

BIOPROSPECTING OF FISH GUT MICROBES

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

by

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **BOOMIKA.V(40230007), MONISHA.V (40230028)** who have done the project work entitled “**BIOPROSPECTING OF FISH GUT MICROBES**” under our supervision from September 2023 to April 2024.

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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, enzymatic and antioxidant activity of fish gut associated actinobacteria isolated from Trevally (*Caranx ignobilis*), Red snapper (*Lutjanus campechanus*), Silver biddy (*Gerres oyena*), Indian mackerel (*Rastrelliger kanagurta*), Indian oil sardine (*Sardinella longiceps*) and Indian Squid (*Uroteuthis duvaucelii*). Bacterial strains are isolated from Trevally (*Caranx ignobilis*), Red snapper (*Lutjanus campechanus*), Silver biddy (*Gerres oyena*), Indian mackerel (*Rastrelliger kanagurta*), Indian oil sardine (*Sardinella longiceps*) and Indian Squid (*Uroteuthis duvaucelii*) fish gut samples by adopting standard spread plate method on SCA, ISP2 and NA media. A total of 2 actinobacterial colony was found out of 34. Antimicrobial activity of actinobacterial strain isolated from Trevally gut sample was determined by agar plug and well diffusion methods. Actinobacterial strain from Squid gut (TFB 11) was showed promising enzymatic activity. Crude extract from the strain TFB 11 was produced by adopting agar surface fermentation and extracted using methanol. It antioxidant property of 52.73% scavenging effect. Based on their phenotypic characteristics, strain TFB 11 was identified as Actinomycetes. The findings suggested that the fish associated actinobacteria is a promising source for multifunctional bioactive compounds for the development of novel therapeutics drugs.

Keywords: Fish Gut, Bioprospecting, Pigments, Actinobacteria, Antioxidant, Anti-TB and Anti-biofilm.

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LIST OF ABBREVIATIONS

ACRONYM	EXPANSION
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>
MHA	Muller hinton agar
NA	Nutrient agar
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PSB	Phosphate saline buffer
RF	Retention factor
RNA	Ribonucleic acid
RPM	Rotation per minute
SCA	Starch casein agar
Sp.	Species
TLC	Thin layer chromatography
UV	Ultraviolet-visible spectroscopy

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CHAPTER 1

INTRODUCTION

1.1 Fish Gut Microbiota

The ecosystem that makes up the gut microbiota of fish is dynamic and diverse, consisting of protists, fungus, bacteria, viruses, and archaea. Numerous factors, including as host genetics, food, environmental factors, and interspecies interactions, contribute to this complexity. Every type of fish has its own distinct community of microorganisms that are adapted to meet its particular physiological and ecological needs (Merrifield and Ringø, 2014). The extraordinary adaptability of fish gut microbiota is highlighted by their diversity, since they can thrive in a wide range of settings, from pure freshwater streams to the saline depths of the ocean. A complex balance between mutualism and competition lays at the basis of the relationship between fish and their intestinal bacteria. Beneficial microorganisms support general health and vigor by assisting in the digestion of complex nutrients, the manufacture of important vitamins, and the regulation of host immunological responses. On the other hand, pathogenic bacteria are a continual menace that test the host's fortitude and demand strong defenses (Merrifield and Ringø, 2014). The constant interplay between host biology, environmental variables, and microbial interactions is reflected in the richness and complexity of the gut microbiota of fish. Different fish species and populations have distinct microbial signatures due to factors like host genetics, environmental features, and diet composition that impact the gut microbiota. The complexity of the gut ecosystem is further enhanced by the interactions between bacteria, which include cooperation, competition, and niche specialization.

1.2 Significance of Bioprospecting

The methodical investigation of biological resources, such as plants, animals, microbes, and their genetic material, is known as bioprospecting. The goal is to find novel substances, genes, or processes that may find use in a variety of businesses. (Johny *et al.*, 2021, Kim *et al.*, 2021) In order to maximize the potential of bioactive chemicals or biological systems found in nature for industrial, medicinal, agricultural, or biotechnological uses, this method entails locating, separating, and characterizing

them. One particularly promising method for finding novel bioactive chemicals with a variety of industrial and clinical applications is bioprospecting of fish gut microorganisms. Fish species' gut microbiota is a rich source of microbial variety since each species has its own distinct community of bacteria that are tailored to its own ecological niche and eating preferences. To survive in the intricate and dynamic environment of the fish gut, these microbial communities have developed unique metabolic pathways, which have resulted in the production of a wide range of bioactive compounds with possible uses. It has been demonstrated that fish gut microorganisms create a wide variety of catalytically active enzymes, which have enormous industrial application potential. These enzymes, which include Amylase Lipase Urease and proteases, are essential for metabolism, nutritional digestion, and host-microbe interactions in the gastrointestinal tract. Researchers can find novel biocatalysts for use in a variety of biotechnological processes, including waste treatment, food processing, pharmaceutical manufacturing, and the creation of biofuels, by isolating and characterizing these enzymes. Bioactive chemicals obtained from fish gut microorganisms may be useful for medication development and discovery in the field of therapeutic applications. For instance, it has been demonstrated that antimicrobial peptides made by gut bacteria may be used in place of traditional antibiotics to treat infections that are resistant to drugs. In a similar vein, secondary metabolites possessing anti-inflammatory or anti-cancer qualities may provide new drug targets for human illness treatment.

1.3 Biodiversity Hotspot

The variety of fish species found in marine environments is especially remarkable, as innumerable species can be found residing in a wide range of habitats, from shallow coastal waters to the deepest parts of the ocean floor. Particularly known for their astounding biodiversity, coral reefs are home to a wide variety of fish species that are all perfectly adapted to the complex microhabitats that coral formations provide (Kim *et al.*, 2021). Marine fish ecosystems display unmatched diversity and complexity, with colourful reef fish darting among the corals and majestic predators patrolling the open ocean. In order to ensure the sustainable use of natural resources and protect the future of bioprospecting endeavors, it is imperative to conserve marine biodiversity.

The loss of biodiversity poses a danger to the availability of genetic variety for bioprospecting as human activities, such as overfishing, habitat destruction, pollution, and climate change, continue to put pressure on marine ecosystems. Thus, maintaining the vast biodiversity of marine fish species and the microbial communities they support depends on conservation initiatives that safeguard marine environments, lessen the effects of human activity, and support sustainable fisheries management.

1.4 Adjusting to Severe Circumstances

The extraordinary resilience and evolutionary flexibility of fish gut microorganisms is demonstrated by their capacity to adapt to a wide range of environmental situations. These microbial communities have developed complex strategies to survive in a variety of settings, such as those with fluctuating salinity, temperature, and pollution exposure. These adaptations not only help fish gut microorganisms to endure harsh environments but also stimulate the synthesis of bioactive substances and enzymes that are extremophilic in nature (Meklat *et al.*, 2011).

1.4.1 Temperature

One important environmental component that affects the metabolism and microbial activity in the fish gut is temperature. Fish species live in environments with a wide range of temperatures, from tropical oceans to frigid polar waters, which exposes their gut microorganisms to temperature changes. In order to maintain optimal enzyme activity and metabolic efficiency over a range of temperature regimes, gut bacteria may respond by going through physiological changes. Certain bacteria create thermophilic or cold-adapted enzymes that can work in extremely hot conditions, helping their fish hosts consume and metabolize nutrients.

1.4.2 Salinity

Another important environmental factor that influences the make-up and functionality of the gut microbiota of fish is salinity, especially for species that live in estuaries and coastal areas where salt levels are very variable. These fish species' gut microorganisms may display osmoregulatory adaptations, such as modifying cell membrane permeability or storing compatible solutes, to deal with variations in salt concentrations. Furthermore, some gut microorganisms create halotolerant enzymes,

which may continue to function in saline environments and facilitate effective dietary nutrient digestion and metabolism.

1.4.3 Pollutant exposure

Fish gut bacteria face a great deal of difficulty when exposed to contaminants, especially in areas where xenobiotics, heavy metals, or organic pollutants are present. Gut microbes may go through detoxification mechanisms, like enzymatic breakdown or sequestration of harmful compounds, in response to pollution stress. Certain microorganisms generate specific enzymes that are essential to detoxification processes, like glutathione-S-transferases and cytochrome P450 monooxygenases. Furthermore, some gut microorganisms may generate bioactive substances that have the ability to bind metals or act as antioxidants, providing defense against metal toxicity and oxidative stress.

1.5 Biotechnological Applications

1.5.1 Enzyme production for industrial purposes

Fish gut bacteria generate a wide range of enzymes, such as proteases, lipases, amylase and urease, each having specific catalytic characteristics. These enzymes are used in many industrial processes, including the preparation of detergents, the production of textiles, the processing of food, and the creation of biofuel. For instance, the food sector can employ the proteases produced by gut microorganisms for dairy processing and meat tenderization, while lipases are useful for the breakdown of lipids in the process of producing biodiesel. By utilizing these fish gut microbial enzymes, standard chemical procedures can be replaced with more affordable and environmentally friendly options that also consume less energy. (Nielsen, P. H. 2013).

1.5.2 Antimicrobial agents for pharmaceuticals

Numerous bioactive substances with antibacterial qualities, such as bacteriocins, secondary metabolites, and antimicrobial peptides, are produced by fish gut microorganisms. These substances may find use in medicines as innovative antibiotics or antifungal agents. To tackle drug-resistant diseases, for example, antimicrobial peptides made by gut microorganisms may provide an alternative to

conventional antibiotics. Researchers can find novel antimicrobial compounds with strong activity against harmful germs by examining the varied microbial communities found in fish guts. This can aid in the fight against infectious diseases and lower the likelihood of antibiotic resistance.

1.5.3 Bioremediation of environmental pollutants

Fish gut bacteria can use processes called biodegradation and biotransformation to break down a variety of contaminants, including pesticides, heavy metals, and hydrocarbons. When compared to conventional procedures, bioremediation employing fish gut bacteria can offer a sustainable and affordable way to clean up contaminated locations, potentially saving a substantial amount of money on remediation expenses. Bioremediation offers a more environmentally friendly way to clean up polluted surroundings without adding more dangerous chemicals by utilizing fish gut bacteria's innate capacity to digest contaminants.

1.5.4 Nutrient recycling in aquaculture

In aquaculture systems, fish gut bacteria are essential for the digestion and absorption of nutrients, which improves feed efficiency and waste control. By improving nutrient use in aquaculture, optimizing fish gut microbiota can lower feed costs and accelerate growth rates. By limiting nutrient release into nearby ecosystems, effective nutrient recycling lessens the environmental impact of aquaculture operations and helps to mitigate eutrophication and water pollution.

