plates. All these *Streptomyces* sp. were isolated as pure form and preserved at 4 °C for further use.

5.2 MORPHOLOGICAL OBSERVATION OF PURE ISOLATE:

The microscopic and macroscopic technique were performed to describe the potent strain. Cultural morphological characteristics of the pure isolate is listed (Table 1).

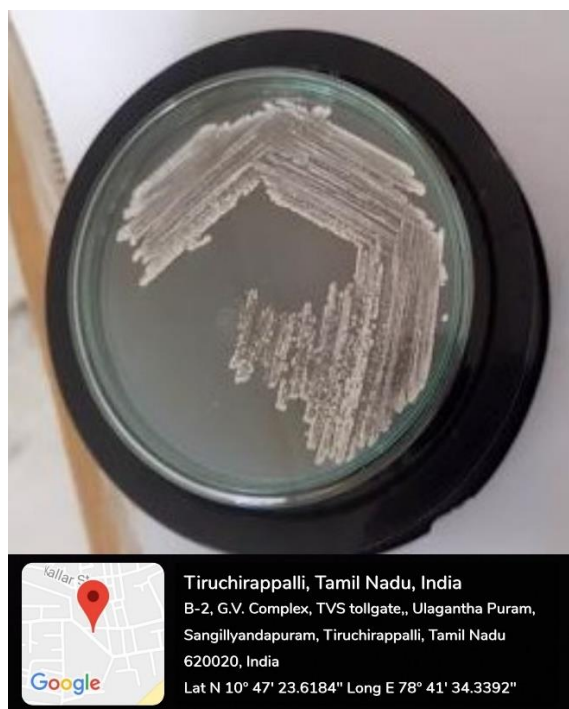


Fig 5.2. Aerial mycelium

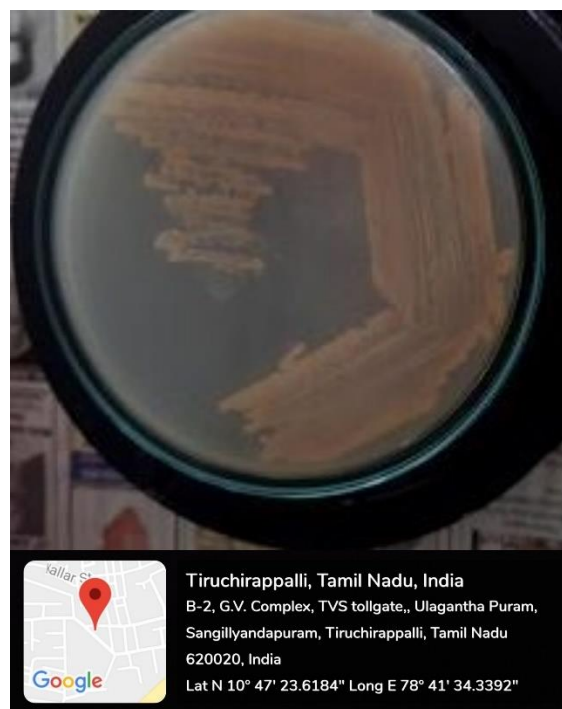


Fig 5.2.1. Substrate mycelium

Table 1: Morphological characteristics of pure isolate

Morphological characteristics	Observation
Temperature	28 °C (Mesophilic)
pH	7.2
Color	Whitish grey
Aerial mycelium	Present
Substrate mycelium	Present
Pigmentation in medium	Yellow diffusible pigment

Spore chain	Spirals
Spore surface	Smooth

The microscopic technique reveals that pure isolate is an actinobacteria that forms well-developed, extensively branched aerial and substrate mycelia concealed with arthrosporic chains at the end. Based on the macroscopic and microscopic characteristics it is indicated that the isolate belongs to *Streptomyces* genus of actinomycetes.

