

BIOPROSPECTING OF FISH GUT ASSOCIATED ACTINOMYCETES PROJECT WORK (PHASE-I)

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

by

TAMIL SELVAM.S (39230052)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & 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**

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This is to certify that this Project Report is the bonafide work of **TAMIL SELVAM.S(39230052)** who carried out the project "**BIOPROSPECTING OF FISH GUT ASSOCIATED ACTINOMYCETES**" under our supervision from September 2022 to April 2023.

Internal Guide

Dr. USHA NANDHINI

External guide

Dr. V. GOPI KRISHNAN

Head of the Department

Dr. V. RAMESH KUMAR

Submitted for Viva-voce Examination held on 11/05/2023

11/05/23
Internal Examiner
External Examiner

DECLARATION

I, **TAMIL SELVAM.S** (Reg. no: 39230052) hereby declare that the Project report entitled "**BIOPROSPECTING OF FISH GUT ASSOCIATED ACTINOMYCETES**" done by me under the guidance of **DR. USHA NANDHINI** (internal guide) and **DR. V. GOPI KRISHNAN** (external guide) at **CENTRE FOR DRUG DISCOVERY AND DEVELOPMENT, COL. Dr.JEPPIAAR RESEARCH PARK, Sathyabama Institute of Science and Technology, Chennai**, is submitted in partial fulfilment of the requirements for the award of a Bachelor of Technology degree in **Biotechnology**.

DATE : 11/05/2023

PLACE : CHENNAI



SIGNATURE OF THE CANDIDATE

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ABSTRACT

Currently, marine microorganisms comprise precious, vital source as new and promising bioactive metabolites with significant biological activities. Historically, marine invertebrates have been also a prolific source of unique natural products with a diverse array of biological activities. In general, marine sediment derived actinobacteria are reported as promising source for novel bioactive compounds, whereas reports on bioactive compounds from fish gut associated actinobacteria are scanty. The present study aims to investigate antimicrobial, anti-TB, antibiofilm and antioxidant activity of fish gut associated actinobacteria isolated from Indian Squid (*Uroteuthis duvaucelii*). Actinobacterial strains are isolated from Indian Squid (*Uroteuthis duvaucelii*) and Indian mackerel (*Rastrelliger kanagurta*) fish gut samples by adopting standard spread plate method on SCA media. A total of 1 actinobacterial colony was found out of 39. Antimicrobial activity of actinobacterial strain isolated from Squid gut sample was determined by agar plug and well diffusion methods. Actinobacterial strain from Squid gut (SQA 4) was showed promising antimicrobial activity against *Staphylococcus aureus* (14mm). We have done the co-culture method also, in that the antimicrobial activity was increased *Staphylococcus aureus* (16mm) and *Candida albicans* (12mm) respectively. Crude extract from the strain SQA 4 was produced by adopting agar surface fermentation and extracted using ethyl acetate. The crude extract showed 31% inhibition against *Mycobacterium smegmatis* at 200µg/ml concentration and 61% inhibition against *Mycobacterium tuberculosis* H37Rv at 200µg/ml. It showed antibiofilm activity of 72.5% inhibition against *Acinetobacter baumannii* and antioxidant property of 62.1% scavenging effect. Based on their phenotypic characteristics, strain SQA 4 was identified as *Streptomyces* sp. The findings suggested that the fish associated actinobacteria is a promising source for multifunctional bioactive compounds for the development of novel therapeutics drugs.

Keywords: Actinobacteria, Fish gut microbiota, anti-TB, antimicrobial and antioxidant.

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LIST OF ABBREVIATIONS

ACRONYM	EXPANSION
<i>A. baumannii</i>	<i>Acinetobacter baumannii</i>
<i>A. caviae</i>	<i>Aeromonas caviae</i>
<i>A. salmonicida</i>	<i>Aeromonas salmonicida</i>
AIA	Actinomycetes Isolation Agar
<i>C. albicans</i>	<i>Candida albicans</i>
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
ISP2	International Streptomyces Project 2
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PEG	Polyethylene glycol
RF	Retention factor
RNA	Ribonucleic acid
RPM	Rotation per minute
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SCA	Starch casein agar
Sp.	Species
TLC	Thin layer chromatography
UV	Ultraviolet-visible spectroscopy

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CHAPTER 1

INTRODUCTION

1.1 Actinomycetes

Actinomycetes are aerobic gramme-positive bacteria that develop branching filaments, hyphae, or asexual spores and have a high G+ C (Guanine+ Cytosine) content. Though they are categorized as bacteria they stand out sufficiently to be studied as separate group. Ferdinand Cohn initially found actinomycete in 1875, and later Harz gave it the name actinomyces (ray fungus) in 1877. Gasperini was the first to identify it as having the ability to eradicate germs and fungi in 1890. Actinomycetes grow well on simple laboratory media with different chemical composition but their growth is much slower than that of other bacterial groups. Most actinomycetes produce leathery, smooth-surfaced, cottony colonies in solid mediums. (Gopikrishnan *et al.*, 2019)

The prokaryotic organisms known as actinomycetes are categorized as bacteria but stand out sufficiently to be studied separately. They are a crucial part of the bacterial ecosystem, particularly in environments with high pH, high temperatures, or water stress. Actinomycetes' elongated cells that branch into filaments or hyphae give them a fungi-like appearance. Actinomycete hyphae are significantly smaller than fungal hyphae, making them distinguishable from them other as hyphae (Balagurunathan *et al.*, 2020). (Pepper, Gerba and Gentry, 2015)

Actinomycetota are of significant interest because of their function in the soil, but there is still much to learn about them. They might be more prevalent in fresh waters (Ghai *et al.*, 2011) while now being mostly understood as soil bacteria. Actinomycetota, one of the major bacterial phyla, comprises Streptomyces, one of the largest bacterial genera. (Yunianta and Yusof, 2012) Actinomycetes are prokaryotic organisms that are classified as bacteria, but are unique enough to be discussed as an individual group. The entire quantity of actinomycete is often one to two orders of magnitude lower than the total number of bacteria. They are a crucial part of the bacterial ecosystem, particularly in environments with high pH, high temperatures, or water stress.

Actinomycetes' elongated cells that branch into filaments or hyphae give them a fungi-like appearance. Actinomycete hyphae are significantly smaller than fungal hyphae, making them distinguishable from them other as hyphae. Extreme habitats can contain a variety of unusual growth conditions, such as high pressure, salt, alkaline and acidic pH, high temperatures, and high and low temperatures. Due to their distinctive mechanisms for adjusting to their harsh environments and their capacity to produce unusual bioactive chemicals, microorganisms from harsh habitats have drawn a lot of interest (Meklat *et al.*, 2011). Despite the excitement, actinomycetes that thrive in challenging environments haven't been thoroughly investigated since the pioneering *Actinopolyspora halophila* was accidentally discovered (Subramani and Aalbersberg, 2013). In a range of settings, such as salt soil, alkaline soil, salty seas, and the ocean, researchers have been searching for uncommon actinomycetes (Jiang *et al.*, 2016).

1.2 Rare habitants of actinomycetes

In recent years, re-isolation of known actinomycetes and antibiotics has commonly resulted from research of actinomycetes from common environments. Instead, bioprospecting of uncharted or understudied ecosystems, such as those in the sea, the desert, forests, caves, and hills, has proven to be an effective method for obtaining a vast array of bioactive compounds from novel bioactive actinomycetes, including anti-TB metabolites. Actinomycetes were bioprospected from understudied environments in India with a focus on their antitubercular activity is blooming part and many studies are undergoing (Manikkam *et al.*, 2014)

Actinomycetes are a diverse group of bacteria that are found in a wide variety of habitats, including soil, water, and plant surfaces. Some rare habitants of actinomycetes include:

1.2.1 Deep-sea sediments

Actinomycetes have been found in deep-sea sediments, which are characterized by high pressure, low temperature, and low oxygen levels. These bacteria have unique adaptations that allow them to survive in these extreme conditions.

1.2.2 Caves

Actinomycetes have been found in caves, where they play an important role in the decomposition of organic matter. Some actinomycetes are also able to produce bioluminescence, which can create stunning visual displays in cave environments.

1.2.3 Hot springs

Actinomycetes have been found in hot springs, which are characterized by high temperatures and extreme pH levels. These bacteria have unique adaptations that allow them to survive in these harsh conditions.

1.2.4 Symbiotic relationships

Some actinomycetes form symbiotic relationships with other organisms, such as plants and insects. For example, some actinomycetes live in the roots of plants and help them to absorb nutrients from the soil.

1.2.5 Arctic and Antarctic regions

Actinomycetes have also been found in the cold and harsh environments of the Arctic and Antarctic regions. These bacteria are able to survive in the freezing temperatures and low nutrient conditions of these regions.

1.3 Streptomyces

Streptomyces is the biggest genera of Actinomycetota and the type genus of the family Streptomycetaceae (Kämpfer, 2006). Streptomyces bacteria have been classified as more than 500 different species (DS, 2016). Streptomyces, like the other Actinomycetota, have gram-positive bacteria and high GC content in their genomes (Madigan and Martinko, 2005). Most streptomyces generate spores and are primarily found in soil and decaying vegetation. They are distinguished by their distinctive "earthy" odour, which is caused by the synthesis of a volatile metabolite called geosmin.

Streptomyces has a wide range of applications in biotechnology, agriculture, and medicine due to its capacity to evolve distinctive structural and biological variants (van der Meij *et al.*, 2017). We can construct a basic picture of this process using both the findings from their research and those from earlier publications. C. Hardisson and M.B. Manzanal were the first to explore sporogenesis in *Streptomyces* on a substantial number of species (seven). The two key stages in the sporogenesis of *Streptomyces* are the formation of the arthrospore and the synthesis of the sporulation septum. We hypothesise that this genus has three primary types based on our ultrastructural examination of the sporulation septum development. (Hwang *et al.*, 2014)

1.4 FISH GUT

Fish gut is a complex microbial community, consisting of bacteria, yeast, viruses, archaeans and protozoans in their Gut. These microbes influence various host functions including development, digestion, nutrition, disease resistance and immunity (Merrifield and Ringø, 2014). These recent advances have substantially improved our knowledge of bacterial community profiles in the fish intestinal microbiota in response to a variety of factors affecting the host, including variation in temperature, salinity, developmental stage, digestive physiology and feeding (Ghanbari, Kneifel and Domig, 2015). One important aim of gastro intestine is mucosal tissues, including the skin, olfactory system, gills and also the gut are in direct contact with the environment and thus are the first contact points of the microbes with their host. Fish's gut microbiota can come from a variety of places. It is well accepted that the microbiota of eggs, the water used for larval rearing, and feeding patterns all affect the complexity and reliance of bacterial colonization processes throughout the early stages of larval fish development. It is now understood that the original invaders are species-specific, with changes in the glycoproteins found on the egg surface influencing variations (Larsen, Mohammed and Arias, 2015). The bacteria that come into contact with the eggs and have a chance to colonize them are also determined by the local water's microbial community. The chorion associated bacteria, the first creatures to occupy the developing GUT, are consumed by sterile larvae as soon as they hatch. (Egerton *et al.*, 2018). The newly hatched larvae's gastrointestinal tract frequently contains a few

microbes. Fish larvae first acquire future bacterial homes when they begin ingesting water to control their osmoregulation. The microbiota then becomes even more diverse as a result of nutrition.(Hansen, Strom and Olafsen, 1992)

Fish make up approximately half of all vertebrate varieties. There are over 34,000 different species of fish known. They play a significant role in aquatic systems. (Johny *et al.*, 2021, Kim *et al.*, 2021). Fish is the primary source of protein for humans across many regions of the globe. This is especially true in the majority of developing countries (Latha and Mohan 2013). Fish and other aquatic animals have a special and closely relationship with their surroundings and the microorganisms that live there. Microorganisms abound in the world's oceans According to studies, 3.6×10^{30} microbial cells account for more than 90% of all oceanic biomass. Fish can have a mutualistic or pathogenic relationship with the microorganisms in their environment (Gómez and Balcazar 2008; Sullam *et al.*, 2012). According to various research findings, microorganisms inhabit the digestive tract, gills, skin, and organs that emit light. Additionally, bacteria may be present in the fish internal organs like the liver, kidney, and spleen (Austin, 2002). The microbiota in the fish gut forms an intricate environment with a diverse spectrum of species. The habitat environment, together with other elements including the developmental stage, season, nutritional status, host genetics, and diet composition, has the potential to have a substantial impact on the microbiota (Kim *et al.*, 2021). Early in the 1930s, researchers started studying the fish gut and skin microbial communities to learn more about these intricate bacteria (Uma *et al.*, 2020). Previously, fish were found to have a small number of bacteria in their stomachs, which were thought to be from their environment or nutrition (Cahill, 1990; Ringø *et al.*, 1995). These conclusions were established after conducting experiments using culture-dependent methodologies.

However, we now realize that in the laboratory conditions used at the time, only 10% of microbes might be isolated and cultured (Amann *et al.*, 1995). Since then, scientific methods have greatly improved, and today it is believed that cultivable bacteria make up less than 0.1% of the whole microbial population in the GIT of various fish species (Zhou *et al.*, 2014). Nevertheless, numerous current researchers continue

to publish findings from culture-based methods, extrapolating microbiota function from information gathered from investigations of the growth of bacteria carried out in laboratory environments (Clements *et al.*, 2014). For the analysis of fish microbiota, a broad range of culture-independent approaches are now accessible. They include clone libraries, quantitative real time PCR (qPCR), finger-printing methods such as denaturing gradient gel electrophoresis (DGGE) and temporal temperature gradient electrophoresis (TTGE), and fluorescent in situ hybridization (FISH). These techniques are employed to ascertain the composition of the microbiota, measure overall microbial levels, quantitatively analyze taxa, ascertain the frequency of specific taxa, and assess host-bacterial relationships along the brush border mucosa (Zhou *et al.*, 2014; Wang *et al.*, 2018).