1.6 Technological Advances

The host fish's general growth, metabolism, eating habits, and immune response are all impacted by the fish's gut microbiota. The rise of bacteria resistant to antibiotics and difficult-to-treat fish illnesses calls for the potential use of gut microbes that benefit fish, which could result in the use of these microbes as probiotics. Antibiotic resistance, which impacts the gut microbiota of aquatic creatures, is one of the major biological and ecological issues brought on by the extensive and methodical use of antibiotics. Probiotics, also referred to as beneficial bacteria, are suggested as an efficient and sustainable substitute for antibiotics. Prebiotics are also thought to improve the growth and well-being of the host by reducing the number of intestinal pathogens and/or

altering the way that health-related bacterial metabolites evolve. With the advent of complex bioinformatics tools and sequencing technologies, it is now possible to research these gut bacteria using metagenomic analysis. This review discusses the information related to the fish gut microbiome, their function, and their importance in aquaculture. A wealth of information was obtained from various metagenomic studies. This information includes the effect of the gut microbiome on the physiology of fish, the composition of gut microbes in different fish, and factors affecting the gut microbial composition of the fish. (G. G., van Leeuwen, C. H. A., & Wilkinson, D. M. 2023)

1.7 Sustainability and Conservation

The marine environment, which makes up over 70% of the planet, is the most diverse ecosystem. More than 95% of marine biomass is made up of microbial species. Included in the water column, sediments, or associations are viruses, bacteria, phytoplankton (photosynthetic planktons), and zooplankton (grazing planktons). Some of the most adaptable organisms tolerating extreme salinities, temperatures, and pressures are found in marine ecosystems because they are constantly exposed to anthropogenic stressors and encompass a variety of naturally occurring extreme environmental conditions (e.g., in polar regions, hydrothermal vents, etc.). A large deal of genetic and functional variety is driven by these adaptive capacities, which facilitate the synthesis of molecules that support biotechnological applications. In this regard, marine microorganisms are gaining a lot of attention since it is thought that they could be a valuable source of molecules for the creation of medications that treat serious disorders. For example, high-value lipids, pigments, and exopolysaccharides are among the innovative potential offered by algae-driven products, which are of particular interest. Additionally, a variety of marine fungal and bacterial species have been shown to generate a broad range of novel chemicals with potential for use as antibiotics. Biomolecules generated from marine yeast have also demonstrated intriguing characteristics for a range of possible uses, such as the synthesis of biosurfactants (Yang& Wei, Q. 2022).

CHAPTER 2

LITERATURE SURVEY

2.1 Introduction

The gut microbiota of fish is composed of a wide variety of microorganisms, including viruses, bacteria, fungus, and archaea. Together, these microbes and their host develop complex symbiotic connections that influence behaviour, immune system regulation, and nutrition metabolism. Additionally, fish's gut microbiota is essential for mediating interactions with their surroundings, impacting things like illness resistance, pollutant degradation, and nutrient cycling. The objective of this thesis is to elucidate the complex dynamics of fish gut microbial activity and its implications for ecology. Through the use of an interdisciplinary approach that combines microbial physiology, molecular ecology, and ecosystem science, this research aims to address fundamental questions about the functions of particular microbial taxa, the drivers of gut microbiota composition, and the effects of gut activity on ecosystem processes.

2.1.1 FISH GUT

Trophic studies are essential in biology and ecology, aiding in the observation of individual organisms and the modeling of ecosystem function. When analyzing fish gut contents, we typically collect samples that offer a snapshot of their diet at a particular moment. While there is a common belief that more detailed data on the composition of gut contents yield more information, we aim to shed light on some key issues with this approach (Gajardo *et al.*, 2016). Specifically, we question the reliability of quantitative methods, such as those measuring the biomass or volume of each prey type collectively, and propose that the frequency of occurrence (%F) provides a more robust and easily interpretable measure of diet composition. Our concern lies with the potential for significant errors inherent in bulk methods, stemming from various sources. From our observations, we find that the contents of most guts cannot be definitively categorized into prey types due to the presence of partially digested and unidentifiable material. Even if separation is feasible, the composition of gut contents at a given time can be influenced by numerous factors unrelated to the actual diet composition. As a result, bulk methods often yield ambiguous interpretations, despite appearing quantitative. While more detailed approaches like prey reconstruction may be necessary for certain research inquiries, %F offers a reliable alternative for

describing dietary composition in many studies. It overcomes many limitations of more intricate methods and provides significant logistical and economic advantages.

2.1.2 Current Approaches

In recent years, there has been a notable increase in research exploring the microbial communities inhabiting the guts of fish, which has enhanced our understanding of the intricate relationships between these microbes and their host fish. These gut microbes play crucial roles in bolstering the digestive and immune systems of fish, and their composition is influenced by various factors associated with the host. However, previous studies have primarily focused on describing the structure of fish gut microbiomes, providing limited insight into the specific effects of these factors and the significant functional capabilities of the gut microbiome. In this review, we have emphasized recent advancements in our knowledge of the diversity and functions of fish gut microbiomes. Additionally, we have examined current methodologies employed in studying these microbiomes, aiming to consolidate the accumulated knowledge and draw accurate conclusions for potential applications. Our review of the literature suggests that future research should pivot towards functional microbiomics to enhance the sustainability and productivity of aquaculture operations.

2.1.3 The Gut Microbiota of Fish

Animals host a diverse array of microorganisms in their gastrointestinal (GI) tract, including bacteria, yeast, viruses, archaea, and protozoans. These microbes play essential roles in various aspects of the host's physiology, such as development, digestion, nutrition, disease resistance, and immune function. A key objective of research on GI microbiota is to establish a scientific basis for developing effective strategies to manipulate these microbial communities, thereby improving host health and enhancing productivity. It explores topics such as microbiota dynamics during early developmental stages, the influence of environmental and host factors on the establishment of bacterial populations within the gut microbiota, and the significance of these microbial communities for host health, development, and nutrition (Amillano-Cisneros *et al.*, 2022).

2.1.4 The role of fish disease resistance in the gut microbiome

The gut microbiota of fish has been found to play a critical role in their ability to resist diseases. Studies have shown that the use of antibiotics reduces the diversity of the gut microbiome in Atlantic salmon, leading to increased susceptibility to the fish

pathogen *Aeromonas salmonicida* (Egerton et al., 2018). Additionally, comparing the gut microbiomes of healthy fish and those affected by *Vibrio anguillarum* disease in European sea bass (*Dicentrarchus labrax*) highlighted the importance of the fish gut microbiome as a complex and dynamic ecosystem crucial for their overall health and well-being. Understanding the composition and functions of the gut microbiome in different fish species is vital for developing strategies to enhance fish health and improve aquaculture practices.

2.1.5 Diversity of gut microbiomes in marine fishes

Microorganisms within the gastrointestinal tract of creatures play imperative parts in nourishment assimilation, homeostasis and resistant reaction control. All inclusive, there are 33,700 angle species, speaking to nearly half of all vertebrate differing qualities and a wide run of physiologies, ecologies and life histories. To examine intestine microbiomes with tall scope, we performed 16S rRNA quality amplicon sequencing with 115 tests of 20 common marine angle species. The fish gut microbiome may be a surprisingly basic community with moo microbial differing qualities (a greatest of 300 amplicon grouping variations as it were) and has up to 70% of obscure species in a few angle species. The intestine microbial community structure was essentially formed by the combined impact of host-associated variables, counting the angle taxon ($p < .001$, $R^2 = 0.16$, $\omega^2 = 0.04$), bolstering propensity ($p < .001$, $R^2 = 0.06$, $\omega^2 = 0.02$) and trophic level ($p < .01$, $R^2 = 0.04$, $\omega^2 = 0.01$), in spite of the fact that the impact was unobtrusive with a little impact estimate. The centre intestine microbiomes of distinctive nourishing propensities were moreover already found in animal-associated and comparing environment tests. Certain vitality digestion system pathways were improved in herbivore/omnivore and zooplanktivore/zoobenthivore angles, though lipid digestion system and glycan digestion system were enhanced in zoobenthivore/piscivore angles. Moreover, substantial ordered inconstancy was found between the intestine microbiomes of angle and creatures, demonstrated by their moo degree of shared microbiota. The information and perceptions detailed in this clear the way for encourage examinations on the co-evolution of angle intestine microbiomes and the physiological capacities of intestine microorganisms and the advancement of probiotics for moving forward the sustenance and wellbeing of aquaculture angle species.

2.1.6 Environmental and ecological factors

Symbiotic bacteria play a vital role in assisting their hosts with nutrient acquisition from their diet, often evolving in tandem and being acquired independently by hosts at similar trophic levels. Although these patterns suggest significant involvement of living factors, the impact of the non-living environment on symbiotic community composition remains relatively unexplored. In our study, we investigated how both living and non-living factors influence the gut bacterial communities of fish across different groups, feeding levels, and environments. Through analyzing 25 16S rRNA libraries using phylogenetic and statistical methods, we found that factors such as salinity, feeding habits, and possibly the evolutionary relationships among hosts have a notable influence on the composition of fish gut bacteria. Interestingly, when compared with bacterial communities from other environments, fish gut communities tended to share similarities with those from mammals and insects. Additionally, a closer examination of individual bacterial types revealed connections between fish gut microbes and those found in other animals, particularly noticeable in herbivorous fish where many gut bacteria were closely related to those found in mammals. Our findings suggest that fish have more specialized gut communities than previously thought. Furthermore, they suggest a trend where fish and mammals may have independently acquired similar bacterial communities, hinting at a potential evolutionary link between fish and modern gut-fermenting mammals.

2.1.7 Bacterial symbiosis in the fish gut and its role in health and metabolism

The density of bacterial communities in the fish gut is notably higher compared to the surrounding water, indicating that the gastrointestinal tract (GIT) offers a favorable environment for their survival. These GIT bacteria can be categorized broadly into two groups: autochthonous, which are capable of colonizing the mucosal surface, and allochthonous, which are free-living. From the perspective of the host, autochthonous bacteria are considered more significant as they provide both nutritional support and aid in preventing diseases. Among their various functions, autochthonous bacteria are known to produce a variety of extracellular enzymes, inhibit the attachment of pathogens, and secrete bacteriocins. The majority of bacterial species in the gut cannot be cultured in laboratory settings, prompting the use of sophisticated techniques such as Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), and Next Generation Sequencing (NGS) to explore microbial communities in the gut. In this review, we have summarized the

impact of gut bacteria in fish, with a specific focus on extracellular enzyme production by gut microbiota, bacterial composition, mechanisms of attachment to the epithelial surface, and their role in disease prevention.