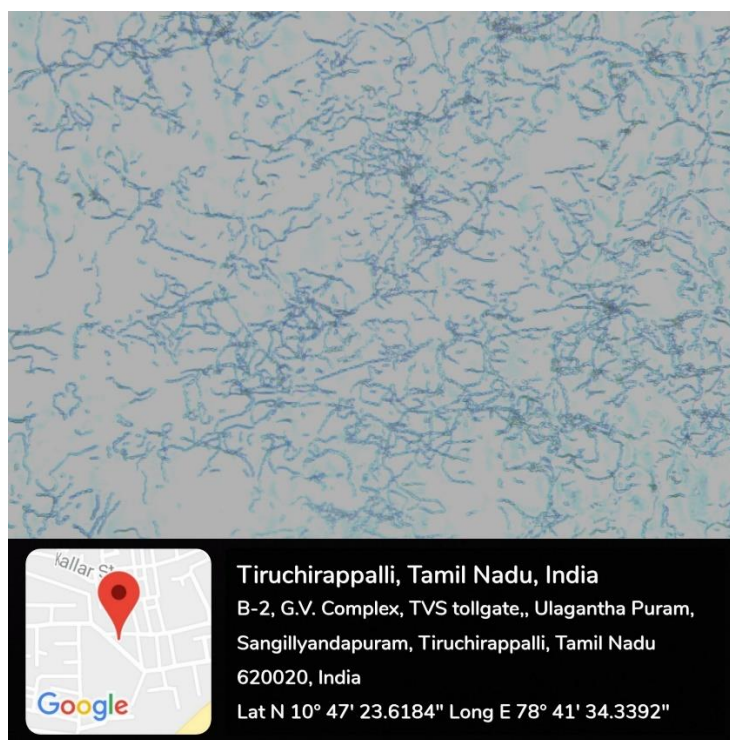


Fig 5.2.2. Microscopic image of *Streptomyces* sp.

5.3 ANTIBIOFILM ACTIVITY OF *STREPTOMYCES* CRUDE EXTRACT AGAINST *C. ALBICANS* BIOFILM:

Ethyl acetate extract of *Streptomyces* sp. isolate showed significant inhibition on the biofilm formation of *C. albicans*, with a significant increase in inhibition with the increase of *Streptomyces* sp. extract concentration (10-50 $\mu\text{L mL}^{-1}$).