The most recent molecular analysis technique, known as next-generation sequencing (NGS), is more routinely utilized in fish investigations. Over the past few decades, studies on fish gut microbiota have significantly risen as a result of the advancement of such breakthrough molecular technologies and the explosive expansion of aquaculture. The fish gut microbiota is vital to the host survival since it actively participates in the metabolism of nutrients, the prevention of possible infections, and the immune system strengthening (Larsen *et al.*, 2015). More recent studies have shown that the bacteria found in fish guts are a promising source of enzymes, secondary metabolites, and aquaculture probiotics (Vignesh *et al.*, 2019).

1.5 Tuberculosis

Mycobacterium tuberculosis (MTB) is the bacterium that causes the chronic granulomatous illness tuberculosis, which is highly contagious and airborne. It is a disease that mostly targets the lungs. The history of tuberculosis is very old and it has afflicted many human beings. Recent molecular genetics research has revealed that the progenitor of *M. tuberculosis* existed 3 million years ago. (Ahmad, 2011) It is crucial to understand the genetics and physiology of *M. tuberculosis* and similar mycobacteria in order to logically create novel antitubercular medicines. Understanding how *M. tuberculosis* interacts with its host is crucial for understanding how these bacteria get past the host's defenses and cause disease. Using the methods presented in this

review, genes from *M. tuberculosis* that are or may be involved in virulence are identified. Future research should identify more bacterial targets for the development of vaccines, medicines, and more specialized diagnostic tools using some of these genes and the proteins they encode as well as newly identified ones.

1.5.1 *M. smegmatis*

Mycobacterium smegmatis is an acid-fast bacterial species in the phylum Actinomycetota and the genus *Mycobacterium*. It is usually 3.0 to 5.0 µm long, bacillus-shaped, and stainable using the Ziehl-Neelsen method and the fluorescent auramine-rhodamine method. Lustgarten discovered a bacillus with a staining look similar to tubercle bacilli in syphilitic chancres. Following this, Alvarez and Tavel discovered organisms like those described by Lustgarten in regular vaginal secretions (smegma). This organism was later given the name *M. smegmatis*. (GORDON and SMITH, 1953)

When conducting laboratory experiments, *M. smegmatis* is helpful for the examination of other *Mycobacteria* species. Considering that it is a "fast grower" and non-pathogenic, *M. smegmatis* is frequently utilized in research on the *Mycobacterium* genus. A biosafety level 1 laboratory is all that is needed to operate with the straightforward model of *M. smegmatis*, which also has a quick time to double. Researchers used *M. smegmatis* as a model for mycobacterial species due to the time and expensive infrastructure needed to deal with pathogenic species.

The cell wall of *Mycobacterium smegmatis* and other mycobacterial species is similar to that of *Mycobacterium tuberculosis*. Like *M. tuberculosis*, it has the ability to aerobically oxidize carbon monoxide. In most synthetic or complicated laboratory media, *M. smegmatis* is easily cultivable and forms noticeable colonies in 3-5 days. It is a particularly desirable model organism for *M. tuberculosis* and other mycobacterial diseases because of these characteristics. Additionally, *M. smegmatis* mc2155 is employed for mycobacteriophage cultivation (King, 2003).

1.5.2 H37Rv

The tuberculosis strain of *M. tuberculosis* known as H37Rv has received the greatest attention in laboratory studies.(Camus *et al.*, 2002) Dr. Edward R. Baldwin originally isolated it from a 19-year-old patient in New York in 1905, who had persistent pulmonary tuberculosis. It was originally known as strain H37 and was kept alive for many years by the Trudeau Sanatorium through the serial passage of cultures. Depending on the growth medium, it was eventually discovered to have varying virulence in animal models(KUBICA, KIM and DUNBAR, 1972). Then, purposefully created strains with varying virulence, H37R became less virulent after growing in acidic media and H37S became more virulent in guinea pigs after growing in alkaline media (with R denoting resistance to environment and S denoting sensitivity to environment).(Steenken, Oatway and Petroff, 1934) Later, the more dangerous strain was given the new moniker H37Rv—R for rough morphology and v for virulent. The strain was used for many laboratory studies and became the standard for tuberculosis. It was later designated as the neotype for the species(Cole *et al.*, 1998)

Fishes have concerned with a wide variety of bacterial taxa and other microbes, which frequently mirror the microflora of the aquatic environment. The gut microbiota of fish can be considered as an organ, which is in charge of vital physiological processes essential to maintain the health of its host. Prior research had suggested that there were few bacteria in fish guts, but more recent research, using cutting-edge molecular methods like NGS technologies, has painted a different picture and revealed that there can be found as much as 10⁹ CFU/g of bacteria cells in the gut of some species. Many of these fish associated microorganisms play uncertain roles, and further research should focus on this area. The health and sustainability of aquaculture would be improved by our increased knowledge of the fish gut microbiota in relation to diet modifications and the effects of microbial ecology alterations on bioactivity research. For both recreational and commercial fisheries, it is crucial to comprehend the bacterial makeup of fish as it is proven to be a big impact on host health, resistance to diseases, and spoiling rates. Finding new bioactive compounds is crucial to combating the threat posed by bacteria that are multidrug resistant. Because natural products include a variety of chemical scaffolding that cannot be equaled by synthetic libraries, they are regarded as a useful resource for drug development. At the moment,

marine microorganisms are a priceless, important source of novel, and promising bioactive metabolites with significant biological activity (Prabha *et al.*, 2023).

CHAPTER:2

LITERATURE SURVEY

2.1 Introduction

The impacts of the fish gut microbiome on the fish include changes those on the fish's overall size, metabolism, feeding habits, and immune response. The need for probiotics is driven by the emergence of antibiotic-resistant bacteria and the difficulty of treating fish ailments. Gut microorganisms that have a good impact on fish could be used as probiotics to combat these problems. Antibiotics have been used extensively and systematically, which has resulted in serious biological and ecological issues, such as the development of antibiotic resistance that affects the gut microbiota of aquatic creatures. Probiotics, also referred to as helpful bacteria, are suggested as an efficient and environmentally friendly antibiotic substitute

2.2 Gut Microbiome Composition

Several studies have used high-throughput sequencing of the 16S rRNA gene to examine the composition of the gut microbiome in several fish species. A study by (Gajardo *et al.*, 2016) discovered that Proteobacteria, Firmicutes, and Actinobacteria dominated the gut microbiome of Atlantic salmon (*Salmo salar*), and another study by (Yang *et al.*, 2023) discovered that Proteobacteria, Firmicutes, and Bacteroidetes dominated the gut microbiome of Nile tilapia (*Oreochromis niloticus*).

2.2.1 Dietary influences on fish's gut microbiota

It is well established that diet has significant effects on how fish's gut microbiota develops. Research on feeding Japanese flounder (*Paralichthys olivaceus*) with a diet containing probiotics increased the abundance of probiotic bacteria in the gut and feeding rainbow trout (*Oncorhynchus mykiss*) with a diet containing prebiotics increased the abundance of lactic acid bacteria in the gut. (Gajardo *et al.*, 2016)

2.2.2 The gut microbiome's role in fish disease resistance

Fish disease resistance is also known to be influenced by the gut microbiota. It was discovered that antibiotic use decreased the diversity of the gut microbiome in

Atlantic salmon and increased the fish pathogen *Aeromonas salmonicida* susceptibility of the fish.(Egerton *et al.*, 2018). The gut microbiome of healthy fish and fish affected with the disease *Vibrio anguillarum* in European sea bass (*Dicentrarchus labrax*) were compared and concluded that fish's gut microbiome is a sophisticated and dynamic ecosystem that is essential to their overall health and welfare. Knowing the make-up and purpose of the gut microbiome in different fish species is important for developing strategies to promote fish health and improve aquaculture practices.(Amillano-Cisneros *et al.*, 2022)

Development of fish gut microbiota

The intestinal microbiota of fish may originate from a variety of sources. It is commonly acknowledged that bacterial colonization mechanisms throughout the initial phases of larval fish development are complicated and reliant on the microbiota of eggs, the water used for larval rearing, and feeding habits. The original invading bacteria are now known to be species-specific, with variations in the glycoproteins present on the egg surface controlling variances (Larsen, 2014). Additionally, the microbial population of the water in the area determines which bacteria come into contact with the eggs and have a chance to colonize them. When sterile larvae hatch, they consume the chorion associated bacteria, which are the first organisms to inhabit the developing GIT (Egerton *et al.*, 2018). The gastrointestinal tract of the freshly hatched larvae frequently has a few microorganisms in it. When fish larvae start drinking water to regulate their osmoregulation, they acquire subsequent bacterial habitats for the first time. Through feeding, the microbiota then diversifies even more. (Hansen and Olafsen, 1999). Composition of fish gut microbiota Fishes are diversely group of gut microbiota, including protists, fungi, yeasts, viruses, bacteria, and archaea. However, the majority of the microbial community in the gut of fish is composed of bacteria and has so far been mainly studied in this area (Egerton *et al.*, 2018). In contrast to terrestrial vertebrates, fish have obligate anaerobes, facultative anaerobes, and aerobic bacteria as the main colonizers of the GIT (Llewellyn *et al.*, 2014). According to research, the fish gut contains between 10⁷ and 10¹¹ microorganisms per g-1 of intestinal material (Nayak, 2010). Proteobacteria, Firmicutes, Actinobacteria,

Fusobacteria, Bacteroidetes, and Verrucomicrobia are some of the bacteria that inhabit the fish GIT, according to studies using NGS (Ringø *et al.* 2006; Desai *et al.*, 2012; Li *et al.*, 2013; Carda-Diequez *et al.*, 2014). The most prevalent phyla of these are Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Fusobacterium. The majority of the microbiota in marine fish is composed of facultative anaerobes, including *Vibrio*, *Acinetobacter*, *Pseudomonas*, *Corynebacterium*, *Flavobacterium*, *Micrococcus*, and *Alteromonas*. Contrarily, *Aeromonas*, *Pseudomonas*, and *Bacteroides* type A predominate in the microbial community of species of freshwater fish, while Enterobacteriaceae: *Plesiomonas*, *Micrococcus*, *Clostridium*, *Acinetobacter*, *Bacteroides* type B, and *Fusarium* are less common species. (Wang *et al.*, 2018). Thirteen samples of fish from four different species in the Antarctic were studied for their fish gut microbiota, which was identified into 804 genera and 36 phyla. Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes are found to be the most prevalent phyla, and *Rhodococcus*, *Acinetobacter*, *Thermus*, *Mycoplasma*, *Propionibacterium*, and *Streptococcus* have also encountered the most prevalent species (Song *et al.*, 2016). According to cultivation-based approaches, Ascomycota and Basidiomycota were the two most often reported fungal phyla. Up to 107 CFU of yeasts were presented per gram of gut material. Protozoa and Archaea in fish intestines received little attention (Bruijn *et al.*, 2018).

Fish gut microbiome and production of secondary metabolites

Pathogens have been reported to exhibit high resistance to numerous antibiotics due to mutations brought by the widespread and careless usage of antibiotics (Sharma *et al.*, 2016). The oldest and most common infectious agent is *Mycobacterium tuberculosis*, which causes tuberculosis (TB) (Hussain *et al.*, 2017). The emergence and spread of illnesses with extended-spectrum beta lactamases, such as those caused by Gram-negative bacteria such as *Acinetobacter baumannii*, *Escherichia coli*, and carbapenem-resistant *Klebsiella pneumonia* (CR-KP), pose a significant risk to public health (Schwaber *et al.*, 2008; Vatopoulos, 2008). To tackle multidrug resistance (MDR), novel antibiotics with specific molecular frameworks and distinctive modes of action are urgently required. Cancer continues to be one of the biggest health issues

affecting people among lifestyle illnesses. Notably, the second-leading cause of cancer fatalities in women after lung cancer is breast cancer. Combining surgical, radiological, immunotherapeutic, and chemotherapeutic methods is one of the therapeutic choices for cancer treatment (Maqsood *et al.*, 2018). Because of their variety of chemical structures, which cannot be equaled by any synthetic libraries, natural products are viewed as a wonderful repository for developing drugs (Rateb *et al.*, 2018). However, our research findings found depsidones, known mollicellins E and J, and new mollicellins K-N from the fungus *Chaetomium brasiliense* expressed antimalarial activity against *Plasmodium falciparum*. It showed that compound new mollicellins K-N resulted in antimicrobial activities against *Candida albicans* and *Mycobacterium tuberculosis* causing tuberculosis. Moreover, all compounds expressed cytotoxicity to KB, BC1, NCI-H187 and five cholangiocarcinoma cell lines (Khumkomkhet *et al.*, 2009). *Ch. globosum* strain KMITL-N0802 recorded a novel anthraquinone-chromanone compound named chaetomanone and known compounds, chrysophanol, chaetoglobosin C, echinulin and isochaetoglobosin D. With this, Chaetomanone and echinulin reported to be expressed antibacterial against *Mycobacterium tuberculosis* (Kanokmedhakul *et al.*, 2002). Our further research findings on the endophytic *Chaetomium globosum* 7s-1 isolated from a plant species, *Rhapis cochinchinensis* found a new xanthoquinodin B9, three epipolythiodioxopiperazines, chetomin, chaetocochin C, dethio-tetra (methylthio) chetomin, four other compounds, chrysophanol, two known xanthoquinodins, xanthoquinodin A1 and xanthoquinodin A3, emodin, and alatinone. It found that compounds xanthoquinodin A3, chetomin, chaetocochin C and dethio-tetra(methylthio) and chetomin expressed antimicrobial activity towards Gram positive bacteria at concentrations of 0.02 pM to 10.81 μM. Moreover, these metabolites also expressed cytotoxicity against a normal cell line (Vero cell) at IC50 values of 0.04–3.86 μM and cytotoxicity toward KB, MCF-7, NCI-H187 cancer cell lines at IC50 0.04–18.40 μM (Tantapakul *et al.*, 2020). These natural products of secondary metabolites may possible to develop as biomedicine to control those pathogens. Marine vertebrates have historically been found to be a rich resource of distinctive natural compounds, with a wide variety of biological activity. Recent research on microbial populations linked to vertebrates shows that microbes are the