2.2 Marine actinobacteria

Marine actinobacteria is an increasingly important field of research in tropical regions. Actinobacteria from marine environments inhabit sediments, water, as well as other biomass like mangroves and animal substrates. These organisms are significant not only for their taxonomic and ecological roles but also for their production of unique metabolites and enzymes. While earlier studies primarily focused on temperate regions, research on marine actinobacteria in tropical environments has gained momentum over the past two decades. From the Indian peninsula alone, 41 species of actinobacteria belonging to 8 genera have been identified, with the genus *Streptomyces* being the most commonly recorded among marine sources. Despite this, only four of India's nine maritime states have been extensively studied for marine actinobacteria. The majority of research conducted thus far has centered around isolating, identifying, and maintaining these organisms in various culture media, as well as investigating their antagonistic properties against pathogens. However, there remains untapped potential for exploring their biotechnological applications further.

2.2.1 Isolation of Gut Actinobacteria from Fishes

Actinobacteria, a group of unicellular gram-positive bacteria characterized by their high guanine and cytosine content in DNA, are widespread in both terrestrial and aquatic environments. They are often found settling in water bodies due to runoff from land. In recent years, there has been significant interest in gut actinobacteria for their important roles in animal health, including promoting growth, providing protection, and aiding in nutrition. Particularly in fishes and poultry, they contribute to symbiosis by helping control bacterial diseases. Fish gut microbiota plays a crucial role in immunity development, pathogen resistance, and overall growth and nutrition. Actinobacteria are renowned for their production of secondary metabolites with antimicrobial properties. The abundance of gut actinobacteria in fishes is influenced by sediment composition and organic matter present in the water. Most studies on fish gut actinobacteria have focused on detritivore and herbivore feeders. Various methods have been utilized to isolate gut microbes in fishes, including traditional spread plate methods using different agar media, as well as culture-independent techniques like

next-generation sequencing targeting specific regions of the intestinal DNA. This chapter aims to provide insights into the techniques used for isolating gut actinobacteria in fishes using a variety of approaches.

2.2.2 *Actinobacteria isolated from freshwater fish gut microbiota*

Researchers explored the variety of Actinobacteria found in the gut microbiomes of two freshwater fish species, *Schizothorax zarudnyi* and *Schizocypris altidorsalis*, using traditional cultivation methods, rep-PCR, and 16S rDNA sequencing. They cultured 277 isolates using three different agar media and selected 33 strains for further analysis based on rep-PCR profiles, phylogenetic investigation, antimicrobial testing, and diversity of secondary-metabolite biosynthetic genes. The sequencing identified eight genera across six families, with 72% of isolates belonging to Streptomycetaceae. Notably, some Actinobacteria genera found have not been reported in freshwater fish microbiota before. All 33 isolates showed antibacterial activity against both human and fish pathogenic bacteria. The study also examined the potential for synthesizing bioactive compounds by investigating various biosynthetic genes among the isolates. The results highlight the significant diversity of Actinobacteria in fish gut microbiota and their potential for producing biologically active compounds.

2.3 Enzymes from the gut bacteria and their influence on intestinal enzyme activity

The role of gut-associated bacteria in aiding digestion, particularly in cold water fish like Atlantic cod, remains relatively understudied. In this study, we aimed to characterize the enzyme activities of GP21 (*Pseudomonas* sp.) and GP12 (*Psychrobacter* sp.), two bacteria isolated from the gastrointestinal tract of Atlantic cod. Additionally, we investigated whether administering these bacteria through feeds could impact the activity of specific intestinal enzymes. GP21 exhibited the ability to produce amylase, chitinase, cellulase, and protease, while GP12 produced only chitinase and protease. These enzymes were found to be active extracellularly under acidic conditions (pH 2–5) and at temperatures ranging from 15 to 30 °C. Interestingly, the influence of orally administered bacteria on intestinal enzyme activity was observed after 40 days of feeding rather than after 20 days. This suggests that GP21 and GP12,

as potential probiotic organisms, could play a role in supporting digestion in Atlantic cod (Lazado, C. C., Caipang, C. M. A., & Kiron, V. 2012).

The gastrointestinal tracts of nine freshwater teleosts, including catla, rohu, mrigal, silver carp, grass carp, common carp, tilapia, walking catfish, and murrel, were examined to isolate and enumerate aerobic bacterial flora. Various selective media were used to identify amylolytic, cellulolytic, lipolytic, and proteolytic microflora from the culture plates. The isolates were then screened qualitatively based on their ability to produce extracellular enzymes. Selected strains were further quantitatively assessed for amylase, cellulase, lipase, and protease activities. Nearly all bacterial isolates exhibited protease activity, while strains from tilapia, grass carp, and common carp showed significant amylolytic and cellulolytic activities. The highest lipase activity was observed in a strain isolated from silver carp. This study highlights the presence of a distinct microbial source of digestive enzymes—amylase, cellulase, lipase, and protease—in addition to endogenous sources in fish gut. The findings from this investigation may contribute to the development of more cost-effective feed formulations for carp by incorporating enzyme-producing bacterial isolates as probiotics.

2.3.1 Antioxidant enzymes activity

To investigate how euryhaline fish respond to varying salinity levels, we examined the gut microbiota composition and antioxidant enzyme activity in the same group of barramundi (*Lates calcarifer*) raised under two distinct salinity conditions (seawater and freshwater) in a controlled laboratory setting. We used high-throughput Illumina sequencing to analyze the gut bacterial composition. Biochemical indicators such as acid phosphatase (ACP), catalase (CAT), glutathione peroxidase (GSH-Px), lipid peroxide (LPO), lysozyme (LZM), malonaldehyde (MDA), peroxidase (POD), and superoxide dismutase (SOD) were measured. The salinity variations did not significantly alter the major composition of barramundi gut bacteria in the circulating aquaculture system. The predominant phyla observed in the barramundi gut microbiota were Proteobacteria and Firmicutes, with *Exiguobacterium*, *Citrobacter*, *Acinetobacter*, and *Pseudomonas* being the dominant genera. Differences in CAT and ACP activity were observed in the barramundi liver between the freshwater and seawater groups. However, GSH-Px, LZM, POD, SOD activity, and MDA and LPO

levels were not significantly affected by salinity changes. This study represents the first high-throughput analysis of gut microbiota diversity in barramundi raised from the same batch under extreme salinity conditions. The findings offer insights for managing animal health in barramundi aquaculture and contribute to further research on the gut microecology of euryhaline fish.

2.3.2 Enzymes-producing marine bacteria

The exploration of marine bacteria capable of producing industrial enzymes in Malaysia remains limited. This study aimed to assess the diversity of such bacteria from collections at the Institute of Marine Biotechnology, University Malaysia Terengganu. Out of 200 bacterial isolates examined, 163 showed successful growth. Those exhibiting high enzyme production, scoring over four, were selected for molecular identification using 16S rDNA. Among them, 161 bacteria were found to secrete amylase (68.7%), lipase (88.3%), and protease (68.7%). Through phylogenetic analysis, three main phyla were identified: Proteobacteria, Firmicutes, and Bacteroidetes, comprising nine genera including *Bacillus*, *Chryseomicrobium*, *Photobacterium*, *Pseudoalteromonas*, *Ruegeria*, *Shewanella*, *Solibacillus*, *Tenacibaculum*, and *Vibrio*. Genetic diversity was observed, particularly within similar marine bacteria species. The microbial community composition appeared to influence both industrial enzyme production and marine bacteria diversity.

2.3.3 Amylase Producing Micro-Organism from Gastrointestinal Tract of marine fish

There is limited understanding of the gut microbiota in fish, and little is known about microbial enzyme activity in fish gastrointestinal tracts. While certain enzymes like cellulase and proteases have been identified in fish guts, amylase stands out as a particularly important enzyme, holding significant value with about a quarter of the total enzyme market. It finds diverse applications in the food, pharmaceutical, and fine chemical industries. This study focuses on isolating, characterizing, and optimizing amylase-producing microorganisms from the gastrointestinal tract of *Catla catla*. Through biological production, the bacterial isolate PAC4 from the fish gut was identified as *Bacillus cereus*, demonstrating the highest amylase production of 92 ± 3 U/ml after 48 hours of incubation. Various physicochemical factors essential for optimal amylase fermentation were identified, including a 3% sodium chloride

concentration, a temperature of 37°C, and a pH of 7. The capacity for amylase production within the intestinal microflora of cultured ayu, carp, channel catfish, Japanese eel, and tilapia specimens was investigated. The average counts of viable aerobes and anaerobes ranged from 1.1×10^6 to 3.7×10^8 cfu g⁻¹ and from 1.3×10^3 to 1.6×10^8 cfu g⁻¹, respectively. Predominant species included *Aeromonas* spp. and Bacteroidaceae across four to five fish species. Out of 206 strains examined, 65 (31.6%) produced ≥ 0.01 U amylase ml⁻¹. The proportion of producers varied among bacterial families, genera, and fish species. While 56% of anaerobes produced amylase, only 20% of aerobes did so. Efficient amylase production was observed in over 50% of *Aeromonas*, Bacteroidaceae, and *Clostridium* strains, while *Acinetobacter*, coryneforms, Enterobacteriaceae, *Moraxella*, *Plesiomonas*, and *Streptococcus* strains did not demonstrate significant amylase production. Notably, 12 strains exhibited high amylase production (≥ 0.05 U ml⁻¹), predominantly from *Aeromonas* (11 strains) and one from *Pseudomonas*. The proportion of high amylase producers in Japanese eel was lower compared to the other four fish species (2–30%). These findings strongly suggest that intestinal microflora-derived amylase plays a crucial role in starch digestion in freshwater fish to some extent.

2.3.4 Protease-producing bacterium

The gastrointestinal tracts (GITs) of aquatic organisms have been recognized as promising sources for identifying probiotic candidates beneficial for aquaculture. Thus, this research aimed to isolate protease-producing bacteria from the GITs of hybrid abalone (*Haliotis rubra* x *Haliotis laevigata*) and evaluate their potential as probiotics for abalone. Ten bacterial strains with proteolytic activity were isolated, all belonging phenotypically to the genus *Bacillus*. Among these, strain MA228 exhibited the highest capacity to hydrolyze casein and gelatine. Analysis of its 16S rRNA sequence (accession number MG976611) revealed a 99% similarity to *Bacillus amyloliquefaciens*. Further in vitro experiments demonstrated that strain MA228 exhibited robust viability and stability in manufactured pellets stored at 4 °C, seawater with a salinity of 35 mg/L, and simulated abalone gastrointestinal conditions. Additionally, in vitro assays indicated that the protease produced by this probiotic candidate significantly improved protein digestion in abalone pellet manufacturing. These findings suggest that *B. amyloliquefaciens* MA228 holds promise as a probiotic to enhance protein digestibility in hybrid abalone.