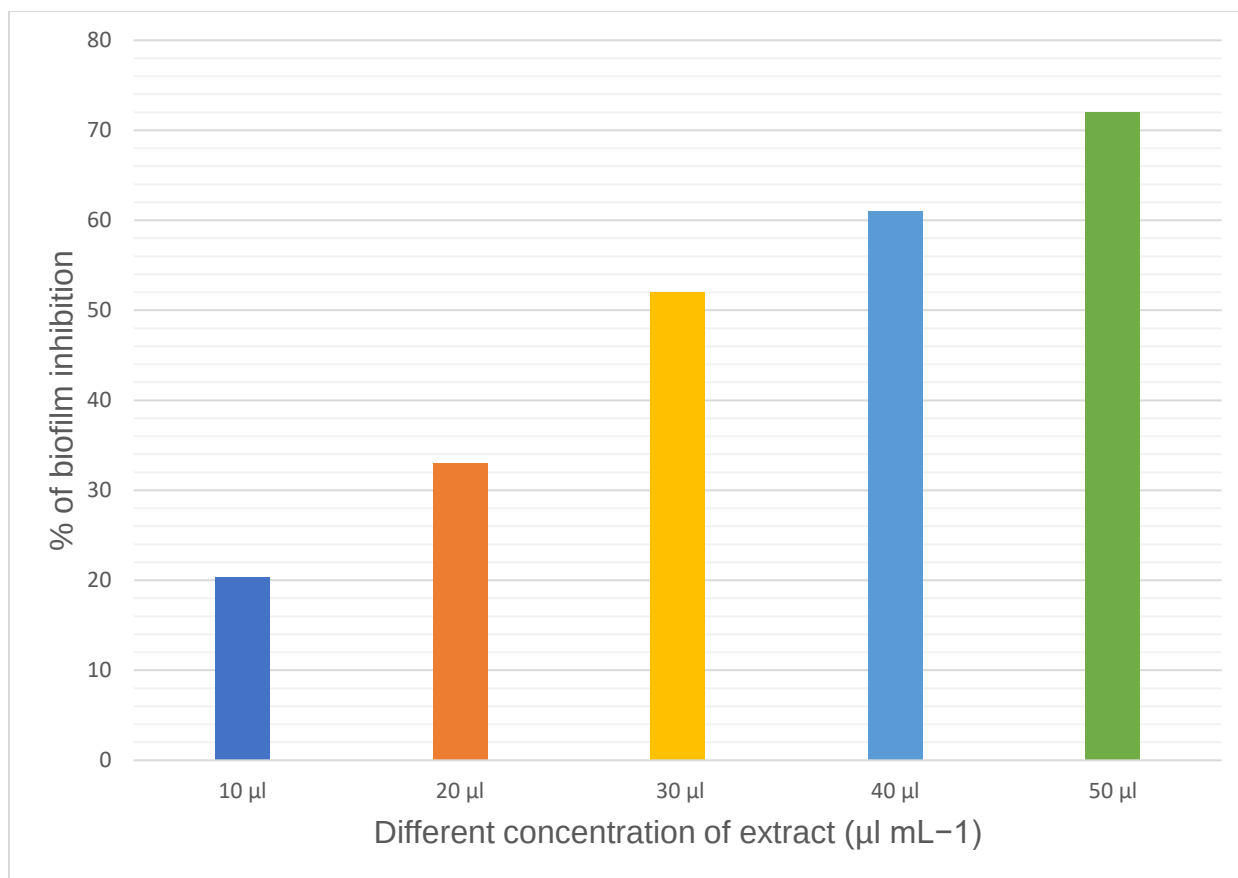


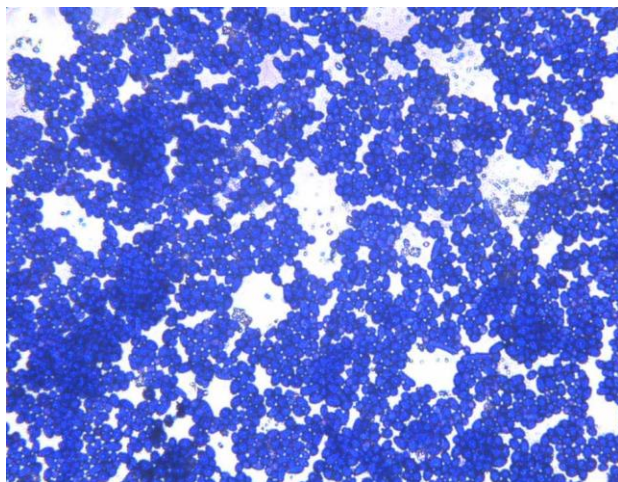
Fig 5.3. Antibiofilm effect of *Streptomyces sp.* extract against *C. Albicans* biofilm

The highest inhibition in *C. Albicans* biofilm of 72% at 50 $\mu\text{l mL}^{-1}$ concentration (Fig. 5.3). The 50% biofilm inhibition was indicated at 30 $\mu\text{l mL}^{-1}$ concentration of *Streptomyces sp.* extract which was specified as the biofilm inhibitory concentration (BIC50). For concentrations of 20 and 10 $\mu\text{l mL}^{-1}$, the crude extract had very less effect on the growth of the strain but inhibited the formation of biofilm by 33% and 20% respectively. Our present study results that the secondary metabolites of *Streptomyces sp.* at BIC have only antibiofilm activity against test organism *C. albicans*.

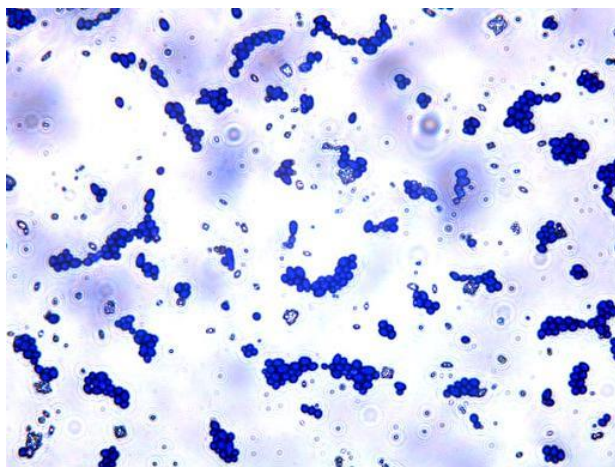
5.4 LIGHT MICROSCOPIC OBSERVATION OF *C. ALBICANS* BIOFILM:

The biofilm inhibition potential of the *Streptomyces sp.* extract was studied on glass slide surface using light microscopic technique. Light microscopic observation was performed for *C. Albicans* biofilm in the absence and presence of *Streptomyces sp.* extract (BIC50)

were examined using in vitro crystal violet assay. Light microscopic observation revealed that the presence of increased yeast cells on the surface layer of the control cell when compared to the treated cells which indicates reduction in the biofilm formation in the treated slides (Fig. 5.4).