real source of many of these chemicals. The microbially rich habitat of the marine system has not been sufficiently studied to yield unique chemical substances such as antibiotics, enzymes, vitamins, medicines, and biosurfactants (Bhatnagar and Kim, 2010; Shekhar *et al.*, 2015). The ability of actinobacteria to produce a wide variety of secondary metabolites with fascinating potential bioactivities makes them well-known among marine microbes (Vignesh *et al.*, 2021). Nineteen bacterial strains were discovered during the preliminary investigation of the intestinal flora of Garra mullia (Sykes), and they displayed a great range of enzymatic activity, morphology, and biochemical properties. The factors that control diversity as determined by analysis of principal components and correspondence analysis demonstrate that bacterial isolates have adapted to use a variety of resources, such as sugar and citrate, and also exhibit endurance to a variety of environmental factors, including pH, salinity, and bile in vitro conditions (Rudresh *et al.*, 2010). Actinomycetes found in Indian Mackerel (*Rastrellinger kanagurata* Cuvier) produce metabolites that prevent the growth of histamine-producing bacteria. These byproducts can be utilized to preserve seafood. (Choudhury *et al.*, 2008). In addition to having antibacterial action against ESBL Klebsiella pneumonia ATCC 13882 and *Mycobacterium tuberculosis*, the secondary metabolites from *Streptomyces enissoceasilis* SFA from shark fish also had anticancer activity against breast cancer cells (Vaishali *et al.*, 2021). Lipid-producing bacteria isolated from the guts of different freshwater and saltwater oily fish from the western coastal region of India have been found to produce PUFA (Masurkar *et al.*, 2014). From the distal (DI) and proximal (PI) areas of the fish gut, Dutta and Ghosh (2015) isolated bacteria that produce extracellular enzymes. These strains may produce the extracellular enzymes lipase, cellulase, phytase, amylase, and xylanase. By using the cross-streaking and double-layer methods, additional research on antagonism against seven possible fish pathogens indicated some of the strains to be antagonistic against one or more pathogens (Dutta and Ghosh 2015). Study of *Streptomyces* spp. in the fish gut microbiota of three environmental biotopes, including fresh-water (*Cyprinus carpio* [common carp]), estuarine (*Oreochromis mossambicus* [tilapia]), and marine (*Epinephelus diacanthus* [grouper]), which have a greater relationship with muddy soils for the synthesis of antibacterial components against human pathogens *Vibrio cholerae*

proved that these extracts have the capacity to produce effective antibacterial components against the pathogen (Deepa *et al.*, 2012). The actinobacterial strains from *R. kanagurta* showed antimicrobial and quorum sensing, anticancer, and anti TB activity. The actinobacterial strains from *P. microdon* showed antimicrobial activity (Vignesh *et al.*, 2019). Due to significant financial loss, biofouling on ship surfaces poses a serious danger to the marine industry. Tributyltin has been used to combat biofouling, which is the root of the environmental problem. According to research, coral-associated marine bacteria and aquatic Actinobacteria have the ability to reduce the development of biofouling (Waturangi *et al.*, 2017).

2.3 Tuberculosis

Nearly one-third of the world's population have been estimated to be infected with tuberculosis (TB), which is caused by *Mycobacterium tuberculosis*. (Mukhopadhyay and Ganguly, 2013)

According to statistics, India ranks 17th out of 22 nations with a high burden of TB (Kashyap *et al.*, 2013). The main issue in TB control is the emergence of drug resistance among *M. tuberculosis* isolates and long-term therapy utilizing a combination of medications for its treatment. New antitubercular medications are therefore urgently needed to combat drug-resistant *M. tuberculosis* strains (Ginsberg, 2010). The new anti-TB medications should have fewer side effects, better pharmacokinetic characteristics, extensive and robust efficacy against drug-resistant strains, and/or be able to shorten the overall length of treatment (de Souza, 2008). Secondary metabolites derived from microorganisms have been utilized for decades to treat tuberculosis. The phylum Actinobacteria (order Actinomycetales) caters to aerobic filamentous Gram-positive bacteria with true aerial hyphae known as actinomycetes (Ma *et al.*, 2020). Actinomycetes are widespread soil organisms that have a remarkable capacity to create secondary metabolites, such as antibiotics, that are clinically beneficial. About 50% of the identified microbial bioactive compounds come from actinomycetes members. (Bérdy, 2012)

2.3.1 Models for Measuring *M. tuberculosis* Virulence

The pathogenicity of *M. tuberculosis* is investigated in tissue culture, primarily employing macrophages, and more recently, using dendritic cells and pneumocytes using model animals. Although tissue culture models are simpler to use and produce results more quickly, they are only effective in the early phases of infection. The ability to study all TB stages makes animal models eventually superior. The choice of animal models is crucial for virulence investigations, and each of the three main model mice, guinea pigs, and rabbits has benefits and drawbacks. Because of their genetics, which have been extensively studied and include inbred strains with immune system mutations, the availability of reagents to measure cytokine levels, and their lower maintenance costs in comparison to other animal models, mice are the most frequently used in vivo models (Birkness *et al.*, 1999). The fact that there exist inbred strains of mice with significantly varied levels of resistance to *M. tuberculosis* and other infections, which enables the identification of relevant loci, is another significant benefit of using mice in research. One such study (Cole *et al.*, 1998) which examined the offspring of a cross between the relatively resistant B6 strain and the relatively sensitive C3H strain of mice, identified the locus known as *sst1* as having a significant impact on tuberculosis susceptibility. However, these studies demonstrated that susceptibility to or resistance to *M. tuberculosis* is a complicated characteristic and that additional, undisclosed loci must also be involved. A group of recombinant congenic mouse strains created by mating strains A/J and C57/BL6 are currently being employed in an ambitious and major investigation to chromosomally map regions imparting resistance or sensitivity to bacterial illnesses (Kramnik *et al.*, 2000) These mouse strains may soon be employed to map the additional loci affecting host resistance to *M. tuberculosis* infections. Although the course of TB in mice differs from that in people in that the granulomas developed are not as distinct, mice are more similar to humans in that they are often less responsive to the disease than other animal models and can develop chronic infection. The stages of the disease, particularly the early stages of granuloma formation, in guinea pigs are similar to those in humans because they are very susceptible to *M. tuberculosis* infection (KUBICA, KIM and DUNBAR, 1972)

2.3.2 H37Rv Strain

H37Rv is the most widely used *Mycobacterium tuberculosis* strain, and its genome is globally used as the *M. tuberculosis* reference sequence (Bermudez and Goodman, 1996)

CHAPTER 3

AIM AND OBJECTIVE OF THE PRESENT STUDY

3.1 Aim

To study the diversity, distribution and bioprospecting of actinobacteria from fish gut.

3.2 Objective

- Isolation and characterization of actinobacteria from fish gut.
- In vitro screening of actinobacterial culture against mycobacterial and non-mycobacterial pathogens and selection of potential strains.
- Production and media optimization of bioactive compounds from potential actinobacterial culture.
- Extraction of Bio-active compounds from the crude extract.
- Characterization and taxonomy of potential actinobacterial culture.

CHAPTER 4

MATERIALS AND METHODS

4.1 Sample Collection

Fishes are collected from the and sent immediately to the laboratory to avoid contamination. Fish samples Indian mackerel (*Rastrelliger kanagurta*) and Indian Squid (*Uroteuthis duvauceli*) are the selected fishes (figure5.1) were collected from Ennore coastal area. Each fish was sent immediately to the laboratory to avoid contamination.

4.2 Media Requirement

Starch casein agar media

ISP2 agar (international streptomyces project media).

ISP2 broth

Nutrient agar

Nutrient broth

Tween 80 agar

Carboxy methyl cellulose agar

Starch agar

Skim milk agar

4.3 Isolation of Actinobacterial Strains

All fishes were washed thoroughly under distilled for few minutes to remove organic debris. The fish is dissected and whole intestine is collected and washed with phosphate saline buffer (PBS) then mashed into homogenous sample using motor and pestle with no water or 1ml of PBS. The resultant homogenates were serially diluted from 10^{-1} to 10^{-5} and plated on starch casein agar media using L-rod by spread plate method. The plates were incubated at room temperature for 28 days for the growth of actinobacterial colonies (Vadivel *et al.*, 2021).

4.4 Recovery and Preservation of Actinobacteria

After incubation, all the cultures were observed for growth, consistency, and colony formation, aerial mycelium and substrate mycelium. The colonies showing actinobacterial morphology were recovered from the isolation plates using sterile L-shaped needle and inoculated into freshly prepared yeast extract malt extract agar (ISP2) agar plates and preserved using ISP2 agar slants as well as in 30% glycerol. (Manikkam *et al.*, 2014)

4.5 Characterization of Actinobacteria

Cultural characterization was done by inoculating the actinobacterial culture into ISP2 Agar medium. All the plates were incubated for 7-14 days at 28°C. Cultural characteristics recorded include growth, colony consistency, aerial mass colour, presence of reverse side pigment and soluble pigment production

4.6 Screening of Actinobacteria for The Production of Enzymes

All the bacterial and actinobacterial cultures will be screened for extracellular enzymatic activities such as cellulase, Protease, lipase and amylase by agar plate method.

4.6.1 Amylase test

Bacterial isolates were Inoculated on starch agar medium containing glucose, peptone, yeast extract, and starch are incubated at 30°C for 48h. after incubation, plates were flooded with iodine solution. Positive culture exhibited clear zone around the colony against the blue-black background due to hydrolysis of starch (Bhutani *et al.*, 2021).

4.6.2 Lipase test

Bacterial isolates producing lipase were screened using tween 80 agar media. Cultures were spot inoculated and incubated at room temperature. Zone of clearance around the colony indicated production of lipase enzyme.

4.6.3 Protease activity

The proteolytic or caseinolytic activity was tested in 10% skim milk plate medium. Bacterial isolates were Inoculated on Skim milk agar after 48hrs of incubation at 30°C transparent clear zones around the growth were considered as an indication of casein activity degradation

4.6.4 Cellulase test

Cultures were inoculated on Carboxy methyl cellulose agar media containing K_2PHO_4 , $MgSO_4$, $7H_2O$ and Carboxy methyl cellulose (CMC) are incubated at 30°C for 48h. after incubation plates were flooded with (0.1% w/v) Congo red for 1h at room temperature followed by destaining with 1M NaCl till suitable contrast is obtained. The clear zone around the culture indicates the production of cellulase enzyme (Bhutani *et al.*, 2021)

4.7 In Vitro Screening for Anti-microbial Activity Against bacterial pathogens

4.7.1 Test organisms

Test pathogens used in this study include standard strains *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Aeromonas caviae*, *Aeromonas salmonicida*, *Acinetobacter baumannii*. *Candida albicans* and *Pseudomonas aeruginosa*. Standard strains were obtained from The Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh. All the clinical pathogens were obtained from Christian Medical College, Vellore, Tamil Nadu and maintained on nutrient agar slants except *C. albicans* which were maintained on Saboraud's Dextrose Agar (SDA) slants.

Antimicrobial activity of actinobacterial cultures was tested by adopting agar plug method (Manikkam *et al.*, 2014)). Actinobacterial cultures grown on ISP2 agar plates were taken. Test pathogens with 0.5 McFarland standards were inoculated into nutrient agar plates and SDA plates respectively using sterile cotton swab. Agar plug with 9 mm diameter were cut from the ISP2 agar grown with actinobacterial cultures were placed over ISP2 agar and SDA plates which were seeded with test pathogens. All the plates were incubated at 37°C for 24 hours. The inhibition zone of bacterial growth was observed and recorded in millimetre (Troussellier *et al.*, 2017). The actinobacterial strain showing the maximum zone of inhibition was selected as the potential strain for future studies.

4.8 Antimycobacterial Activity of Fish Gut Actinobacteria

Antagonistic activity of actinobacteria against *M. smegmatis* was tested using agar plug method. 7H9 agar media was prepared and the test pathogen *M. smegmatis* was seeded with sterile cotton swab to form a uniform mat. Agar plug of 6mm size was cut from actinobacteria cultured ISP2 plate and placed over 7H9 plate immediately after seeding and were sealed with parafilm and incubated for 48 hours at room temperature. The plate is cross checked for contamination by Ziehl Neelsen staining method (Chen *et al.*, 2012) Zone of inhibition was expressed in millimetre in diameter. The Actinobacterial strain SQA 4 which showed maximum inhibition was selected as potential strain for further studies.

4.9 Effect of Medium Consistency and Incubation Period of Bioactive Compounds

Based on the antibacterial results, selected potential actinobacterial strain were tested for bioactive metabolite production in submerged and solid medium. The selected actinobacterial cultures were inoculated into YEME agar plates as well as in each 100 ml of YEME broth. The plates were incubated at 28°C in incubator for 10 days. All the flasks were incubated at 28°C in a rotary shaker for 10 days. Bioactive compound production in solid and submerged medium was assessed from first to eighth day of incubation using *S. aureus* as test organisms. The solid medium was tested by adopting agar plug method (Bennett *et al.*, 2008) and submerged medium was tested by using agar well diffusion method against test organism seeded on nutrient agar plate.