2.3.5 Lipase production from fish gut bacteria

Lipases play a crucial role in various applications across industries such as food, detergents, pharmaceuticals, and chemical synthesis. They are sourced from microbial, plant, and animal origins, with microbial lipases garnering more attention due to their distinct properties and scalability for large-scale production. Despite advancements, there remains a demand for lipases with enhanced catalytic capabilities and stability, especially in challenging environments. Marine organisms offer a promising avenue for discovering such lipases, as marine enzymes have demonstrated utility in process optimization and innovation. The production of lipases is influenced by factors like the composition of the culture medium, including carbon and nitrogen sources, as well as environmental conditions like temperature and pH. This review aims to identify various types of lipases sourced from marine organisms, elucidate their roles, and discuss their unique characteristics and potential applications.

2.3.6 urease production by fish gut bacteria

Ureotelic elasmobranchs, which need nitrogen for both protein growth and urea-based osmoregulation, likely face nitrogen limitations in their natural environments. While mechanisms for retaining and scavenging nitrogen in various organs like the gills, kidney, rectal gland, and gut are known, the latter remains less understood. In vitro experiments with intestinal sac preparations from Pacific spiny dogfish sharks showed significant urea reabsorption from the lumen post-feeding, accompanied by increasing mucosal fluid ammonia concentrations over time. Blocking urea reabsorption with phloretin led to a notable rise in luminal ammonia accumulation. A sensitive assay using [14C] urea indicated microbial urease's potential role in ammonia production. Urease activity was detected in chyme/intestinal fluid and epithelial tissue of both fed and fasted sharks but absent in gall-bladder bile. Urease levels varied among individuals but were generally higher in chyme than in epithelia and greater in fed sharks compared to fasted ones. Comparable urease activities were found in Pacific spotted ratfish, a ureotelic holocephalan, but much lower in ammonotelic teleosts. In dogfish, urease activity was inhibited by acetohydroxamic acid and boiling. Blocking urease activity with acetohydroxamic acid in gut sac preparations shifted net ammonia accumulation to absorption, suggesting microbial urease's crucial role in nitrogen

handling. It converts some urea-N to ammonia, which can then be reabsorbed for amino acid synthesis or reconversion to urea.

2.3.7 Pectinase from a Fish Gut Bacterium

In this study, we isolated 11 gut bacteria from *Systomus sarana*, commonly known as olive barb, a freshwater fish species. Upon screening, these strains were found to produce extracellular pectinase enzyme. Among them, strain SS6 exhibited the highest pectinase production, reaching 208.731 U/ml. Through molecular characterization, we identified SS6 as *Aeromonas guangheii*. We optimized the culture conditions for SS6 using response surface methodology with a Box–Behnken design. The optimal conditions included 2.11% maltose, 2.20% yeast extract, pH 6.5, and a temperature of 27.3 °C with a 32-hour incubation period, resulting in enhanced pectinase production of 371 U/ml. The purified pectinase had a molecular weight of approximately 50 kDa, as determined by 10% 2-D PAGE. Nine peptides were identified from the purified enzyme through MALDI-TOF-MS analysis, with the mass spectrum of the peak at 2211 of peptide indicating the reference pectinase protein. We PCR amplified the referenced gene primer (pectate lyases) and analyzed its nucleotide sequence. The *exo-pelA* gene was cloned into the *pest* vector and found to be overexpressed in *Escherichia coli* BL21. The ORF encoded a mature protein consisting of 425 amino acids (1236 nucleotides) with a predicted molecular weight of approximately 48.7 kDa. These findings underscore the potential of fish gut microbes as a source of biotechnologically important enzymes.

2.3.8 Glutaminase production from fish gut bacteria

L-glutaminase is a vital anticancer substance widely utilized worldwide for targeting cancer cells by depriving them of L-glutamine. *Halomonas meridiana*, a marine bacterium isolated from the Red Sea, was identified as a potent producer of L-glutaminase. Optimization of L-glutaminase fermentation was achieved at 36 hours, pH 8.0, 37°C, and 3.0% NaCl concentration, with 1.5% glucose and 2% soybean meal. The purified enzyme exhibited a specific activity of 36.08 U/mg and a molecular weight of 57 kDa according to SDS-PAGE analysis. It demonstrated significant activity at pH 8.0 and 37°C. Kinetic parameters indicated a high affinity for its substrate, with K_m and V_{max} values of 12.2×10^{-6} M and 121.95 $\mu\text{mol/mL/min}$, respectively. The anticancer efficacy of the enzyme was notable, showing significant toxicity against

colorectal adenocarcinoma cells LS 174 T (IC₅₀ 7.0 µg/mL) and HCT 116 (IC₅₀ 13.2 µg/mL). In HCT 116 cells, early apoptosis was more pronounced, whereas late apoptosis was more prevalent in LS 174 T cells. Additionally, induction of nuclear fragmentation by L-glutaminase was higher in HCT 116 cells compared to LS 174 T cells. This study highlights the potential of *Halomonas meridiana*-derived L-glutaminase as an effective agent against colorectal cancer (Mostafa et al., 2021)

2.3.9 cellulase production from fish gut microbes

In this study, two cellulolytic bacterial strains were isolated from the gut of an estuarine fish, *Etroplus suratensis*. Identification of the strains was conducted using both conventional methods and 16S rRNA gene sequencing, revealing them to be *Bacillus altitudinis* and *Bacillus licheniformis*. The cellulase production capacity of these strains was optimized by varying nutritional parameters such as carbon sources, nitrogen sources, phosphate sources, surfactants, and metal ions. Among the carbon sources tested, fructose and carboxymethyl cellulose (CMC) induced the highest cellulase production in both strains. Ammonium hydrogen carbonate and ammonium sulphate were found to enhance cellulase production to the greatest extent in *Bacillus altitudinis* and *Bacillus licheniformis*, respectively, among the nitrogen sources tested. Similarly, the addition of sodium dihydrogen orthophosphate led to maximum cellulase production in both strains. Triton X-100 was identified as the most effective surfactant for inducing cellulase production by both *Bacillus altitudinis* and *Bacillus licheniformis*. Among the tested metal ions, magnesium sulphate and manganese sulphate resulted in the highest enzyme production by *Bacillus altitudinis* and *Bacillus licheniformis*, respectively.

2.4 Tuberculosis

An estimated one-third of the global population is infected with tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mukhopadhyay and Ganguly, 2013). India ranks 17th among 22 nations with a high TB burden (Kashyap et al., 2013). The primary challenge in TB control is the emergence of drug-resistant *M. tuberculosis* strains, requiring prolonged therapy with multiple medications. There is an urgent need for new antitubercular drugs to combat drug-resistant strains effectively (Ginsberg, 2010). These medications should ideally have fewer side effects, improved

pharmacokinetics, robust efficacy against drug-resistant strains, and the potential to shorten treatment duration (de Souza, 2008). Secondary metabolites from microorganisms have been a cornerstone in tuberculosis treatment for decades. Actinobacteria, belonging to the order Actinomycetes, comprise aerobic filamentous Gram-positive bacteria, commonly known as actinomycetes (Ma et al., 2020). Actinomycetes are ubiquitous soil organisms known for their ability to produce secondary metabolites, particularly antibiotics, which have proven clinically valuable. Approximately 50% of identified microbial bioactive compounds are sourced from actinomycetes members.

2.4.1 Investigation of anti-TB activity employing actinobacteria

Infectious diseases like tuberculosis and other lung-related opportunistic infections present significant public health concerns. Due to the resistance of these pathogens to current drugs, there's an urgent need for new antibiotics. Actinobacteria, known for their diverse pharmaceutical and biotechnological uses, are rich sources of natural products. Discovering actinobacterial metabolites relies on isolating them from unexplored environments, with fish being a promising but underexplored source, as suggested by literature. In this study, we screened 13 gut-associated actinobacterial strains from fish for their ability to combat mycobacterial infections. We optimized fermentation methods to enhance bioactive metabolite production, conducted various tests including determining minimum inhibitory concentrations, characterized the metabolites, and analyzed them using gas chromatography-mass spectrometry. Strain MCA2 showed promising activity against *Mycobacterium smegmatis*. We extracted the bioactive metabolite from strain MCA2 using agar surface fermentation and ethyl acetate extraction. This metabolite demonstrated antitubercular activity by significantly reducing relative light units against *Mycobacterium tuberculosis* H37Rv. Based on its physical traits, strain MCA2 was identified as *Streptomyces* sp.

CHAPTER 3

AIM AND OBJECTIVE OF THE PRESENT STUDY

3.1 AIM

- To isolate and identify microbes from gastrointestinal tract of the marine fish.
- To study the various activity of the isolated microbes.

3.2 OBJECTIVE

- Isolation and identification of microbes from fish gut
- Screening of bacteria and selection of potential strain
- Enzyme Activity: bacterial cultures were screened for extracellular enzymes such as cellulase, pectinase, protease, amylase, lipase and urease by agar plate method.
- Production and extraction of pigment from marine actinobacterial strain.
- Characterization and taxonomy of potential actinobacterial culture.
- To perform Anti-infective assays: Anti-Biofilm, Anti-Mycobacterial assay

CHAPTER 4

MATERIALS AND METHODS

4.1 Sample Collection

Fishes were collected and sent immediately to the laboratory to avoid contamination. Fish samples Trevally (*Megalaspis cordyla*), Red snapper (*Lutjanus Campechanus*), Indian mackerel (*Rastrelliger kanagurta*), Indian oil sardine (*Sardinella longiceps*) and Indian Squid (*Uroteuthis duvauceli*) are the selected fishes were collected from East coastal area.

4.2 Media Requirement

- Starch casein agar media
- ISP2 agar (international streptomyces project media).
- ISP2 broth
- Nutrient agar
- Nutrient broth
- Amylase agar
- Protease agar
- Lipase agar
- Urease agar
- Muller Hinton agar

4.3 Isolation of 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^{-3} to 10^{-5} and plated on SCA, ISP2 and NA media using L-rod by spread plate

method. The plates were incubated at room temperature for a minimum of two days for the growth of bacterial colonies .

4.4 Recovery and Preservation of bacteria

Following the incubation period, we examined all the cultures for growth, consistency, colony formation, aerial mycelium, and substrate mycelium. The colonies were collected from the isolation plates using a sterile inoculation loop. These colonies were then inoculated into freshly prepared yeast extract malt extract agar (ISP2) and Nutrient agar plates. The cultures were preserved using both Nutrient agar slants and a 30% glycerol solution.

4.5 Characterization of bacteria

Cultural characterization was done by inoculating the bacterial culture into Nutrient Agar medium. All the plates were left to incubate for 3-5 days at a temperature of 28°C. Cultural characteristics recorded include growth, colony consistency, aerial mass colour, presence of reverse side pigment and the production of soluble pigments.