Control



Treated

Fig 5.4. Light microscopic images ($\times 400$) revealed the antibiofilm activity of *Streptomyces* sp. extract at their BIC50 ($3 \mu\text{L mL}^{-1}$) against *C. albicans*

5.5 CHARACTERIZATION OF *STREPTOMYCES* SP. EXTRACT:

5.5.1 Fourier Transform Infrared (FTIR) spectral analysis

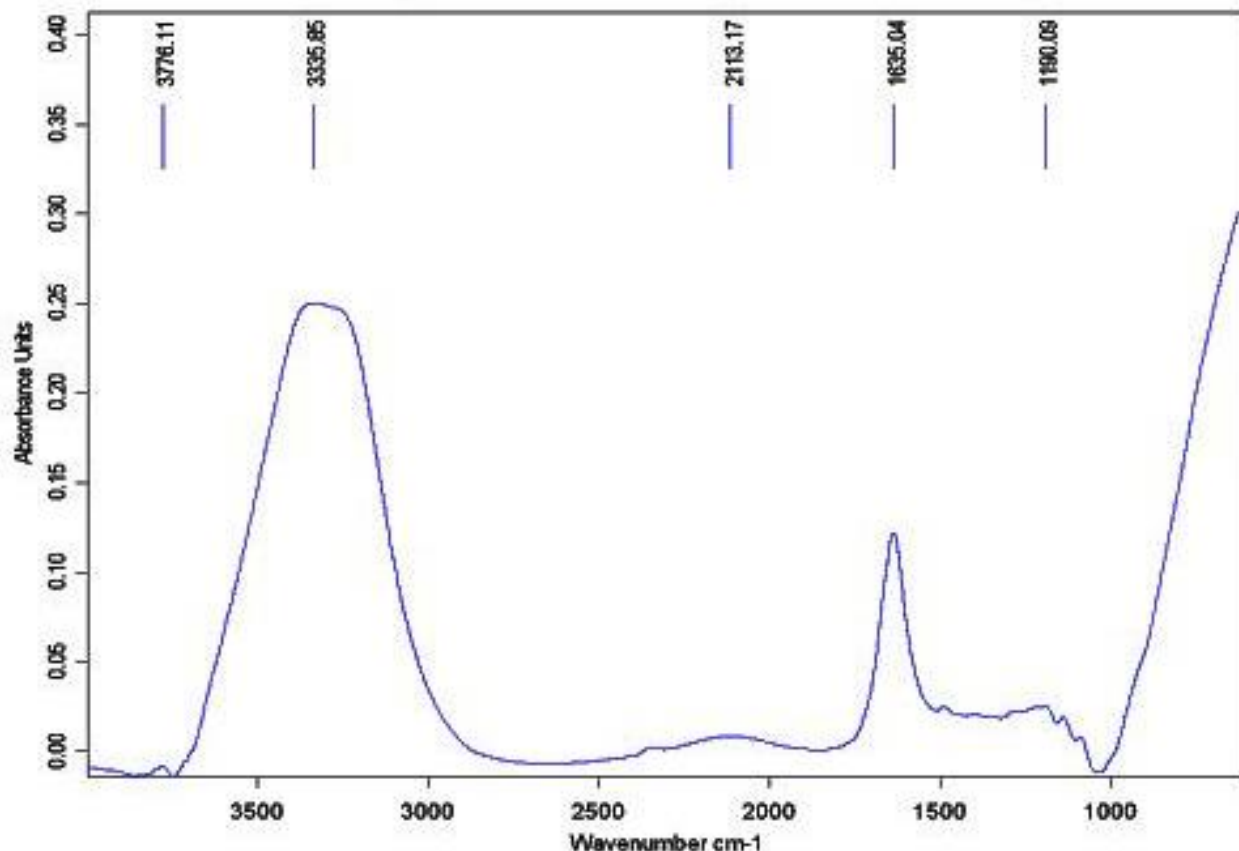


Fig 5.5. FTIR spectrum of secondary metabolites of *Streptomyces* sp. ethyl acetate extract from marine soil sediment