4.10 Co-Culture Method

Co-culture of different microbes simulating the natural state of microbial community may produce potentially new compounds because of nutrition or space competition. Co-culture of microorganisms which involves the cultivation of two or more microorganisms in the same confined environment may produce potentially new compounds by stimulating the silent genes or gene clusters of one partner or increase the yields of previously described metabolites (Joo *et al.*, 2011). Actinomycetes strain was grown separately in a culture test tube containing 40 ml of ISP2 broth medium. Inducer strain *E. coli* was grown separately overnight in nutrient broth medium. 20 µl

culture of inducer strain both alive and heat-killed cells (121°C, 15 min) were inoculated after 12 h of culturing actinomycetes strains (20 ml) for the enhancement of antibacterial activity. All the culture tubes were then shaken on a rotary shaker (220 rpm) at 31°C for 5 days. After 5 days, the culture filtrate was separated by centrifugation at 4000 rpm for 10 minutes at 10° C and clear supernatant was separated. Antibacterial activity of the cell-free supernatant was performed in triplicate by the agar well diffusion technique in Mueller-Hinton agar plates using *E. coli* as the test organism. The inoculums (*E. coli*) equivalent to 0.5McFarland standard were spread on the MHA plates with a sterile swab moistened with the bacterial suspension. Wells were made using stainless steel borer. Culture supernatants (100µl) loaded in respective wells. All the plates were incubated at 37°C for 24 h. The antibacterial activity was then evaluated by measuring the diameter of the zone of inhibition (ZOI) around the well. Characterization of secondary metabolites carried out for 3 cell-free culture supernatants SQA 4, SQA 4-live *E. coli* and SQA 4- head killed *E. coli* cultures were done. Same procedure is repeated for Co-culture of *Staphylococcus aureus*, *Candida albicans* and *Mycobacterium smegmatis*.

4.11 Production and Extraction of Bioactive Compounds from Potential Actinobacterial Strains

Bioactive compound from actinobacterial culture was produced by agar surface fermentation (Manikkam *et al.*, 2014).The actinobacterial culture was inoculated on ISP2 agar plates and incubated at 30°C for 10 days for the production of secondary metabolites. Mass production of the potential strain is carried out based the results of the antibacterial activity of potential strain on both YEME agar and YEME broth on different incubation period. To study the effect of solvent on extraction of bioactive metabolites, different extracts such as n-hexane, chloroform, ethyl acetate were tested against *S.aureus* ((da Cunha *et al.*, 2018)

4.12 Microplate Alamar Blue Assay

MABA is used for measuring cell proliferation and viability by monitoring the oxidation/reduction state of the environment of cellular growth. As with the tetrazolium salts, Alamar blue is a soluble redox dye that is stable in culture medium and nontoxic

(Ansar Ahmed, Gogal and Walsh, 1994). The oxidized dye is blue and nonfluorescent; upon reduction it turns pink and fluorescent, therefore, growth can be determined by a visual colour change or by using a fluorometer. Alamar blue has been used to assess the susceptibility of *M. tuberculosis* antimicrobials in several laboratories (Yajko *et al.*, 1995; Collins and Franzblau, 1997; Franzblau *et al.*, 1998)

MABA is used to check activity against *M. tuberculosis* and *M. smegmatis* for crude extract of SQA 4.

4.13 Free Radical Scavenging Assay

The free radical scavenging capacity of the crude extract of SQA4 strain was measured based on the method delineate by Brand-Williams with slight modification (Brand-Williams, Cuvelier and Berset, 1995). The whole assay is done in 96 well microplate .100µl of 0.1 mM DPPH solution in methanol was mixed with 100µl of crude extract solution of varying two-fold dilution concentrations (200 µg/ml, 100, 50, 25... till 0.39), Blank sample was prepared and L-Ascorbic acid was used as the standard. These wells were thoroughly mixed and kept in the dark for 30 min and their absorbance was measured at 517 nm using UV-Vis spectrophotometer. The inhibition % was calculated using the following formula.

$$\text{Inhibition \%} = \frac{A_c - A_s}{A_c} \times 100$$

Ac-Absorbance of control reaction

As-Absorbance of the std. sample

4.14 Anti-Biofilm Assay

A loopful of test pathogens were inoculated in TSB tubes respectively and adjusted to 0.5 McFarland standards. The tubes were incubated at 37 °C for 24 hrs. In 96 well plate, 100 µl of sterile TSB was suspended in all wells except the outer wells.100 µl of samples at 200 µg/mL concentrations were added to 1st wells and mix thoroughly using micropipette. Two-fold dilution is done by transferring 100 µl of diluted sample from 1st well to 2nd well and 2nd well to 3rd, till the desired concentration is achieved. Add 50 µl of standard bacterial inoculum to all the wells except control wells. Distilled water

was added to the outer well of the plate. Control wells were maintained [positive control (PC): media + pathogen, blank (B): only media to check sterility] Incubate the plates at 37 °C for 24 hrs. After incubation, spent culture was removed by gently tapping the plate aseptically. Rinse the plate with 1X PBS for twice. Plates were air dried at room temperature. Add 200 µl of 0.1% CV to all the wells and incubated for 20-30 mins at room temperature. Rinse the plate again with distilled water for twice. The plates were completely air dried. Solubilize the stained wells with 200 µl of 95% ethanol. OD reading is taken at 570nm using UV spectrometer. Calculate the percentage of biofilm inhibited using the formula mentioned below

$$\% \text{ biofilm inhibited} = \frac{\text{Control}_{570} (\text{PC}) - \text{Biofilm inhibit}_{570} (\text{T})}{\text{Control}_{570} (\text{PC})} \times 100$$

PC – OD value of positive control at 570 nm.

T - OD value of test sample at 570 nm.

4.15 Optimization of Bioactive Compounds Production (Media Optimization)

It is a method used to determine the parameter that needs to be altered in order to obtain a higher yield during production, since ISP2 is being used regularly, the strain tends to grow accustomed to the media and its composition, therefore the yield obtained for the strain in standard ISP2 is a saturated yield, whereas when the components of the media are slightly tweaked by increasing or decreasing the concentration, the yield produced may vary a reduction or increase in yield can be observed.

4.15.1 Classical method (One factor at a time)

Effect of critical medium components and culture conditions on bioactive compounds production from the strain SQA 4 was determined by adopting classical one factor at a time method. To screen for a suitable substrates and environmental conditions the strain SQA 4 was inoculated into basal medium containing different carbon source, nitrogen source, minerals, pH, seawater and temperature. After incubation, agar plug from all the media was tasted against the *S. aureus*.

4.16 Determination and Purification of The Biologically Active Compounds

4.16.1 Thin Layer Chromatography

Active compounds were determined by thin layer chromatography (TLC). The plates were placed in TLC chamber pre-saturated with different solvents like butanol, acetone, n-hexane, chloroform, ethyl acetate and so on. Crude extracts in ethyl acetate were spotted 2 cm from the bottom of a precoated aluminum sheet of silica gel. The TLC sheets were removed when the solvent front has moved to 15 cm from the original extract position and subsequently allowed to dry. After drying, the spots on the developed plates were visualized under visible (white), short UV (254 nm), and long UV (366 nm) light. As post derivatization, the sheets were sprayed with vanillin sulfuric acid for colour reaction and allowed to dry. A visualizer and a scanner were used for photo documentation at UV 254 nm and UV 366 nm and under the visible light before and after application of the vanillin sulfuric acid spray. The movement of each separating spot of the extract was expressed by its retention factor (R_f) (Augustine, Bhavsar and Kapadnis, 2005). Values were calculated for each spot using the following formula:

$$R_f = \frac{\text{component length}}{\text{liquid length}}$$

R_f = retention factor

component length = Distance travelled by the substance from reference line (mm)

liquid length = Distance travelled by the solvent front from reference line (mm)

4.17 Characterization and Taxonomy of Actinobacterial Strain

4.17.1 Micromorphology:

Micromorphological characteristics of the potential strain SQA 4 was studied by adopting slide culture method. About 2ml of melted ISP2 agar medium mixed with the spores of the strain SQA 4 was poured over the surface of sterile microscopic slides as a thin layer. The slides were then kept in sterile petri plates and incubated at 30°C for

10 days. Then the slides were observed under bright field microscope at 40X magnification. The recorded microscopic characteristics include the presence/absence of aerial mycelium, substrate mycelium, mycelia fragmentation and the details of spore chain morphology.(Balagurunathan *et al.*, 2020)

These actinobacterial strains were tested for their antimicrobial activity against *S. aureus* and *M. smegmatis* by agar plug method as described above.

4.17.2 Physiological characteristics

4.17.2.1 Effect of carbon and nitrogen sources

About 1% of various ether sterilized sugars were added and poured into petri plates. Actinobacterial strain SQA 4 was inoculated on different sugar containing ISP2 agar plates. Growth was recorded after 7 days of incubation at 30°C. carbon source used in this study include D-glucose, cellulose, lactose, starch and mannitol (Hi media) (Sreenivasan, Manikkam and Manigundan, 2022). The Effect of different nitrogen sources like yeast extract, malt extract, peptone, KNO₃ and soy bean meal was also studied using the above procedure. All the plates were observed for growth after 7 days of incubation at 30°C. All the plates were observed for growth after 7 days of incubation.

4.17.2.2 Effect of pH

Effect of pH on growth was determined by inoculating the actinobacterial strain SQA 4 onto ISP2 agar medium adjusted to different pH values viz. 1,5,7,9 and 11. All the plates were observed for growth after 7 days of incubation at 30°C. Effect of temperature on growth was determined by inoculating the actinobacterial strain SQA 4 onto ISP2 agar plates and incubating at different temperatures viz. 5°C, 30°C, 37°C and 40°C. All the plates were observed for growth after 7 days of incubation.

4.18 Molecular Characterization of Actinobacterial Strain SQA 4

The molecular characterization of strain SQA 4 was carried out at BioServe Biotechnologies (India) Pvt Ltd., Bangalore. Genomic DNA of the potential actinobacterial strain SQA 4 extracted using solute ready genomic DNA kit. The 16SrRNA gene was amplified by PCR using primers 8F (5'-

AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Patel, 2001). The reaction mixture (50 µl) contained 100 ng of 2 µl genomic DNA, 5 µl of 10x buffer, 4 µl of BSA (1 mg/ml), 1 µl of dNTP mixture (2.5 mM), 1 µl of each primer (10 µM) and 3.5 U of Taq DNA polymerase. PCR condition followed as 6 min at 94°C followed by 35 cycles of 45 s at 94°C, 45 s at 55°C and 1.5 min at 72°C, followed by 8 min extension at 72°C. PCR amplification was performed in an Eppendorf Master cycler Gradient. Sequences were edited and contig was assembled in DNA baserv.3 and compared with GenBank sequences by BLAST analysis. The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank using CLC sequence viewer 6.0 programs. The alignment was constructed to phylogenetic tree using neighbor joining in MEGA7 software. (Bahobail, Gad El-Rab and Amin, 2016). The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses based on 1000 resampling of the neighbor joining data set (Clarridge, 2004).

4.19 Characterization and taxonomy of potential actinobacterial strain

Based on phenotypic 16S ribosomal RNA (16S rRNA) study, the potential endophytic actinobacterial strain SQA4 was characterized. (Shirling and Gottlieb, 1966)

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Isolation of Actinobacteria from Fish Gut

Totally 39 different bacterial colonies were isolated from gut of fishes collected from Ennore, Chennai. Gut was removed from the fishes and homogenized using a surface sterilized mortar and pestle. (Fig:5.2). Seeded serial dilution plates were observed after incubation (Table 5.1)

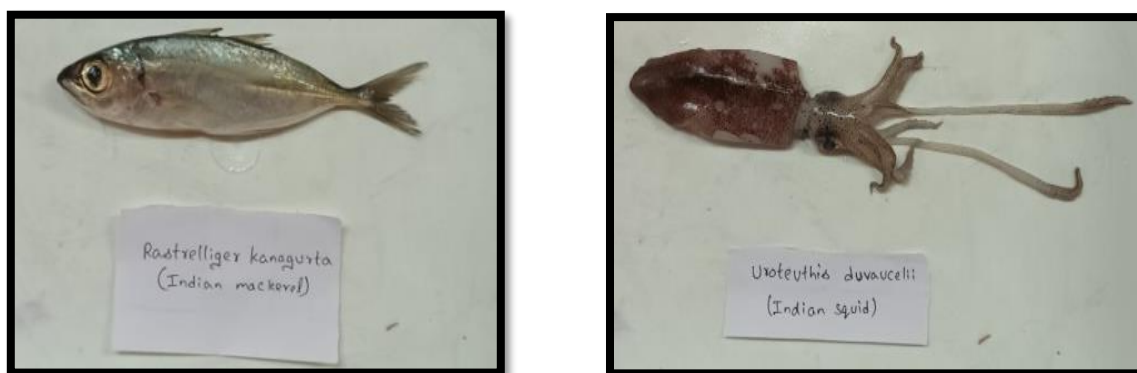


Fig:5.1: Fish samples selected for actinobacterial isolation



Fig:5.2: dissected gut from Fish samples

5.2 Preliminary Identification

Bacterial cultures were grouped based on the morphological characteristics such as growth, consistency, aerial mass color, reverse side pigment, Soluble pigment,

aerial mycelium, substrate mycelium. Most of the cultures are non-actinomycetes (pasty and leathery consistency). A culture has shown powdery consistency and is considered as actinobacteria (Table:5.2) (fig:5.3). Aerial mycelium and Substrate mycelium was seen on the culture which was viewed under bright microscopy(Fig:5.4). Previously Sivakumar *et al.*, 2007; Gopikrishnan *et al*, 2019;2020 reported the fish gut associated actinobacteria from both fresh water and marine fishes.

Table 5.1: Colony count

S.NO	Gut sample	Media	Dilution	WEEK 1	WEEK 1	WEEK 1
1	Indian mackerel	SCA	10 ⁻³	20	47	53
2	Indian mackerel	SCA	10 ⁻³	33	74	TNTC
3	Indian mackerel	SCA	10 ⁻⁴	15	28	34
4	Indian mackerel	SCA	10 ⁻⁴	17	34	47
5	Indian mackerel	SCA	10 ⁻⁵	9	18	20
6	Indian mackerel	SCA	10 ⁻⁵	8	19	23
7	Indian squid	SCA	10 ⁻³	17	34	47
8	Indian squid	SCA	10 ⁻³	19	45	57

9	Indian squid	SCA	10^{-4}	1	1	4
10	Indian squid	SCA	10^{-4}	8	19	36
11	Indian squid	SCA	10^{-5}	5	14	25
12	Indian squid	SCA	10^{-5}	7	18	27



Fig:5.3: Identified actinomycetes recovered on ISP2 agar plate.