4.6 Screening of bacteria for The Production of Enzymes

The extracellular enzymatic activities, such as cellulase, protease, lipase, Pectinase, Glutaminase, Urease and amylase, of all bacterial cultures will be assessed using the agar plate method.

4.6.1 Amylase test

Bacterial isolates were Inoculated on starch agar medium containing glucose, peptone, yeast extract, and starch, and then left to incubate at 30°C for 48 hours.

4.6.2 Lipase test

Bacterial isolates with lipase producing capabilities were screened using tween 80 agar media. The Cultures were spot inoculated and subsequently incubated at room temperature.

4.6.3 Protease activity

Proteolytic or caseinolytic activity was tested using a 10% skim milk plate medium. Bacterial isolates were Inoculated on Skim milk agar after 48 hours of incubation at 30°C.

4.6.4 Cellulase test

Cultures were inoculated on Carboxy methyl cellulose agar media containing KH_2PO_4 , K_2HPO_4 , MgSO_4 , NaCl , FeSO_4 , Agar and Carboxy methyl cellulose (CMC), and then incubated at 30°C for 48 hours.

4.6.5 Urease test

Bacterial isolates were inoculated in the urease agar media containing peptone, incubated at 30 degree C for 48 hrs.

4.6.6 Glutaminase test

Bacterial isolates were introduced into a glutaminase agar medium comprising potassium chloride (KCl), magnesium sulphate (MgSO_4), potassium dihydrogen phosphate (KH_2PO_4), ferrous sulphate (FeSO_4), zinc sulphate (ZnSO_4), sodium chloride (NaCl), L-Glutamine, and Phenol red. The cultures were then subjected to incubation at 30°C for a duration of 48 hours.

4.6.7 Pectinase test

Bacterial isolates were introduced into a pectinase agar medium containing Ammonium phosphate ($(\text{NH}_4)_2\text{HPO}_4$), Potassium dihydrogen phosphate (KH_2PO_4), Magnesium sulphate (MgSO_4), Pectin, and Agar, with the pH adjusted to 4.5. Subsequently, the cultures were incubated at 30°C for a period of 48 hours.

4.7 PRODUCTION AND EXTRACTION OF PIGMENT FROM MARINE ACTINOBACTERIAL STRAIN

4.7.1 SOURCE

This study delves into the exploration of pigment production by the actinobacterial culture TFB11, isolated from the gut of marine fish in the East Coastal Area. The culture undergoes subculturing and is preserved on ISP2 agar slants. For long-term storage, it is kept in 30% glycerol broth.

4.7.2 Characterization of Actinobacteria

The actinobacterial culture underwent cultural characterization by being introduced to ISP2 agar medium, with subsequent incubation lasting 7-14 days at 28°C. The documented cultural traits included observations on growth, colony consistency, coloration of the aerial mass, the existence of pigmentation on the reverse side, and the generation of soluble pigments.

4.7.3 Production of pigments and Bioactive metabolites

The process involves initiating agar surface fermentation for the production of pigments from actinobacterial cultures. Actinobacterial cultures are introduced to ISP2 agar plates and then kept in incubation at 28°C for a period of 10 days. Throughout this incubation phase, secondary metabolites, including pigments, are released into the agar medium by the actinobacteria.

4.7.4 Solvent Extraction

After incubation, mycelial growth was removed aseptically using sterile spatula. The agar medium was cut into pieces and extracted overnight at room temperature using different solvents (1:2 ratio) such as methanol and ethyl acetate. The solvent extracts were concentrated using Eppendorf concentrator plus and quantified.

4.8 Antioxidant Assay – DPPH

DPPH (1,1-diphenyl-2-picrylhydrazyl) was dissolved in 3.3 ml of methanol and protected from light using aluminum foil. Following this, around 150µl of DPPH solution was added to 3ml of methanol, and the absorbance was promptly measured at 517 nm for establishing the baseline reading. Various concentrations (100, 200, 300, 400, and 500µg) of both the crude pigment and a standard compound (ascorbic acid) were then separately added to 3ml portions of methanol. Subsequently, 150µl of DPPH was added to each reaction tube. Absorbance readings were taken after 15 minutes at 517 nm using a UV-Visible spectrometer, specifically the Cyberlab Model, with methanol acting as the blank. The percentage of DPPH radical scavenging activity for each sample was then calculated using a predefined formula.

4.9 Determination and Purification of The Biologically Active Compounds

4.9.1 Thin Layer Chromatography

Active compounds were identified using thin layer chromatography (TLC). TLC plates were introduced into a chamber pre-saturated with various solvents such as butanol, acetone, n-hexane, chloroform, and ethyl acetate. Crude extracts dissolved in ethyl acetate were applied as spots 2 cm from the bottom of a silica gel-coated aluminium sheet. The TLC sheets were removed once the solvent front reached 15 cm from the original spot position and then left to dry. After drying, the spots on the plates were visualized under visible (white) light, short UV (254 nm), and long UV (366 nm) light. Subsequently, the sheets were sprayed with vanillin sulfuric acid for colour development and allowed to dry. Visual documentation was conducted using a visualizer and scanner under UV 254 nm and UV 366 nm, as well as under visible light, both before and after the application of the vanillin sulfuric acid spray. The migration of each separated spot of the extract was determined by its retention factor (R_f). (Augustine et al., 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)

4.9.2 HPLC Analysis of Pigment

The collected crude was given to HPLC for analyzing crude pigments involves the separation of components within the pigment mixture based on their affinity for the stationary phase and the mobile.

4.10 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)

4.11 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 et al., 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 (Collins & Franzblau, 1997; Franzblau et al., 1998; Yajko et al., 1995)

4.12 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 μ l 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.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Sample Collection

Totally 6 fish samples were collected from East Coastal Area, Chennai. Gut was removed from the fishes and homogenized using a surface sterilized mortar and pestle. Seeded serial dilution plates were observed after incubation.



Caranx ignobilis
(Trevally)



Rastrelliger Kanagurta
(Indian mackerel)

Fig:5.1: Fish Samples selected for Isolation



Fig:5.2: Dissected gut from fish sample

5.2 Isolation of strains

The resultant homogenates were serially diluted from 10^{-3} to 10^{-5} and plated on SCA, ISP2 and NA media using L-rod by spread plate method and kept for incubation. And about 34 strains were isolated.

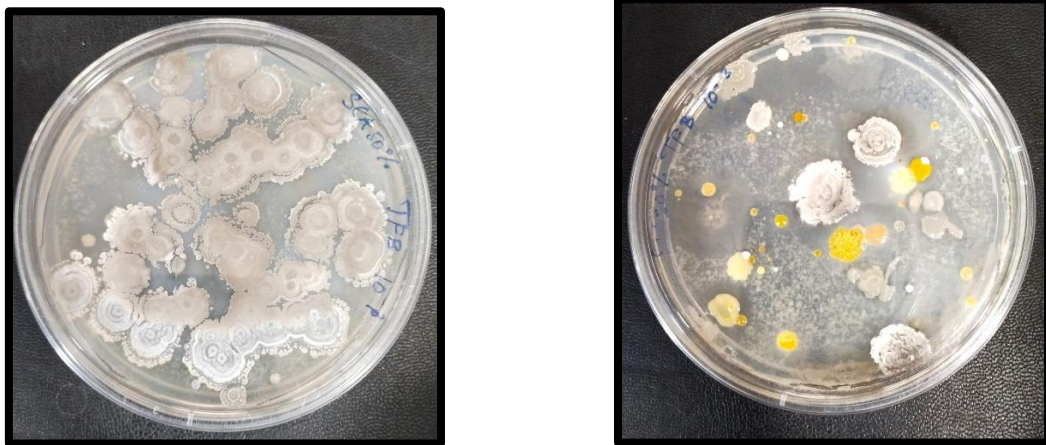


Fig:5.3: Isolation plate

5.3 Recover and preservation of bacteria

The colonies were collected from the isolation plates using a sterile inoculation loop. These colonies were then inoculated into freshly prepared yeast extract malt extract agar (ISP2) and Nutrient agar plates. The cultures were preserved using both Nutrient agar slants.

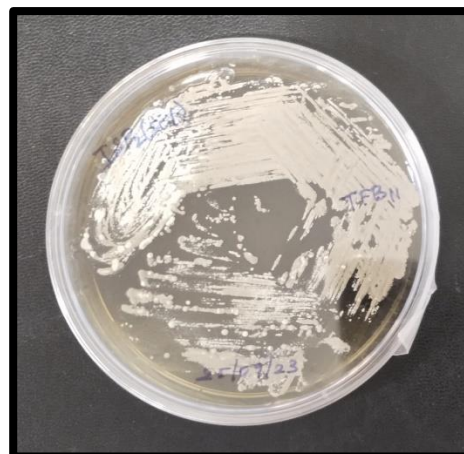


Fig:5.4: Subculture plate

5.4 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. Aerial mycelium and Substrate mycelium was seen on the culture which was viewed under bright microscopy. Previously Sivakumar *et al.*, 2007; Gopikrishnan *et al.*, 2019;2020 reported the fish gut associated actinobacteria from both fresh water and marine fishes.



Fig:5.5: Identified actinomycetes recovered on ISP2 agar plate.

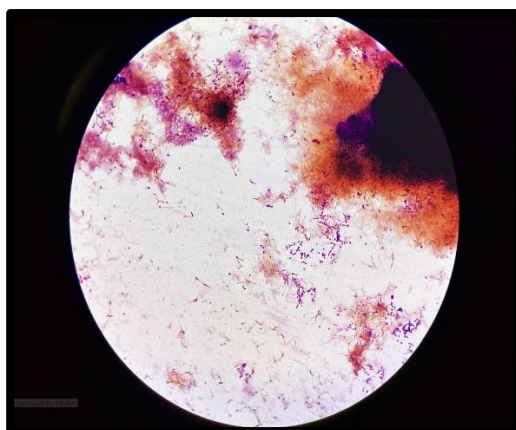


Fig:5.6: Morphology of actinobacterial strain under bright field Microscope

Table 5.1: Morphological Identification

S.N O	STRAIN NO	SAMPLE	ECO SYSTEM	GROW TH	CONSISTE NCY	AM C	RS P	S P
1	TFB 1	Trevally fish gut	Marine water	Good	smooth	-	-	-
2	TFB 4	Trevally fish gut	Marine water	Good	Smooth	-	-	-
3	TFB 8	Trevally fish gut	Marine water	Good	slimy	-	-	-
4	TFB 10	Trevally fish gut	Marine water	Good	powdery	whit e	-	-
5	TFB 11	Trevally fish gut	Marine water	Good	powdery	Gre y	Bla ck	+
6	TFB 12	Trevally fish gut	Marine water	Good	sticky	-	-	-
7	TFB 13	Trevally fish gut	Marine water	Good	sticky	-	-	-
8	TFB 14	Trevally fish gut	Marine water	Good	slimy	-	-	-
9	TFB 15	Trevally fish gut	Marine water	Good	slimy	-	-	-
10	RSA 1	Red snapper fish gut	Marine water	Good	sticky	-	-	-
11	RSA 2	Red snapper fish gut	Marine water	Good	smooth	-	-	-
12	RSA 3	Red snapper fish gut	Marine water	Good	smooth	-	-	-
13	RSA 4	Red snapper fish gut	Marine water	Good	smooth	-	-	-
14	RSA 5	Red snapper fish gut	Marine water	Good	smooth	-	-	-