Fourier transform infrared spectroscopy spectra of the purified extract of *Streptomyces* sp. showed different peaks corresponding to standard library spectra (Fig. 5.5). FTIR spectrum of *Streptomyces* sp. extract exhibited different bands at 3776.11, 3335.85, 2113.17, 1635.04 and 1190.09 cm⁻¹. The peak observed at 3776.11 cm⁻¹ corresponds to hydroxyl group (OH stretching), the broad band at 3335.85 cm⁻¹ was attributed to N-H stretching in Aliphatic secondary amino group. A peak observed at 2113.17 cm⁻¹ indicated double bond CN stretching and the absorption peak at 1635.04 cm⁻¹ corresponds to double bond of polygenic compound. From the FTIR analysis, it was evident that the

crude extract of *Streptomyces* sp. is rich in hydrocarbons substituted with OH, amino, aldehyde, or keto group.

5.5.2 GC-MS analysis

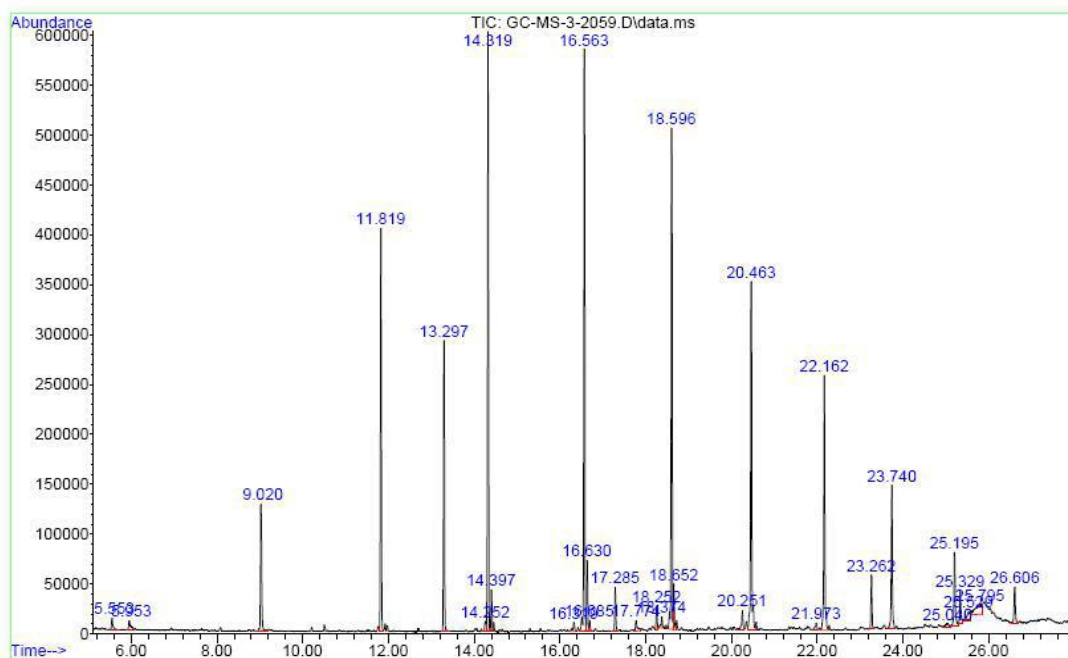


Fig 5.6. GC-MS analysis of secondary metabolites of *Streptomyces* sp. ethyl acetate extract from marine soil sediment

GC-MS was done to identify the specific compounds in *Streptomyces* sp. by comparing its retention factor to the standard. The analysis revealed the presence of 30 compounds with different retention times listed in the below table.

Table 2: List of bioactive constituents identified through GC-MS analysis of *Streptomyces* sp. extract

Peak	Area%	Retention time	Compound
1	0.43	5.553	2-Pentene, 4,4-dimethyl
2	0.47	5.953	1-Decene
3	3.87	9.020	11,13-Tetradecadien-1-ol