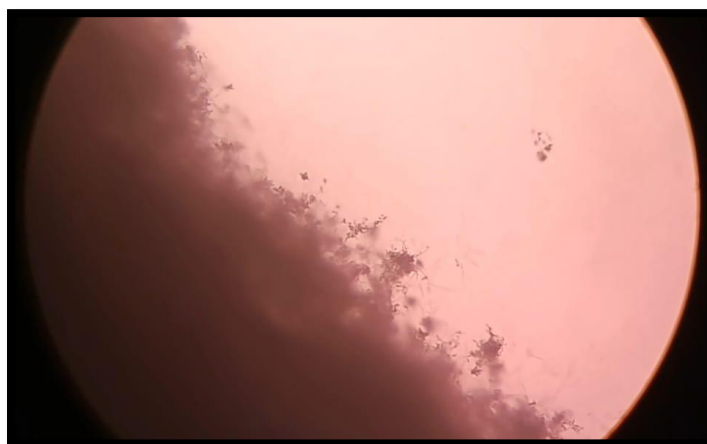


Fig:5.4: Morphology of actinobacterial strain under bright field Microscope

Table 5.2: Morphological Identification

S.No	STRAIN NAME	Growth	Consistency	Aerial mycelium (If so-colour)	Soluble pigment (If so-colour)	Reverse pigment (If so-colour)
1.	IM 1	+	Smooth	-	-	-
2.	IM 2	+++	Smooth	-	+(Pale brown)	+(Pale brown)
3.	IM 3	+++	Slimy	-	-	-
4.	IM 4	-	No form	-	-	-
5.	IM 5	+	Slimy	-	-	+(yellow)
6.	IM 6	+	Pasty	-	-	-
7.	IM 7	+	Smooth	-	-	-
8.	IM 8	+	Smooth	-	-	-
9.	IM 9	-	No form	-	-	-
10.	IM 10	++	Smooth	-	-	-
11.	IM 11	++	Pasty	-	-	-
12.	IM 12	+	Pasty	-	-	-
13.	SQA 1	+++	Smooth	-	-	-
14.	SQA 2	+	Smooth	-	-	-
15.	SQA 3	+	Slimy	-	-	-

16.	SQA 4	+++	Powdery	+	-	-
				(white)		
17.	SQA 5	+	Smooth	-	-	-
18.	SQA 6	++	Pasty	-	-	+
						(orange)
19.	SQA 7	+++	Smooth	-	-	+
						(yellow)
20.	SQA 8	++	Smooth	-	-	-
21.	SQA 9	+++	Smooth	-	-	-
22.	SQA 10	+	Smooth	-	-	-
23.	SQA 11	+	Pasty	-	-	-
24.	SQA 12	++	Pasty	-	-	+
						(brown)
25.	SQA 13	-	No form	-	-	-
26.	SQA 14	+++	Smooth	-	-	-
27.	SQA 15	+++	Slimy	-	-	-
28.	SQA 16	+	Slimy	-	-	-
29.	SQA 17	+	Smooth	-	-	-
30.	IMP 1	+	Slimy	-	-	-
31.	IMP 2	+	Slimy	-	-	-
32.	IMP 3	+	Pasty	-	-	-
33.	IMP 4	+	Slimy	-	-	+
						(white)
34.	IMP 5	++	Smooth	-	-	-
35.	IMP 6	+	Smooth	-	-	-
36.	ISQP 1	+	Smooth	-	-	-

37.	ISQP 2	++	Slimy	-	-	-
38.	ISQP 3	+	Smooth	-	-	-
39.	ISQP 4	++	Slimy	-	-	+ (yellow)

5.3 Screening of actinobacteria for the production of enzymes

5.3.1 Amylase activity

The SQA 4 was quantified as a producer of an industrially important enzyme amylase. They were identified by formation of clear zones against dark blue or dark brown plates respectively (Fig 5.5). The hydrolysis of starch has been practiced on an industrial scale for many years and is gradually replacing the traditional acid hydrolysis.



Fig5.5: Amylase activity

5.3.2 Cellulase activity

Screening of enzyme actinobacterial strains was performed, using the skim milk agar media. In this, it showed promising enzyme activity against cellulase by forming a zone of hydrolysis after adding 1% Congo red and NaOH. SQA 4 showed a very clear zone (Fig 5.6) and this can be taken for the future studies.

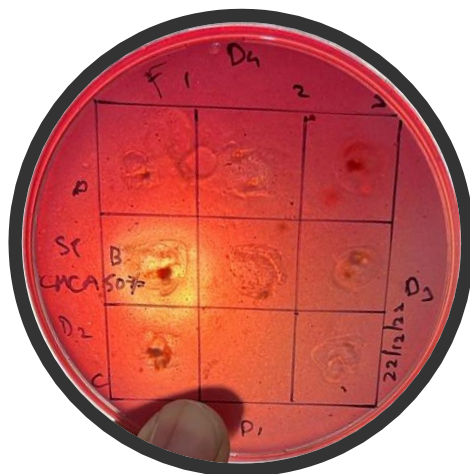


Fig 5.6: Cellulase activity

5.3.3 Protease activity

Proteases represent about 60% of total enzymes market sales. At present, the biggest part of the hydrolytic enzyme market is occupied by the alkali proteases. SQA 4 showed the zone of clearance (Fig 5.7).

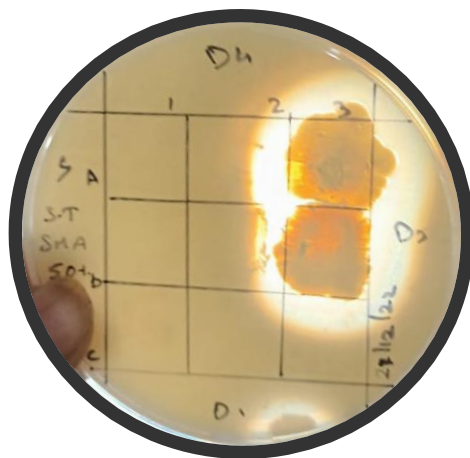


Fig 5.7: Protease activity

5.3.4 Lipase activity

The market for microbial lipase is anticipated to reach USD 590.2 Million by 2023, rising at a CAGR of 6.8% from 2018. It is currently expected to be worth USD 425.0 Million in 2018. The production of modified compounds using lipase enzymes

from microorganisms is just one of the many industrial applications for these conceptually versatile and skilled enzymes. SQA 4 showed the zone of clearance (Fig 5.8). Earlier Sivakumar *et al.*, 2007; Gopikrishnan *et al*, 2019;2020;2021 described the screening of fish gut associated actinobacteria for its enzymes activities. Above studies were less studied in fish gut associated actinobacteria.

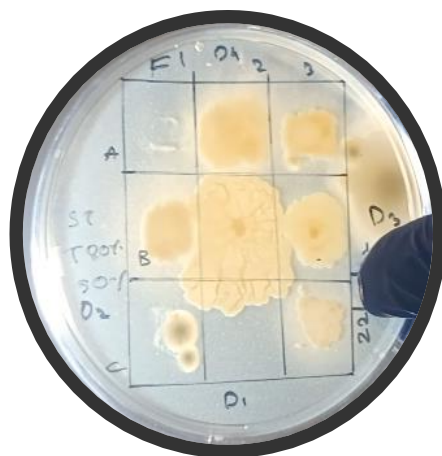


Fig 5.8: Lipase activity

5.4 Screening of Bacteria for Antimycobacterial Activity

Antimycobacterial activity of bacterial cultures were given in (Table 5.3). Of the 39 bacterial strains tested, SQA4 showed maximum zone of inhibition of 14mm for *Staphylococcus aureus*, 10mm for *Candida albicans* and 21mm for *Mycobacterium smegmatis* tested in agar plug method (Table 5.3) (Fig:5.9). Previously Sivakumar *et al.*, 2007; Gopikrishnan *et al*, 2019;2020;2021;2023 and Radhakrishnan *et al.*, 2021 reported the fish gut associated actinobacteria and its antimicrobial activities. Fish gut associated actinobacteria against fish mycobacterial and candida pathogens was less studied. In our study we showed the anti-mycobacterial and antifungal activity of fish associated actinobacteria against fish pathogens (Radhakrishnan *et al.*, 2021).

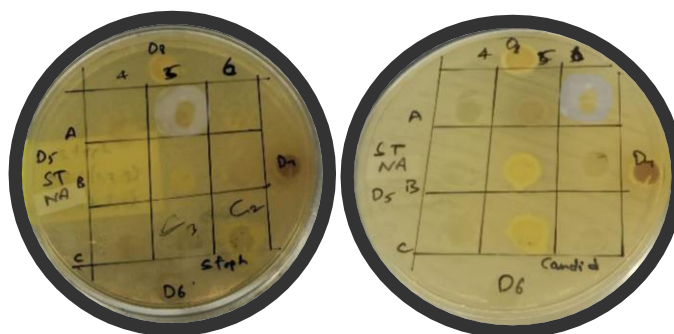


Fig:5.9: SQA 4 Zone of inhibition against test pathogens (*Staphylococcus aureus*, *candida albicans* and *Mycobacterium smegmatis*)

Table 5.3: Antimicrobial activity of bacterial strains by agar plug method

Sn.no	STRAIN NAME	Inhibition zone in mm (bore cutter diameter -9mm)			
		<i>klebsiella pneumonia</i>	<i>Mycobacterium smegmatis</i>	<i>candida albicans</i>	<i>Staphylococcus aureus</i>
1.	IM 1	-	-	-	-
2.	IM 2	-	-	-	-
3.	IM 3	-	-	-	-
4.	IM 4	-	-	-	-
5.	IM 5	-	-	-	-
6.	IM 6	-	-	-	-
7.	IM 7	-	-	-	-
8.	IM 8	-	-	-	-
9.	IM 9	-	-	-	-
10.	IM 10	-	-	-	-
11.	IM 11	-	-	-	-
12.	IM 12	-	-	-	-
13.	SQA 1	-	-	-	-
14.	SQA 2	-	-	-	-
15.	SQA 3	-	-	-	-
16.	SQA 4	-	21	10	14

17.	SQA 5	-	-	-	-
18.	SQA 6	-	-	-	-
19.	SQA 7	-	-	-	-
20.	SQA 8	-	-	-	-
21.	SQA 9	-	-	-	-
22.	SQA 10	-	-	-	-
23.	SQA 11	-	-	-	-
24.	SQA 12	-	-	-	-
25.	SQA 13	-	-	-	-
26.	SQA 14	-	-	-	-
27.	SQA 15	-	-	-	-
28.	SQA 16	-	-	-	-
29.	SQA 17	-	-	-	-
30.	IMP 1	-	-	-	-
31.	IMP 2	-	-	-	-
32.	IMP 3	-	-	-	-
33.	IMP 4	-	-	-	-
34.	IMP 5	-	-	-	-
35.	IMP 6	-	-	-	-
36.	ISQP 1	-	-	-	-
37.	ISQP 2	-	-	-	-
38.	ISQP 3	-	-	-	-
39.	ISQP 4	-	-	-	-

5.5 Selection of Potential Strain

The actinobacterial strain SQA 4 was selected as the potential isolate as it possesses a broad range of antimicrobial activity ranging from 10 mm to 21mm.

5.6 Effect of Medium Consistency and Incubation Period of Bioactive Compounds

Based on the antibacterial results the strain SQA 4 is selected as potential strain. The selected SQA 4 actinobacterial fish gut strain was tested for bioactive metabolite production in submerged and solid medium. The SQA 4 strain is inoculated into YEME agar plates as well as in each 100 ml of YEME broth. This consistency antibacterial test is done to confirm on what media the SQA 4 strain can further be taken for mass production to extract the secondary metabolite crude. The plates were incubated at 28°C in incubator starting from day 2 till 10 days. All the flasks were incubated at 28°C in a rotary shaker for 10 days. Bioactive compound production in solid and submerged medium was assessed from first to eighth day of incubation using *S. aureus* as test organisms. The solid medium was tested by adopting agar plug method (Eccleston et al., 2008) and submerged medium was tested by using agar well diffusion method against test organism seeded on nutrient agar plate (Table 5.4).

Table 5.4: Effect of Fermentation Method on Bioactive Metabolites Production

YEME AGAR				YEME BROTH	
Incubation period	strain	growth	Activity against <i>s.aureus</i>	growth	Activity against <i>s.aureus</i>
Day 1	SQA 4	-	-	-	-
Day 2	SQA 4	++	14mm	+	-
Day 3	SQA 4	+++	15mm	+	-
Day 4	SQA 4	+++	16mm	++	-
Day 5	SQA 4	+++	16mm	+++	-
Day 6	SQA 4	+++	17mm	+++	-
Day 7	SQA 4	+++	17mm	+++	6mm
Day 8	SQA 4	+++	17mm	+++	6mm

Day 9	SQA 4	+++	17mm	+++	7mm
Day 10	SQA 4	+++	17mm	+++	6mm

The mass production of potential SQA 4 strain is carried out in the solid-state fermentation since the consistency and stability of the potential strain is shown in YEME agar fermentation with maximum zone of inhibition in mm.

5.7 Co-culture of Test pathogens

Co-culture method is done on SQA 4 for test pathogens *Staphylococcus aureus*, *Candida albicans* and *Mycobacterium smegmatis* and it triggered the secondary metabolite production for those test pathogens which has resulted in larger zone of inhibitions. (Table 5.5) (fig:5.10, fig:5.11 and fig:5.12).