15	RSA 6	Red snapper fish gut	Marine water	Good	smooth	-	--	-
16	RSA 7	Red snapper fish gut	Marine water	Good	smooth	-	-	-
17	RSA 8	Red snapper fish gut	Marine water	Good	No form	-	-	-
18	IM ₁ 1	Indian Mackerel fish gut	Marine water	Good	smooth	-	-	-
19	IM ₁ 2	Indian Mackerel fish gut	Marine water	Good	smooth	-	-	-
20	IM ₁ 3	Indian Mackerel fish gut	Marine water	Good	slimy	-		-
21	IM ₁ 4	Indian Mackerel fish gut	Marine water	Good	slimy	-	-	-
22	IM ₂ 1	Indian Mackerel fish gut	Marine water	Good	smooth	-	-	-
23	IM ₂ 2	Indian Mackerel fish gut	Marine water	Good	slimy	-	-	-
24	IM ₂ 3	Indian Mackerel fish gut	Marine water	Good	smooth	-	-	-
25	IM ₂ 4	Indian Mackerel fish gut	Marine water	Good	slimy	-	-	-
26	OS ₁ 1	Indian oil sardine fish gut	Marine water	Good	sticky	-	-	-
27	OS ₁ 2	Indian oil sardine fish gut	Marine water	Good	smooth	-	-	-
28	OS ₁ 3	Indian oil sardine fish gut	Marine water	Good	smooth	-	-	-
29	OS ₁ 4	Indian oil sardine fish gut	Marine water	Good	slimy	-		-
30	OS ₂ 1	Indian oil sardine fish gut	Marine water	Good	smooth	-	-	-
31	OS ₂ 2	Indian oil sardine fish gut	Marine water	Good	sticky	-	-	-
32	SQ1	Squid fish gut	Marine water	Good	sticky	-	-	-
33	SQ2	Squid fish gut	Marine water	Good	smooth	-	-	-
34	SQ3	Squid fish gut	Marine water	Good	sticky	-	-	-

5.5 Screening of bacteria for production of enzymes

Table 5.2: Enzyme Activity

S.NO	SAMPLE	AMYLASE	PECTINASE	PROTEASE	GLUTAMINASE	LIPASE	UREASE	CELLULASE
1	TFB 1	+	-	-	-	+	-	-
2	TFB 4	-	-	+	-	+	-	-
3	TFB 8	+	-	-	-	+	+	-
4	TFB 10	+	-	+	-	+	-	-
5	TFB 11	+	-	+	-	+		-
6	TFB 12	+	-	+	-	+	-	-
7	TFB 13	+	-	-	-	+	-	-
8	TFB 14	+	-	-	-	+	-	-
9	TFB 15	+	-	+	-	+	-	-
10	RSA 1	+	-	-	-	+	-	-
11	RSA 2	+	-	-	-	-	+	-
12	RSA 3	-	-	-	-	-	+	-
13	RSA 4	+	-	-	-	+	-	-
14	RSA 5	+	-	-	-	-	-	-

15	RSA 6	+	-	—	-	+	+	-
16	RSA 7	+	-	+	-	+	+	-
17	RSA 8	+	-	-	-	+	-	-
18	IM11	+	-	-	-	-	-	-
19	IM12	+	-	-	-	-	-	-
20	IM13	+	-	-	-	-	-	-
21	IM14	+	-	-	-	-	-	-
22	IM21	+	-	-	-	-	-	-
23	IM22	+	-	-	-	-	-	-
24	IM23	+	-	-	-	-	-	-
25	IM24	+	-	-	-	-	-	-
26	OS11	+	-	-	-	-	-	-
27	OS12	-	-	-	-	-	-	-
28	OS13	-	-	-	-	-	-	-
29	OS14	-	-	-	-	-	-	-
30	OS21	-	-	-	-	-	-	-
31	OS22	-	-	-	-	-	-	-
32	SQ1	+	-	-	-	-	-	-
33	SQ2	-	-	-	-	-	-	-
34	SQ3	-	-	-	-	-	-	-

5.5.1 Amylase test

The production of amylase, an industrially significant enzyme, was measured in TFB 11. Clear zones formed against dark blue or dark brown plates, respectively, served as indicators of their identities. The conventional acid hydrolysis is gradually being replaced by the hydrolysis of starch, which has been carried out on an industrial scale for many years.

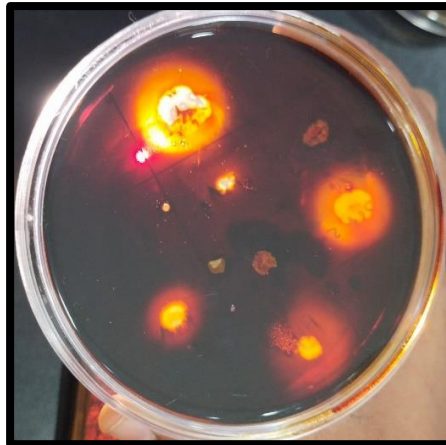


Fig:5.7: Amylase plate

5.5.2 Urease test

Urease activity of an infectious microorganisms can contribute to the development of urinary stones, pyelonephritis, gastric ulceration and other diseases. RSA2 turned into pink and it shows positive.

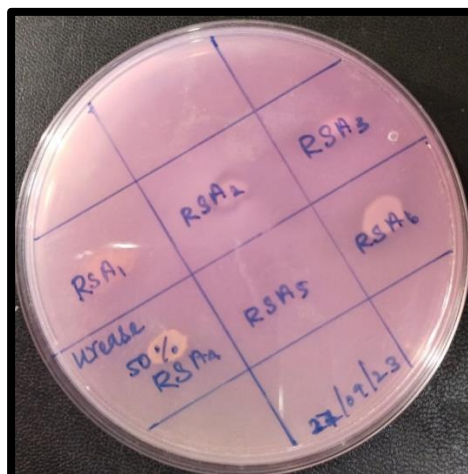


Fig:5.8: urease plate

5.5.3 Protease test

About 60% of the enzyme market's overall sales are made up of proteases. Alkali proteases currently have the largest share of the hydrolytic enzyme market. TFB 11 displayed the clearance zone.



Fig:5.9: Protease plate

5.5.4 Lipase test

By 2024, the microbial lipase market is expected to grow at a compound annual growth rate (CAGR) of 6.8% from 2018 to USD 590.2 million. It is now projected to have a 2018 value of USD 425.0 million. The utilisation of lipase enzymes derived from microorganisms in the synthesis of modified substances represents just one of the numerous industrial uses for these highly diverse and proficient enzymes. TFB 11 displayed the clearance zone. Sivakumar et al. (2007) and Gopikrishnan et al. (2019, 2020, 2021) previously reported screening actinobacteria linked with fish guts for their enzyme activity. Less research was done on fish gut-associated microorganisms in the papers above.

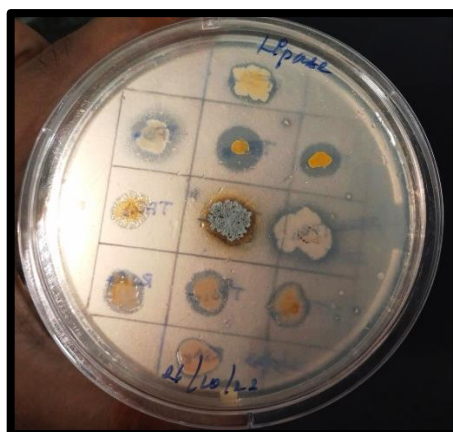


Fig:5.10: Lipase plate

5.6 Extraction of pigment from marine actinobacterial strain

The TFB11 strain's crude components were extracted using methanol and ethyl acetate, with methanol extract exhibiting the highest amount and activity. When n-hexane was extracted, no chemical was found. Methanol and ethyl acetate were used to extract crude components of strain TFB11 from MHA agar, however n-hexane was not used. The maximum amount of methanol was extracted.

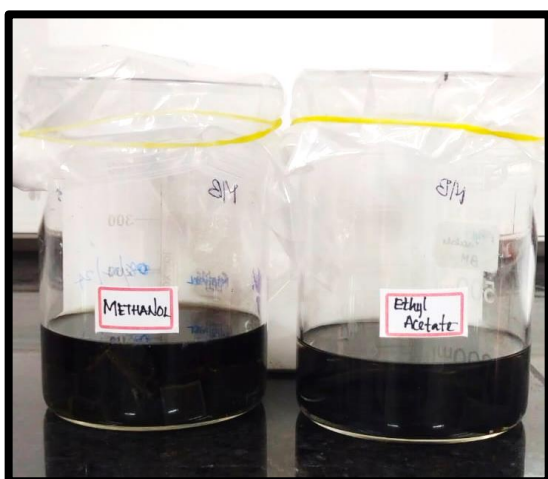


Fig:5.11: Crude Extraction

5.7 Antioxidant assay-DPPH

The free radical scavenging capacity of the crude extract of TFB11 strain was measured based on the method delineate by Their colour change were observed and absorbance was measured at 517 nm using UV- spectrophotometer. The crude extract of TFB11 shows good activity at 60 and 80 µg/mL concentrations with scavenging effect of 52.73% and 49.09%.