4	8.69	11.819	5-Tetradecene
5	7.66	13.297	Phenol, 2,4-bis(1,1-dimethylethyl)
6	0.35	14.252	Cyclohexane, (1-hexyltetradecyl)
7	13.37	14.319	2-Tetradecene
8	0.97	14.397	1-Iodo-2-methylundecane
9	0.38	16.319	1-Decanol, 2-ethyl
10	14.75	16.563	9-Eicosene
11	1.66	16.630	Heptadecane
12	0.32	16.685	5-Ethyl-1-nonene
13	1.11	17.285	Phthalic acid, isobutyl cyclohexyl
14	0.53	17.774	1,2-Benzenedicarboxylic acid
15	0.60	18.252	Dibutyl phthalate
16	0.37	18.374	Dodecane, 3-methyl
17	13.34	18.596	1-Octadecene
18	0.93	18.652	Heptadecane, 9-octyl
19	0.68	20.251	Nonadecane
20	10.46	20.463	1-Docosene
21	0.31	21.973	Tetracontane, 3,5,24-trimethyl
22	6.54	22.162	Heptadecyl heptafluorobutyrate
23	1.31	23.262	1,2-Benzenedicarboxylic acid
24	3.89	23.740	Heptadecyl trifluoroacetate
25	0.31	25.040	1-Bromo-11-iodoundecane
26	2.33	25.195	Cyclooctacosane
27	0.98	25.329	2,6,10,14,18,22-Tetracosahexaene
28	0.68	25.529	Propanenitrile, 3-(5-diethylamino-1-methyl-3-pentynyloxy)
29	1.54	25.795	n-Propyl 11-octadecenoate
30	1.19	26.606	Heptacosyl acetate

Table 2 shows the GC-MS spectrum of ethyl acetate extract of *Streptomyces* sp. which revealed the presence of 30 compounds. Among the 30 compounds, 5 compounds had a high peak percentage with a retention time of 16.563 min (C1), 14.319 min (C2), 18.596 min (C3), 11.819 min (C4), and 20.463 min (C5). Based on the available library reference data, the compounds C1, C2, C3, C4, and C5 were determined as 9-Eicosene, 2-Tetradecene, 1-Octadecene, 5-Tetradecene, and 1-Docosene respectively (Table 2). Eicosane and DBP, isolated from *Streptomyces* sp., were documented for their antifungal activity against *Rhizoctonia solani* (Ahsan et al. 2017). Thus, from the GC-MS spectrum, it is evidenced that most of the compounds identified from *Streptomyces* sp. extract have been expressed for their antimicrobial and antifungal activities.

CHAPTER 6

SUMMARY AND CONCLUSION

The soil sample were collected and *Streptomyces sp.* was isolated and cultured at particular temperature. Morphological characteristics revealed the nature of the isolated bacteria with pH range of 7.2 and aerial mycelium and substrate mycelium was present. In microscopic studies the sample was confirmed as actinobacteria which belongs to the genus *Actinomyces*. The antibiofilm activity of *Streptomyces* showed good inhibition level against *C. albicans* where the highest inhibition rate was 72% of 50 $\mu\text{l mL}^{-1}$ concentration. FT-IR analysis was done to find out the functional groups and it confirmed the presence of hydroxyl group of *Streptomyces*. GC-MS was done for the identification of compounds and the study confirm the presence of marine actinobacteria.

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IDENTIFICATION OF ANTIFUNGAL COMPOUND FROM MARINE ACTINOMYCETES

Submitted in partial fulfilment **of the requirements for the award of Bachelor of Technology degree in** Biotechnology by D HARI HARAN (37230023) / DEPARTMENT OF BIOTECHNOLOGY SCHOOL OF BIO AND CHEMICAL ENGINEERING **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** (DEEMED TO BE UNIVERSITY) Accredited with Grade "A" by NAAC JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119 MARCH- 2021 DEPARTMENT OF BIOTECHNOLOGY **BONAFIDE CERTIFICATE** This is to certify that this Project Report is the bonafide work of D HARI HARAN (37230023) who carried out the project entitled "Identification of antifungal compound from marine actinomycetes" under our supervision from December 2020 to February 2021.

Internal Guide External Guide Dr. Y SWARNALATHA Dr. C KARTHIKEYAN **Head of the Department** Dr. V RAMESH KUMAR

Submitted for Viva
voce Examination held on _____ Internal Examiner External Examiner
DECLARATION I, D Hariharan (37230023), hereby declare that the Project Report entitled "Identification of antifungal compound from marine actinomycetes" done by me under the guidance of Dr. C Karthikeyan at KIRND Institute of research and developments PVT.