Table 5.5: Antibacterial activity of SQA 4

Test pathogen	Zone of inhibition in mm. (Well size: 7mm)	
	General SQA 4 strain	Co-cultured SQA 4 strain
<i>Staphylococcus aureus</i>	14	16
<i>Candida albicans</i>	10	12
<i>Mycobacterium smegmatis</i>	21	27



Fig:5.10: heat killed and live of *S. aureus*



Fig:5.11: heat killed and live of *C. albicans*

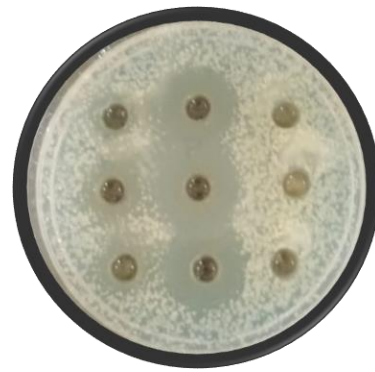


Fig:5.12: general SQA 4, heat killed and live *M. smegmatis*

5.8 Production of Bioactive Metabolites

Crude compounds of strain SQA4 were extracted in chloroform and ethyl acetate, in which maximum quantity and activity was observed in ethyl acetate extract. No compound was obtained in n-hexane extraction. Crude compounds of strain SQA4 were extracted from YEME agar in chloroform, methanol and ethyl acetate but not in n-hexane. Maximum quantity was extracted in ethyl acetate (Fig: 5.13).

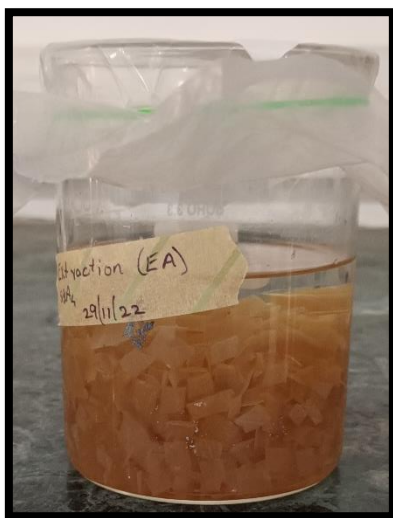


Fig: 5.13: Secondary metabolite extraction of SQA 4

5.9 Microplate Alamar Blue Assay

5.9.1 MABA for *Mycobacterium smegmatis*

The crude extract shows slight activity against *Mycobacterium smegmatis* at 200 and 100 µg/mL concentrations 31% and 12% inhibition. (Table 5.6)

Table 5.6: Optical density reading under UV-spectrometry at 600nm for *M.smegmatis*

Strain name	200 µ/mL			100 µ/mL		
OD values	0.207	0.388	0.399	0.491	0.405	0.366
Avg.	0.331			0.421		
Inhibition	31%			14%		

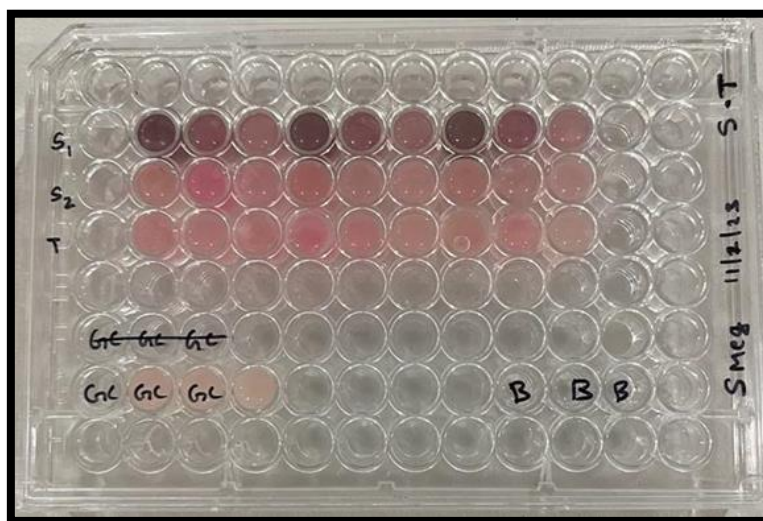


Fig:5.14: MABA for *Mycobacterium smegmatis*.

5.9. MABA for *Mycobacterium tuberculosis* H37 Rv

The crude extract shows promising activity against *Mycobacterium tuberculosis* H37 Rv at 200 and 100 µg/mL concentrations of 61% and 12% inhibition.

Table 5.7: Optical density reading under UV-spectrometry at 600nm for *Mycobacterium tuberculosis* H37 Rv.

Concentration	200 µ/mL			100 µ/mL		
OD values	0.345	0.357	0.355	0.478	0.462	0.475
Avg.	0.352			0.471		
Inhibition	61%			12%		

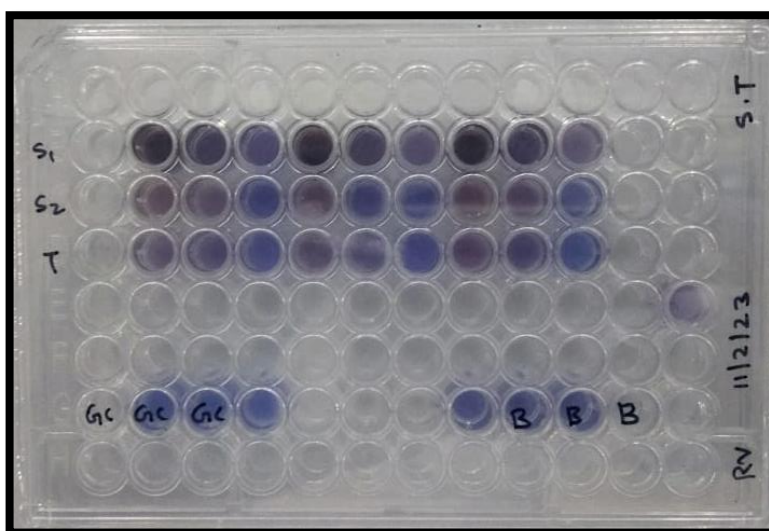


Fig:5.15: MABA for *Mycobacterium tuberculosis* H37 Rv.

5.11 Anti-biofilm Assay

Anti-biofilm Assay is done for extract of SQA 4 for test pathogens *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. (fig 5.16) The crude extract of SQA 4 shows good anti-biofilm activity against *A. baumannii* at 200 and 100 µg/mL concentrations with inhibition of 72.55% and 53.86%.

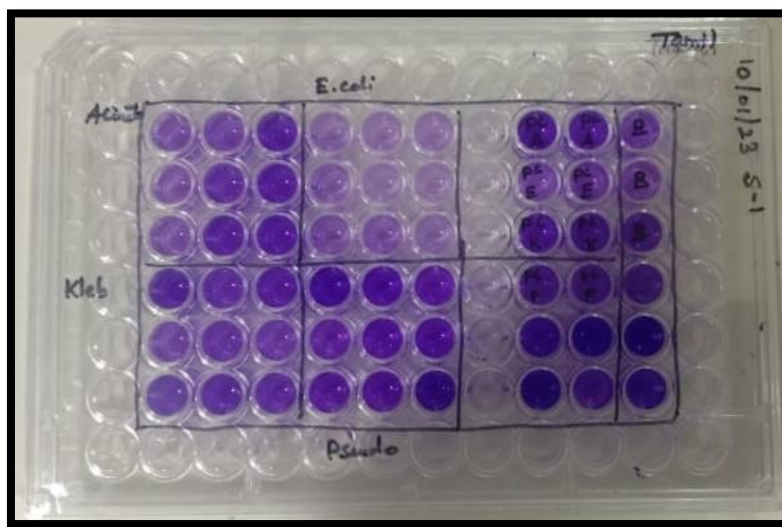


fig 5.16: Anti-biofilm Assay on microplate

Table 5.8: Inhibition of biofilm formation of test pathogens (%)

Concentration μ/mL	<i>A. baumannii</i>			<i>E. coli</i>			<i>K. pneumonia</i>			<i>P. aeruginosa</i>		
	200	100	50	200	100	50	200	100	50	200	100	50
Inhibition %	72. 55	53. 86	28. 28	47. 24	49. 64	54. 85	21. 06	38. 95	42. 86	23. 55	24. 77	23. 31

5.12 Free Radical Scavenging Assay

The free radical scavenging capacity of the crude extract of SQA4 strain was measured based on the method delineate by Brand-Williams et al., (1995) Their colour change were observed and absorbance was measured at 517 nm using UV-Vis spectrophotometer. The crude extract of SQA 4 shows good activity at 200 and 100 μg/mL concentrations with scavenging effect of 62.1% and 50.6%. (fig 5.17)

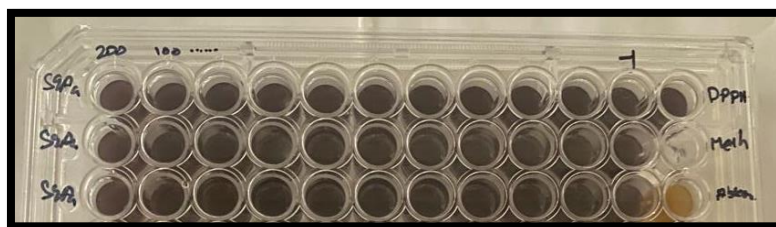


fig 5.17: free radical scavenging on microplate

5.13 Partial Purification and Characterization of Anti-mycobacterial Compounds

In analytical TLC, 5 spots were obtained from crude ethyl acetate extract of SQA 4 when using hexane: Ethyl acetate (1.5:0.5, v/v) as solvent system. The R_f value of separated compounds SQA 4 A-F was calculated as 0.86, 0.57, 0.46, 0.35, 0.21 and 0.15 (Fig: 5.18).

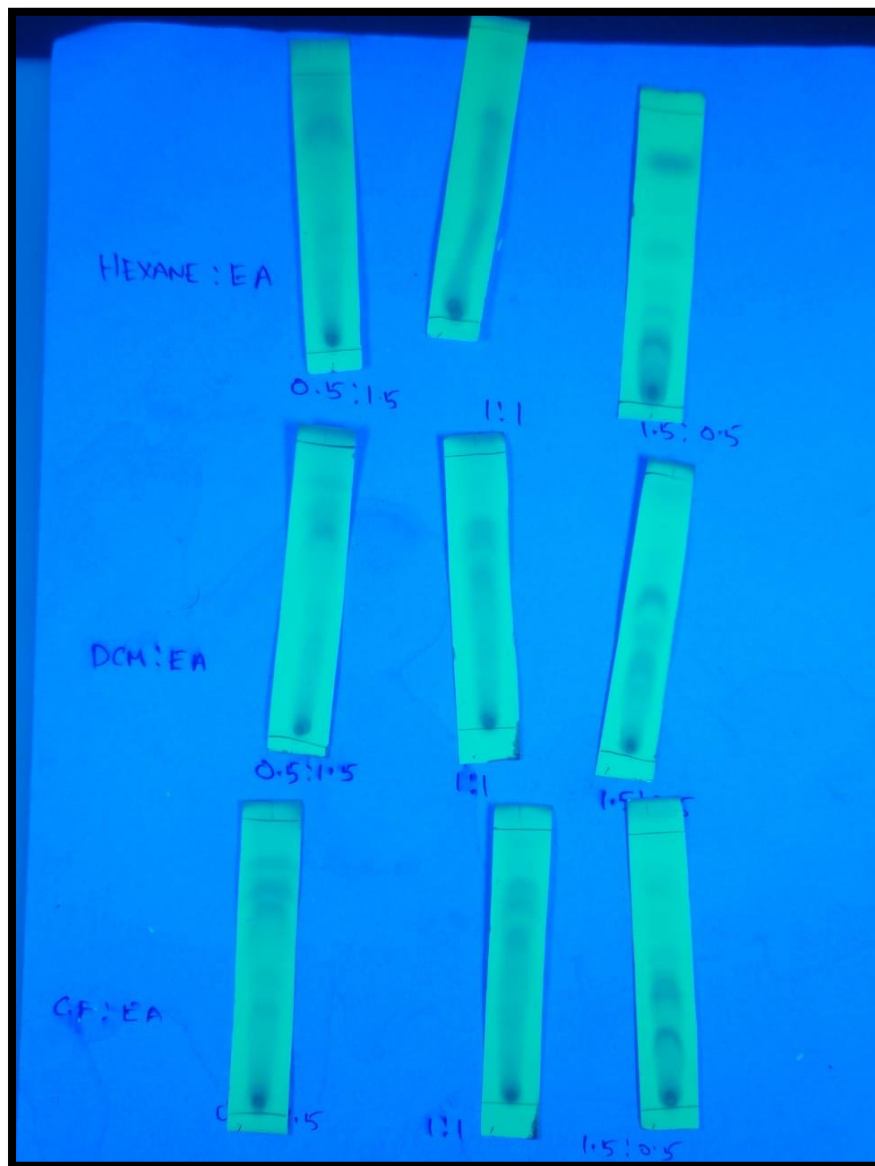


Fig: 5.18: Thin layer chromatography verification of SQA 4 extract.

5.14 Physiological characteristics

The actinobacterial strain SQA 4 was able to grow well utilizing wide range of carbon sources including D-glucose, cellulose, lactose, starch and mannitol. Growth of strain SQA 4 was observed at a wide range of nitrogen sources (including peptone, yeast extract, malt extract, soybean and potassium nitrate). Also, the strain SQA 4 was able to attain good growth in a pH ranges of 1,5,7,9 and 11. (Table :5.6) Based on the

studied phenotypic characteristics SQA 4 actinomycete strains was identified as member of the genus *Streptomyces*.