Table 5.3: Free radical scavenging Activity

SAMPLE CONCENTRATION(µg)	ABSORBANCE	% SCAVENGING
20	0.129	48.18
40	0.108	38.18
60	0.138	52.73
80	0.134	49.09

5.8 Partial Purification and Characterization of Bacterial Compounds

In analytical TLC, 5 spots were obtained from crude methanol extract of TFB 11 when using combinations of methanol, chloroform ethyl acetate (1.5:0.5, v/v) as solvent system. The R_f value of separated compounds TFB 11 A-F was calculated as 0.76, 0.53, 0.36, 0.45, 0.27 and 0.15

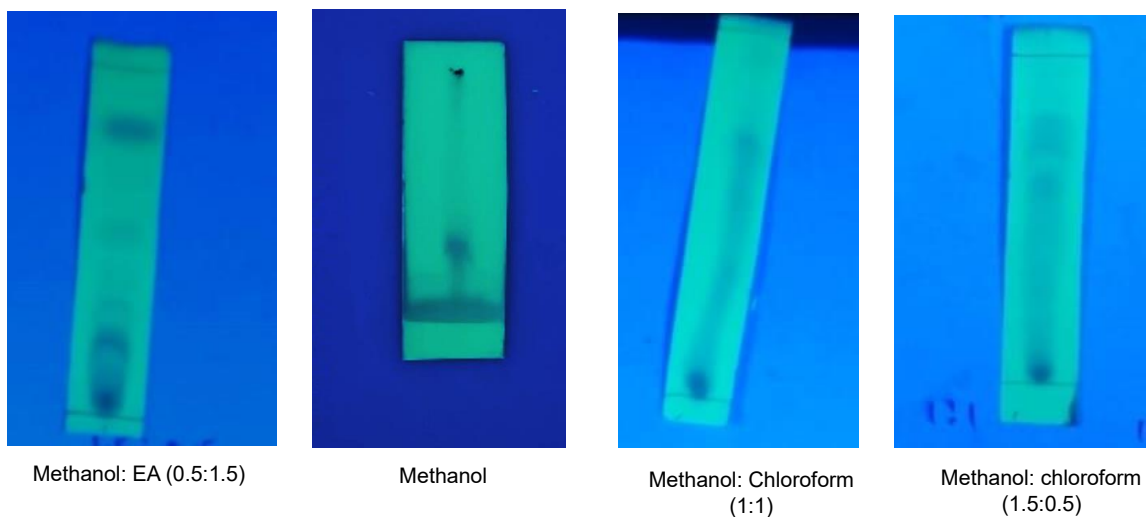
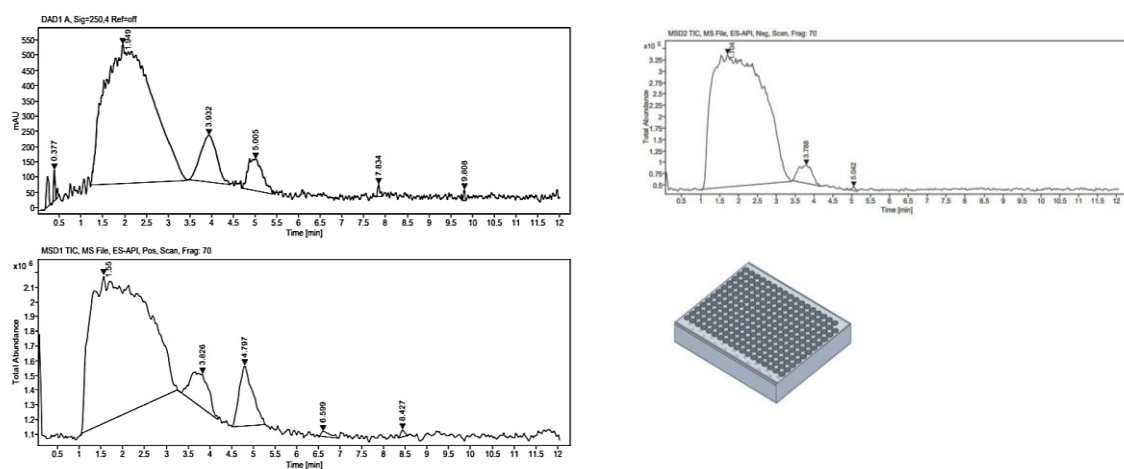


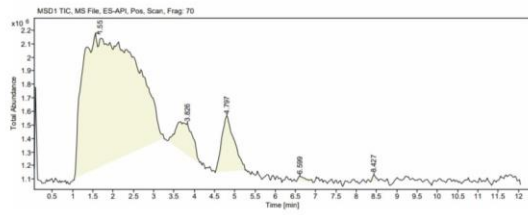
Fig.5.12: Thin layer chromatography verification of TFB 11 extract

5.9 HPLC Analysis of Pigment

Through HPLC (High Performance Liquid Chromatography) the individual components are separated and quantified from the mixture of TFB 11 extract.



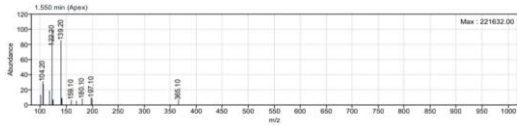
Signal details: MSD1 TIC, MS File, ES-API, Pos, Scan, Frag: 70



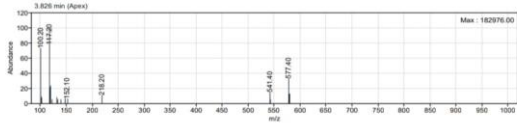
RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
1.550	MSD1 TIC, MS File, ES-API, Pos, Scan, Frag: 70	0.34863		1006048	87246856	84.3
3.826	MSD1 TIC, MS File, ES-API, Pos, Scan, Frag: 70	1.56839	1.66336	234370	6642777	6.4
4.797	MSD1 TIC, MS File, ES-API, Pos, Scan, Frag: 70	0.69776	1.71687	409938	8754204	8.5
6.599	MSD1 TIC, MS File, ES-API, Pos, Scan, Frag: 70	0.23758	4.09902	35142	531651	0.5
8.427	MSD1 TIC, MS File, ES-API, Pos, Scan, Frag: 70	0.92052	6.45982	49324	293620	0.3

Filtered on peak height > 1000 counts

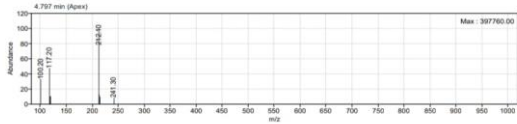
Retention time: 1.55 min Area Percent: 84%



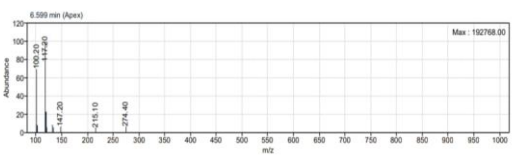
Retention time: 3.826 min Area Percent: 6%



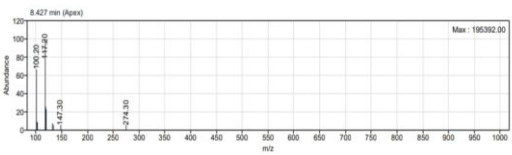
Retention time: 4.797 min Area Percent: 8%



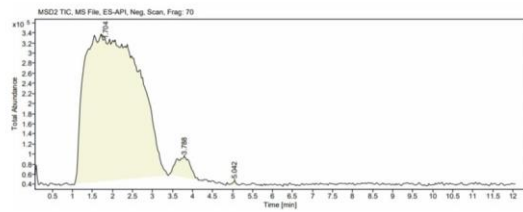
Retention time: 6.599 min Area Percent: 1%



Retention time: 8.427 min Area Percent: 0%



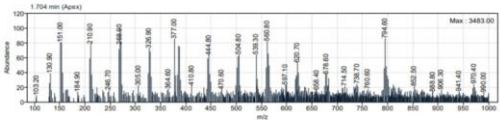
Signal details: MSD2 TIC, MS File, ES-API, Neg, Scan, Frag: 70



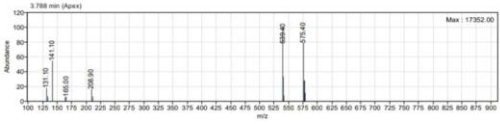
RT [min]	Signal Description	Symmetry	Resolution	Height [count]	Area [count*min]	Rel. Area [%]
1.704	MSD2 TIC, MS File, ES-API, Neg, Scan, Frag: 70	0.44292		291876	27771674	96.3
3.788	MSD2 TIC, MS File, ES-API, Neg, Scan, Frag: 70	1.27416	1.53327	40714	1020301	3.5

Filtered on peak height > 1000 counts

Retention time: 1.704 min Area Percent: 96%



Retention time: 3.788 min Area Percent: 4%



Retention time: 5.042 min Area Percent: 0%

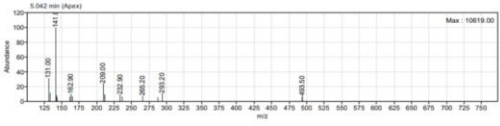


Fig.5.13: HPLC Analysis of TFB 11 extract.

5.10 Anti-biofilm Assay

Anti-biofilm Assay is done for extract of TFB11 for test pathogens, *Pseudomonas aeruginosa*. (fig 5.16) The crude extract of TFB11 shows good anti-biofilm activity against *Pseudomonas aeruginosa* at 200 and 100 µg/mL concentrations with inhibition of 33% and 31%.

Table 5.4: Inhibition of biofilm formation of test pathogen (%)

Concentration µ/mL	<i>P. aeruginosa</i>		
	200	100	50
Inhibition %	33	31	20

5.11 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 5.5: Optical density reading under UV-spectrometry at 600nm for *M.smegmatis*

Strain name	125 µ/mL			250 µ/mL			500 µ/mL		
OD values	1.068	0.731	0.939	0.804	0.731	0.694	0.476	0.46	0.511
Avg.	0.912			0.743			0.482		
Inhibition	1%			20%			48%		

5.12 Sequencing results

The strain TFB11 was subjected to 16sRNA analysis. The PCR amplification of *Streptomyces. sp* TFB11 16sRNA gene yields 1202 base pairs. NCBI BLAST analysis of TFB11 shows 98% 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* TFB11 is designated as *Streptomyces Harbinensis* (fig: 5.14). It's coming under rare forms and further studies like DNA-DNA hybridization are need to done.

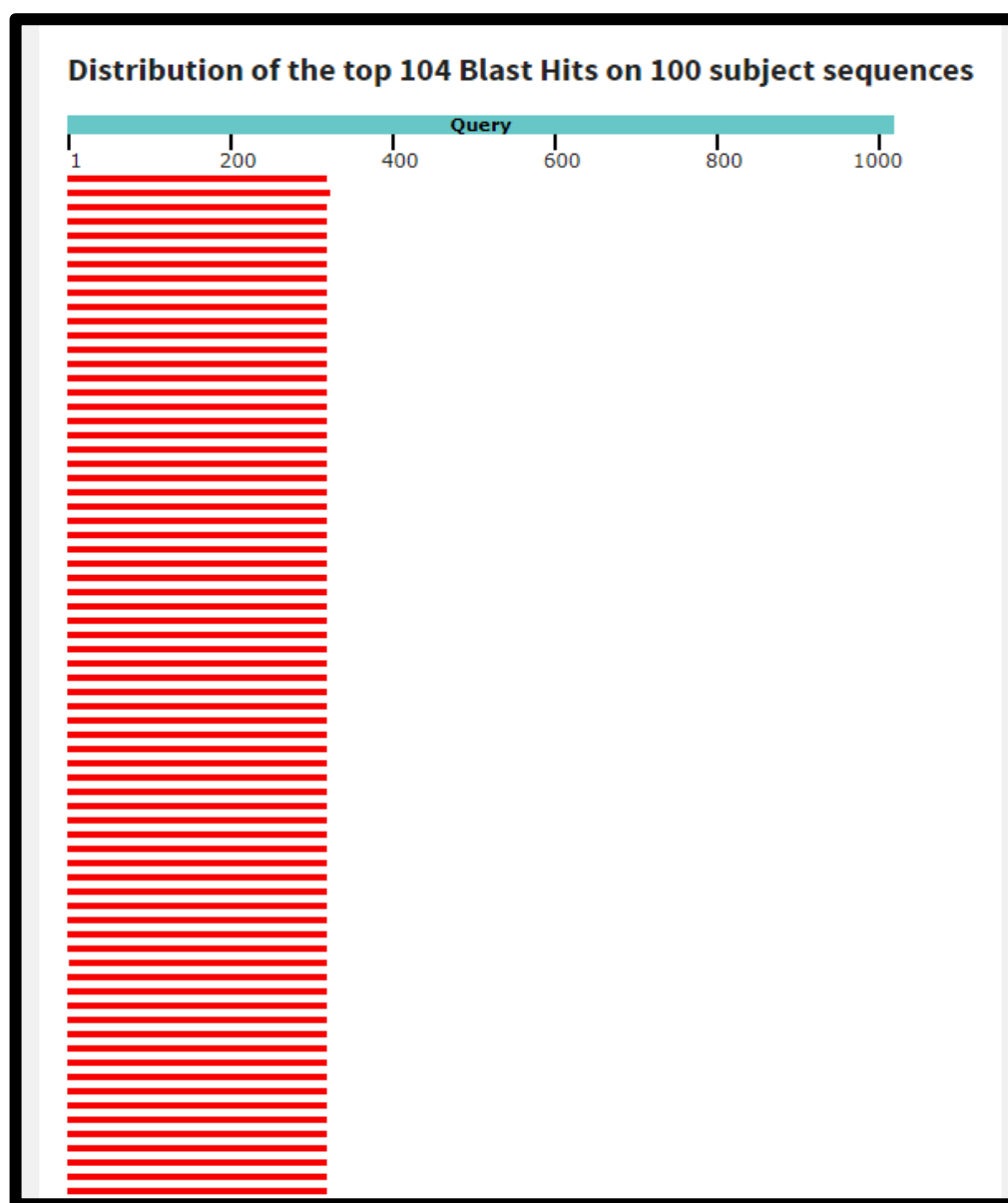


Fig: 5.14: blast results of TFB 11

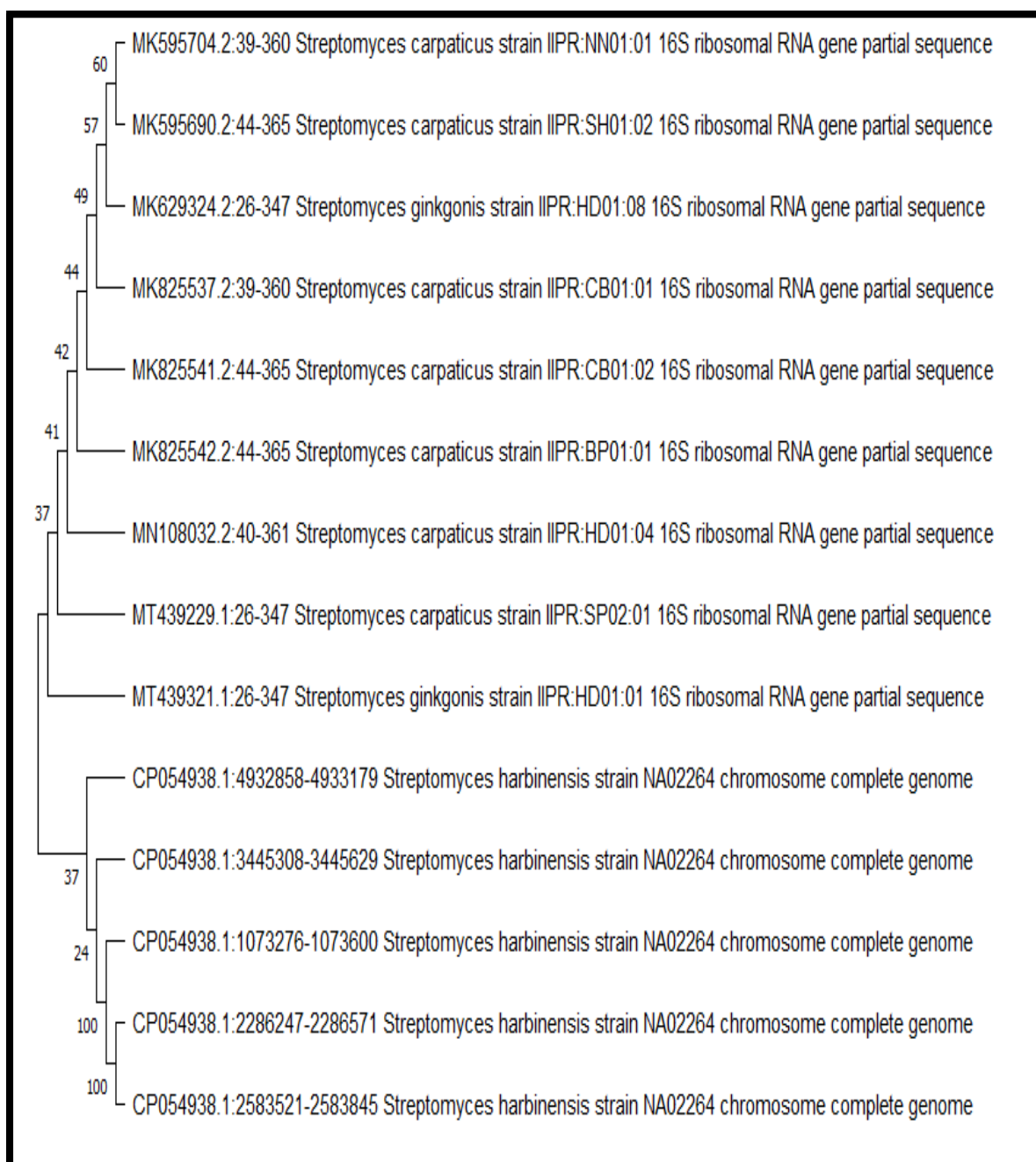


Fig:5.15: Phylogenetic tree

CHAPTER 6

SUMMARY AND CONCLUSION

There is much hope for the finding of bioactive bacteria in the environments that have not yet undergone thorough investigation or scrutiny. Examining the bacteria's enzymatic properties in fish gut samples was the aim of the current investigation. On an ISP2 agar plate, primary 34 cultures were recovered, in that one is actinomycetes. The strain TFB 11 also demonstrated various significant properties such as, Antioxidant activity yielding a significant result, the strain also exhibited properties of enzymes and it produces black pigment. This proves that the strain TFB 11 has great potential. Crude from the strain TFB 11 was produced by adopting agar surface fermentation and extracted by methanol. In the project's early phases, fish from marine sources sampled, and the microbial communities within their guts were isolated. After that, scientists used cutting-edge molecular methods like DNA sequencing to determine and categorize the many microbial species that were there. By identifying interesting genes and metabolic pathways, metagenomic analysis shed light on these microbial communities' functional capacities. Additionally the research looked into the potential of fish gut bacteria for biotechnology in a range of fields, including aquaculture, bioremediation, and medicines. Promising characteristics of several strains included the synthesis of antibacterial chemicals, industrially useful enzymes, and the capacity to break down contaminants. The project's goal was to use these discoveries in real-world settings. Researchers envisioned the creation of novel biotechnological goods and processes with major societal and economic benefits by utilizing the special qualities of fish gut microorganisms. In summary, the study "Bioprospecting of Fish Gut Microbes" has shed light on the variety, ecology, and prospective applications of fish gastrointestinal tract microbial populations in biotechnology. These bacteria have a rich supply of genetic and metabolic variety, which has ramifications for many industries like aquaculture, biotechnology, and environmental remediation. This has been revealed by a thorough investigation of these microbes. regulatory agencies, business, and academia to keep working together in order to guarantee the ethical and sustainable development of biotechnological goods made from fish gut microorganisms. Based on these facts, we conclude that studying the unusual inhabitants yields novel species and they have the

ability to produce secondary metabolites. A prospective source of new actinobacteria is fish intestine.

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ANNEXTURE

Conference - Abstract Publication

Bioprospecting of fish gut microbes

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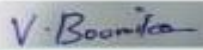
In recent years there has been a growing interest in exploring the fish gut microbes for various biotechnological applications. The gastrointestinal tract of fish shows diverse and complex microbial community that plays a vital role in the host's health, digestion and overall well-being. Fish gut microbes have been a source of antimicrobial compounds in aquaculture and in many pharmaceuticals industries. In fish, the gut microbes living in the gastrointestinal tract influence the physiology, nutrition, lifespan, immunity besides acting as a barrier against pathogens. By understanding this interaction, we can lead the development of novel probiotic and prebiotics, contributing to sustainable aquaculture practices and reduced antibiotic usage. The gut microbiome not only augment the digestive and immune system in fish but is itself shaped by several host-associated factors. Sometimes the fish gut microbes can affect the nutrition, growth, reproduction and population of the host fish. This review provides an in-depth overview of the bioprospecting efforts focused on fish gut microbes, highlighting their bioactive compounds, enzymes, and other valuable biomolecules. It also discusses the challenges and future prospects of fish gut microbe bioprospecting and also highlight the importance of their industrial importance.

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Keywords: Fish Gut, Bioprospecting, Pigments.

GATE

		GRADUATE APTITUDE TEST IN ENGINEERING 2024 अभियांत्रिकी स्नातक अभिक्षमता परीक्षा २०२४ ORGANISING INSTITUTE: INDIAN INSTITUTE OF SCIENCE, BENGALURU	
ADMIT CARD			
Name of the Candidate		BOOMIKA VENKATESAN	
Registration Number		BT24557406161	
Paper Code/Name		BT : Biotechnology	
Date		10th February 2024 (Saturday)	
Time		9:30 AM to 12:30 PM	
Session		Forenoon : S5	
Photo ID Submitted		Aadhaar ID (Number : 544204890598)	
Examination Centre Code: 7406			
Address of the Centre:			
Mohammed Sathak College of Engineering, No.34, Rajiv Gandhi Road OMR, IT Highway, Siruseri, Inside Siphos IT Park, Chennai, Tamilnadu, PIN : 603103, India.			
			
Prof. Chandrasekhar Seelamantula Organising Chairperson, GATE 2024 (on behalf of NCB-GATE, for MoE)		T500T22 0C3A3E876709F073C37F22AA185325A	
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