Table 5.9: Physiological characteristics of endophytic actinobacteria

Characteristics		Growth	AMC (If so colour)	RSP (If so colour)	SP (If so colour)
Utilization of carbon source	D-glucose	+++	White	-	-
	Mannitol	+++	White	-	-
	Starch	+++	White	Pale yellow	-
	cellulose	+++	White	-	-
	Lactose	+++	White	-	-
Utilization of nitrogen source	Peptone	+++	White	-	-
	Yeast extract	+++	White	-	-
	Malt extract	+	White	-	-
	Potassium nitrate	+	White	-	-
	Soya bean	+++	White	Pale yellow	brown
PH	1	-	-	-	-
	5	++	White	-	-
	7	+++	White	-	-
	9	++	White	-	-
	11	+	White	-	-
Temperature	5°C	-	-	-	-

	30°C	+++	White	-	-
	37°C	++++	White	-	-

5.15 Sequencing results

The strain SQA 4 was subjected to 16sRNA analysis. The PCR amplification of *Streptomyces. sp* SQA 4 16sRNA gene yields 1202 base pairs. NCBI BLAST analysis of SQA 4 shows 97% similarity to sequence of *Streptomyces. sp* the evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Nandhini et al.,2018) which further confirms distinct phylogenetic tree constructed with *Streptomyces. sp* SQA 4 is designated as *Phaeobacter sp.* (fig: 5.19). **It's coming under rare forms and further studies like DNA-DNA hybridization are need to done.**

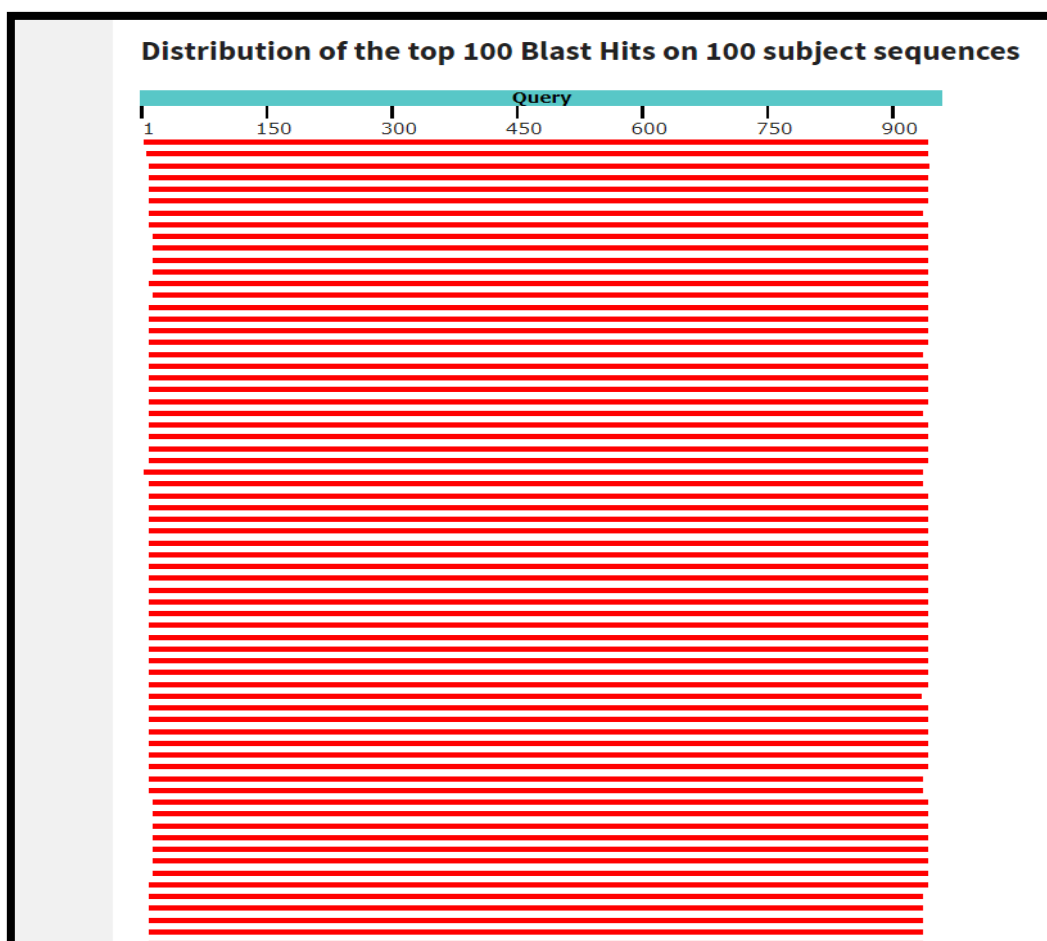


Fig: 5.19: blast results of SQA 4

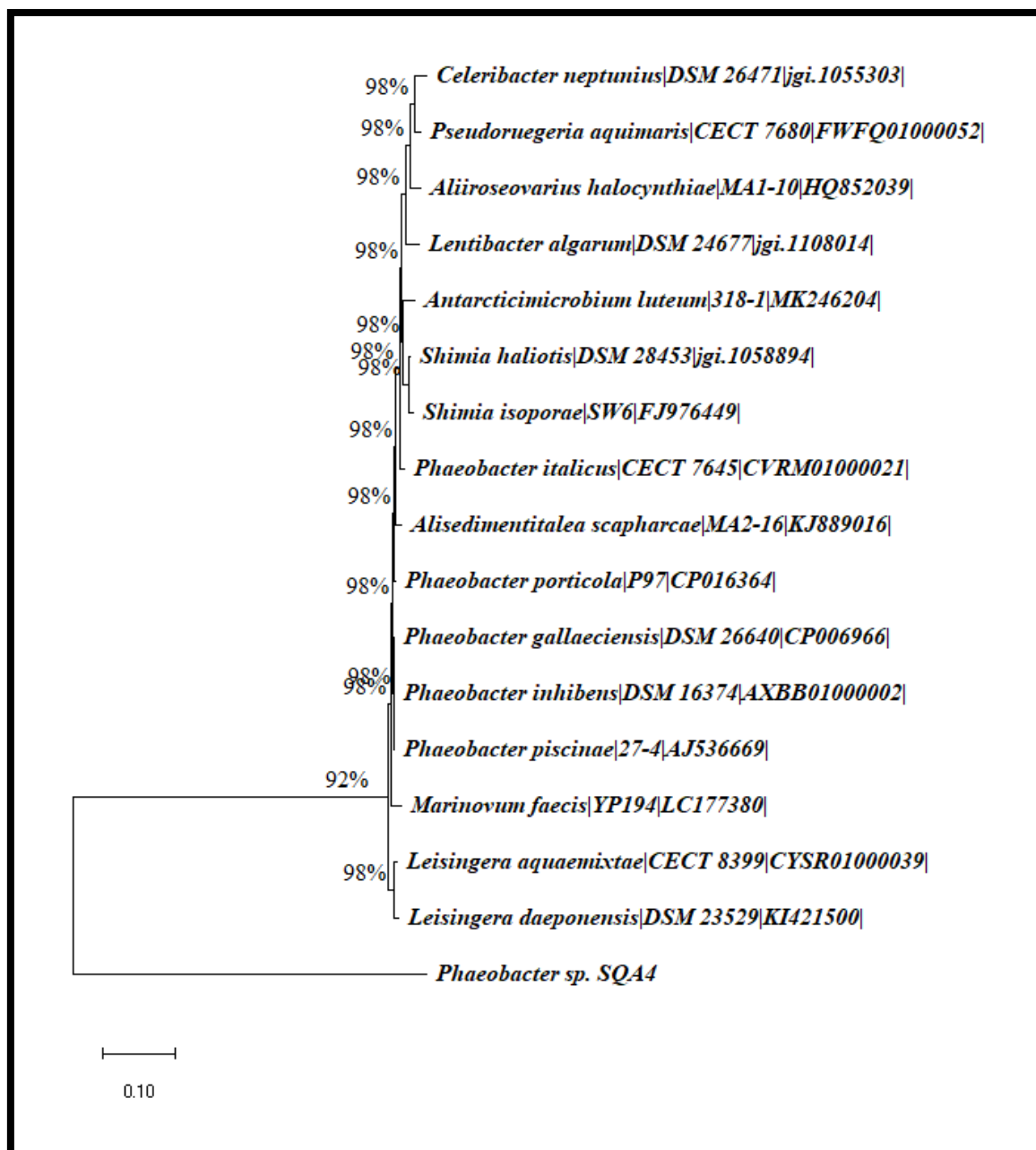


Fig:5.20 Phylogenetic tree

5.16 Chromatograph of SQA 4 crude extract

Through HRMS (high resolution mass spectrometry) the atomic mass of the potential compound was determined.

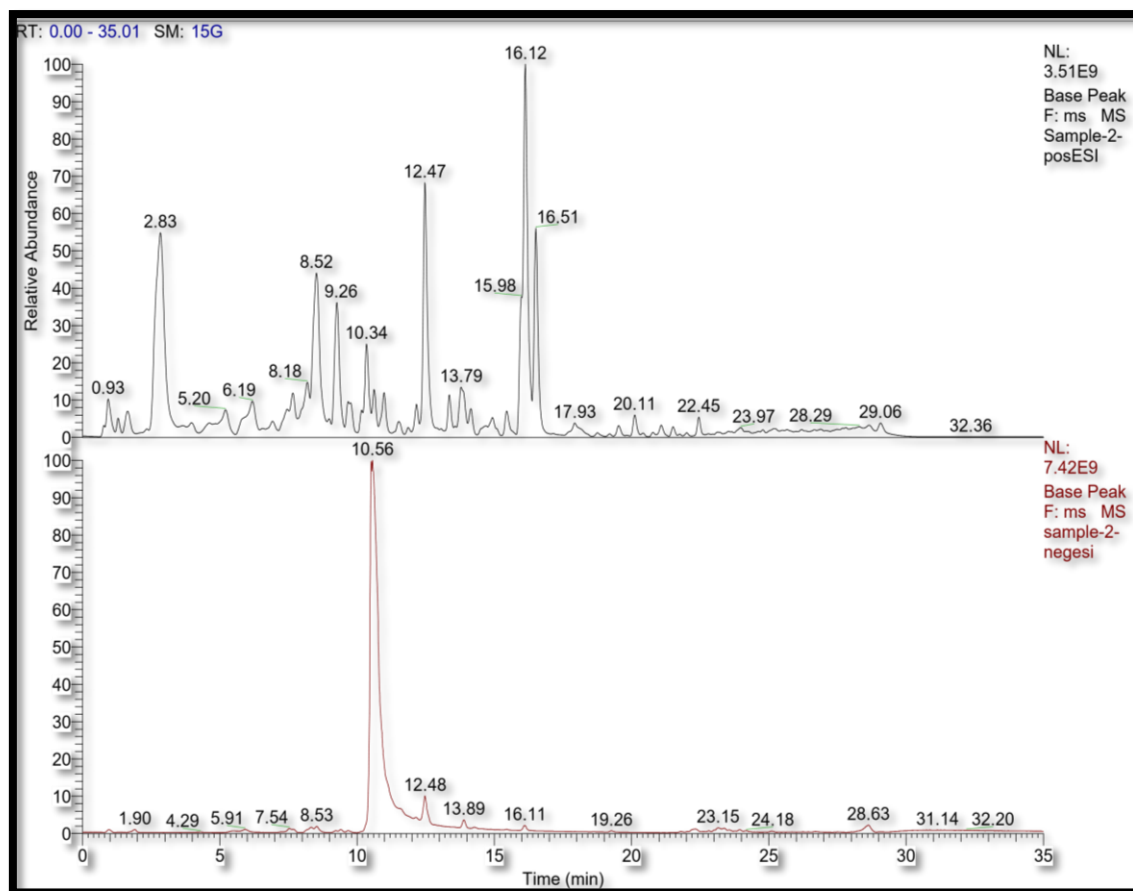


Fig:5.21 Chromatograph of SQA 4

In a nutshell, the results revealed the significant actinomycete diversity from the fish gut microbiota. Obviously, some of the rare-actinomycete isolated in this study may be seen as new candidates representing a valuable source of biologically active compounds with antimicrobial activity and genes for their biosynthesis. Further research including the bioactive assays and molecular characterizations are important to clarify the biotechnological importance and ecological roles of this actinomycete population

CHAPTER – 6

RESULT AND DISCUSSIONS

The ecosystems that have not yet been fully investigated or examined hold great promise for the discovery of bioactive actinobacteria. The goal of the current study was to investigate the antibacterial potential of actinobacteria from Fish gut samples. On an ISP2 agar plate, primary 39 cultures were recovered, in that one is actinomycetes. The agar plug method was used to assess the antimicrobial activity of actinobacterial strain which was named SQA 4. When used against the pathogens *Mycobacterium smegmatis*, *Mycobacterium tuberculosis H37 Rv*, *Staphylococcus aureus*, *Aeromonas caviae*, *Aeromonas salmonicida*, *Acinetobacter baumannii*, *Candida albicans* and *Pseudomonas aeruginosa* actinobacterial strain SQA 4 demonstrated promising activity (10–27 mm Zone of inhibition).

The strain SQA 4 also demonstrated various significant properties such as, Antioxidant activity yielding a significant result, the strain also exhibited properties of anti-biofilm against *E. coli*, *Klebsiella*, *Pseudomonas*, and *Acinetobacter*. This proves that the strain SQA 4 has great potential.

Bioactive metabolites from the strain SQA 4 were produced by adopting agar surface fermentation and submerged fermentation using ISP2 agar and ISP2 broth, respectively. Antimicrobial activity of agar plug from ISP2 agar and cell supernatant from ISP2 broth was tested against *S.aureus* by agar plug and well diffusion method, respectively for every 24 hours up to 8 days. The strain produced antimicrobial compound earlier on ISP2 agar when compared to ISP2 broth.

Crude antimicrobial from the strain SQA 4 was produced by adopting agar surface fermentation and extracted by ethyl acetate.

Microscopic, cultural and physiological and molecular characteristics the SQA 4 strain was studied by adopting standard procedures. Based on the studied phenotypic characteristics, BLAST and phylogenetic tree analysis of SQA 4 comes under

Phaeobacter sp. Further DNA-DNA hybridization, total G-C content and whole genome sequence is needed

Effect of various medium components on antimicrobial compound production by the strains SQA 4 was studied by adopting agar plug method. Various sugars and nitrogen compounds and minerals were found to influence the antimicrobial compound production.

Through HRMS (high resolution mass spectrometry) the atomic mass of the potential compound was determined. Our results concur with the predictions that the fish gut can be a source for novel sp. of actinobacteria with novel bioactive secondary metabolites for biotechnology and medical applications.

With this data we are concluding that examining the rare habitants results in getting novel specius and they are potential in secondary metabolite production. Fish gut is the promising source for novel actinobacteria.

CHAPTER 7

REFERENCES

- 1 Ahmad, S. (2011) 'Pathogenesis, immunology, and diagnosis of latent mycobacterium tuberculosis infection', *Clinical and Developmental Immunology*. Available at: <https://doi.org/10.1155/2011/814943>.
- 2 Amillano-Cisneros, J.M. et al. (2022) 'Loss of gut microbial diversity in the cultured, agastric fish, Mexican pike silverside (*Chirostoma estor*: *Atherinopsidae*)', *PeerJ*, 10. Available at: <https://doi.org/10.7717/peerj.13052>.
- 3 Ansar Ahmed, S., Gogal, R.M. and Walsh, J.E. (1994) 'A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [³H]thymidine incorporation assay', *Journal of Immunological Methods*, 170(2). Available at: [https://doi.org/10.1016/0022-1759\(94\)90396-4](https://doi.org/10.1016/0022-1759(94)90396-4).
- 4 Augustine, S.K., Bhavsar, S.P. and Kapadnis, B.P. (2005) 'A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* PU 23', *Journal of Biosciences*, 30(2). Available at: <https://doi.org/10.1007/BF02703700>.
- 5 Bahobail, A., Gad El-Rab, S.M.F. and Amin, G.A. (2016) 'Locally Isolated Bacterial Strains with Multiple Degradation Potential Capabilities on Petroleum Hydrocarbon Pollutants', *Advances in Microbiology*, 06(11). Available at: <https://doi.org/10.4236/aim.2016.611081>.
- 6 Balagurunathan, R. et al. (2020) 'Characterization and Identification of Actinobacteria', in. Available at: https://doi.org/10.1007/978-1-0716-0728-2_3.
- 7 Bennett, D. et al. (2008) 'A pilot study of resistance to aspirin in stroke patients', *Journal of Clinical Neuroscience*, 15(11). Available at: <https://doi.org/10.1016/j.jocn.2008.01.006>.
- 8 Bérdy, J. (2012) 'Thoughts and facts about antibiotics: Where we are now and where we are heading', *Journal of Antibiotics*. Available at: <https://doi.org/10.1038/ja.2012.27>.
- 9 Bermudez, L.E. and Goodman, J. (1996) 'Mycobacterium tuberculosis invades and replicates within type II alveolar cells', *Infection and Immunity*, 64(4). Available at: <https://doi.org/10.1128/iai.64.4.1400-1406.1996>.

- 10 Bhutani, N. *et al.* (2021) 'Bioprospecting for extracellular enzymes from endophytic bacteria isolated from *Vigna radiata* and *Cajanus cajan*', *Journal of Applied Biology and Biotechnology*, 9(3). Available at: <https://doi.org/10.7324/JABB.2021.9304>.
- 11 Birkness, K.A. *et al.* (1999) 'An in vitro tissue culture bilayer model to examine early events in *Mycobacterium tuberculosis* infection', *Infection and Immunity*, 67(2). Available at: <https://doi.org/10.1128/iai.67.2.653-658.1999>.
- 12 Brand-Williams, W., Cuvelier, M.E. and Berset, C. (1995) 'Use of a free radical method to evaluate antioxidant activity', *LWT - Food Science and Technology*. Available at: [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5).
- 13 Camus, J.C. *et al.* (2002) 'Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv', *Microbiology*, 148(10). Available at: <https://doi.org/10.1099/00221287-148-10-2967>.
- 14 Chen, P. *et al.* (2012) 'A highly efficient Ziehl-Neelsen stain: Identifying de novo intracellular *Mycobacterium tuberculosis* and improving detection of extracellular *M. tuberculosis* in cerebrospinal fluid', *Journal of Clinical Microbiology*, 50(4). Available at: <https://doi.org/10.1128/JCM.05756-11>.
- 15 Clarridge, J.E. (2004) 'Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases', *Clinical Microbiology Reviews*. Available at: <https://doi.org/10.1128/CMR.17.4.840-862.2004>.
- 16 Cole, S.T. *et al.* (1998) 'Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence', *Nature*, 393(NOVEMBER).
- 17 Collins, L.A. and Franzblau, S.G. (1997) 'Microplate Alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*', *Antimicrobial Agents and Chemotherapy*, 41(5). Available at: <https://doi.org/10.1128/aac.41.5.1004>.
- 18 da Cunha, M.C. *et al.* (2018) 'Using response surface methodology to improve the L-asparaginase production by *Aspergillus niger* under solid-state fermentation', *Biocatalysis and Agricultural Biotechnology*, 16. Available at: <https://doi.org/10.1016/j.bcab.2018.07.018>.

- 19 DS, A.-R. (2016) 'Phylogenetic Analysis of Streptomyces spp. Exhibited Different Antimicrobial Activities', *Iraqi Journal of Science*, 57(1).
- 20 Egerton, S. et al. (2018) 'The gut microbiota of marine fish', *Frontiers in Microbiology*. Available at: <https://doi.org/10.3389/fmicb.2018.00873>.
- 21 Franzblau, S.G. et al. (1998) 'Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay', *Journal of Clinical Microbiology*, 36(2). Available at: <https://doi.org/10.1128/jcm.36.2.362-366.1998>.
- 22 Gajardo, K. et al. (2016) 'A high-resolution map of the gut microbiota in Atlantic salmon (Salmo salar): A basis for comparative gut microbial research', *Scientific Reports*, 6. Available at: <https://doi.org/10.1038/srep30893>.
- 23 Ghai, R. et al. (2011) 'Metagenomics of the water column in the pristine upper course of the Amazon river', *PLoS ONE*, 6(8). Available at: <https://doi.org/10.1371/journal.pone.0023785>.
- 24 Ghanbari, M., Kneifel, W. and Domig, K.J. (2015) 'A new view of the fish gut microbiome: Advances from next-generation sequencing', *Aquaculture*. Available at: <https://doi.org/10.1016/j.aquaculture.2015.06.033>.
- 25 Ginsberg, A.M. (2010) 'Drugs in development for tuberculosis', *Drugs*. Available at: <https://doi.org/10.2165/11538170-000000000-00000>.
- 26 Gopikrishnan, V. et al. (2019) 'Isolation, characterization and identification of antibiofouling metabolite from mangrove derived Streptomyces sampsonii PM33', *Scientific Reports*, 9(1). Available at: <https://doi.org/10.1038/s41598-019-49478-2>.
- 27 GORDON, R.E. and SMITH, M.M. (1953) 'Rapidly growing, acid fast bacteria. I. Species' descriptions of Mycobacterium phlei Lehmann and Neumann and Mycobacterium smegmatis (Trevisan) Lehmann and Neumann.', *Journal of bacteriology*, 66(1).
- 28 Hansen, G.H., Strom, E. and Olafsen, J.A. (1992) 'Effect of different holding regimens on the intestinal microflora of herring (Clupea harengus) larvae', *Applied and Environmental Microbiology*, 58(2). Available at: <https://doi.org/10.1128/aem.58.2.461-470.1992>.

- 29 Hwang, K.S. *et al.* (2014) 'Systems biology and biotechnology of *Streptomyces* species for the production of secondary metabolites', *Biotechnology Advances*. Available at: <https://doi.org/10.1016/j.biotechadv.2013.10.008>.
- 30 Jiang, Y. *et al.* (2016) 'Isolation and Cultivation Methods of Actinobacteria', in *Actinobacteria - Basics and Biotechnological Applications*. Available at: <https://doi.org/10.5772/61457>.
- 31 Joo, H.M. *et al.* (2011) 'Lactobacillus johnsonii HY7042 ameliorates Gardnerella vaginalis-induced vaginosis by killing Gardnerella vaginalis and inhibiting NF- κ B activation', *International Immunopharmacology*, 11(11). Available at: <https://doi.org/10.1016/j.intimp.2011.07.002>.
- 32 Kämpfer, P. (2006) 'The Family Streptomycetaceae, Part I: Taxonomy BT - The Prokaryotes', *The Prokaryotes* [Preprint], (Chapter 22).
- 33 Kashyap, R.S. *et al.* (2013) 'Tuberculosis in India: The continuing challenge', *Current Science*, 105(5).
- 34 King, G.M. (2003) 'Uptake of Carbon Monoxide and Hydrogen at Environmentally Relevant Concentrations by Mycobacteria', *Applied and Environmental Microbiology*, 69(12). Available at: <https://doi.org/10.1128/AEM.69.12.7266-7272.2003>.
- 35 Kramnik, I. *et al.* (2000) 'Genetic control of resistance to experimental infection with virulent Mycobacterium tuberculosis', *Proceedings of the National Academy of Sciences of the United States of America*, 97(15). Available at: <https://doi.org/10.1073/pnas.150227197>.
- 36 KUBICA, G.P., KIM, T.H. and DUNBAR, F.P. (1972) 'Designation of Strain H37Rv as the Neotype of Mycobacterium tuberculosis', *International Journal of Systematic Bacteriology*, 22(2). Available at: <https://doi.org/10.1099/00207713-22-2-99>.
- 37 Larsen, A.M., Mohammed, H.H. and Arias, C.R. (2015) 'Comparison of DNA extraction protocols for the analysis of gut microbiota in fishes', *FEMS Microbiology Letters*, 362(5). Available at: <https://doi.org/10.1093/femsle/fnu031>.

- 38 Ma, A. *et al.* (2020) 'Phylogenetic and Physiological Diversity of Cultivable Actinomycetes Isolated From Alpine Habitats on the Qinghai-Tibetan Plateau', *Frontiers in Microbiology*, 11. Available at: <https://doi.org/10.3389/fmicb.2020.555351>.
- 39 Madigan, M.T. and Martinko, J. (2005) 'Brock Biology of Micro-Organisms Brock Biology of Microorganisms', *Ugeskrift for Laeger*, 159(January).
- 40 Manikkam, R. *et al.* (2014) ' Bioactive Potential of Actinomycetes from Less Explored Ecosystems against Mycobacterium tuberculosis and Other Nonmycobacterial Pathogens ', *International Scholarly Research Notices*, 2014. Available at: <https://doi.org/10.1155/2014/812974>.
- 41 van der Meij, A. *et al.* (2017) 'Chemical ecology of antibiotic production by actinomycetes', *FEMS Microbiology Reviews*. Available at: <https://doi.org/10.1093/femsre/fux005>.
- 42 Meklat, A. *et al.* (2011) 'Isolation, taxonomy, and antagonistic properties of halophilic actinomycetes in saharan soils of algeria', *Applied and Environmental Microbiology*, 77(18). Available at: <https://doi.org/10.1128/AEM.00326-11>.
- 43 Merrifield, D.L. and Ringø, E. (2014) *Aquaculture nutrition: Gut health, probiotics and prebiotics*, *Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics*. Available at: <https://doi.org/10.1002/9781118897263>.
- 44 Mukhopadhyay, B. and Ganguly, N.K. (2013) 'Tuberculosis research in India', *Current Science*.
- 45 Patel, J. (2001) '16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory', *Molecular Diagnosis*, 6(4). Available at: <https://doi.org/10.1054/modi.2001.29158>.
- 46 Pepper, I.L., Gerba, C.P. and Gentry, T.J. (2015) 'Introduction to Environmental Microbiology', in *Environmental Microbiology: Third Edition*. Available at: <https://doi.org/10.1016/B978-0-12-394626-3.00001-6>.
- 47 Radhakrishnan, M., Balaji, S. and Balagurunathan, R. (2007) 'Thermotolerant Actinomycetes From the Himalayan Mountain – Antagonistic Potential , Characterization and Identification of Selected Strains', *Appl. Biol.*, 36(1).

- 48 Shirling, E.B. and Gottlieb, D. (1966) 'Methods for characterization of *Streptomyces* species', *International Journal of Systematic Bacteriology*, 16(3). Available at: <https://doi.org/10.1099/00207713-16-3-313>.
- 49 de Souza, M.V.N. (2008) 'Promising Drugs Against Tuberculosis', *Recent Patents on Anti-Infective Drug Discovery*, 1(1). Available at: <https://doi.org/10.2174/157489106775244163>.
- 50 Sreenivasan, A., Manikkam, R. and Manigundan, K. (2022) 'Isolation, Characterization and Antimicrobial Activity of Endophytic Actinobacteria from Medicinal Plants', *Indian Journal of Pharmaceutical Sciences*, 84(5). Available at: <https://doi.org/10.36468/pharmaceutical-sciences.1009>.
- 51 Steenken, W., Oatway, W.H. and Petroff, S.A. (1934) 'Biological studies of the tubercle bacillus: III. dissociation and pathogenicity of the r and s variants of the human tubercle bacillus (H37)', *Journal of Experimental Medicine*, 60(4). Available at: <https://doi.org/10.1084/jem.60.4.515>.
- 52 Subramani, R. and Aalbersberg, W. (2013) 'Culturable rare Actinomycetes: Diversity, isolation and marine natural product discovery', *Applied Microbiology and Biotechnology*, 97(21). Available at: <https://doi.org/10.1007/s00253-013-5229-7>.
- 53 Troussellier, M. et al. (2017) 'Sustaining rare marine microorganisms: Macroorganisms as repositories and dispersal agents of microbial diversity', *Frontiers in Microbiology*, 8(MAY). Available at: <https://doi.org/10.3389/fmicb.2017.00947>.
- 54 Yajko, D.M. et al. (1995) 'Colorimetric method for determining MICs of antimicrobial agents for *Mycobacterium tuberculosis*', *Journal of Clinical Microbiology*, 33(9). Available at: <https://doi.org/10.1128/jcm.33.9.2324-2327.1995>.
- 55 Yang, J. et al. (2023) 'Comparative Analysis of the Symbiotic Microbiota in the Chinese Mitten Crab (*Eriocheir sinensis*): Microbial Structure, Co-Occurrence Patterns, and Predictive Functions', *Microorganisms*, 11(3). Available at: <https://doi.org/10.3390/microorganisms11030544>.

- 56 Yunianta, A. and Yusof, N. (2012) 'Analysis and categorization of e-learning activities based on meaningful learning characteristics', *Johor Bahru: Universiti ...*, 6(9).