Bioactive molecules from the gut bacteria of Goan fish;

Grey mullet (*Mugil cephalus*)

MBT 651: DISSERTATION

Credits: 16

Submitted in partial fulfilment of Master's Degree

M.Sc. Marine Biotechnology

by

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Date: 08-04-2024

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Seal of the School:

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "**BIOACTIVE MOLECULES FROM THE GUT BACTERIA OF GOAN FISH; GREY MULLET (*Mugil cephalus*)**" is based on the results of investigations carried out by me in Masters at the Department of Biotechnology, School of Biological Sciences and Biotechnology, Goa University under the Supervision of **Mrs. Dviti L. Volvoikar** and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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This is to certify that the dissertation report **"BIOACTIVE MOLECULES FROM THE GUT BACTERIA OF GOAN FISH; GREY MULLET (*Mugil cephalus*)"** is a bonafide work carried out by **Ms G. Anvitha** under my supervision in partial fulfilment of the requirements for the award of the Master's degree in the Discipline M.Sc. Marine Biotechnology at School of Biological Sciences and Biotechnology, Goa University.

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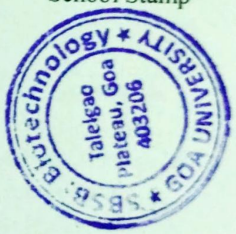

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CONTENTS

PREFACE

In this dissertation, I explore marine biotechnology, focusing on special molecules found in the gut bacteria of grey mullet fish. As I study the vast ocean, I see its potential to give us many different kinds of molecules that can be important for science. I want to understand how these molecules work, like enzymes, which help with digestion; antimicrobials, which fight germs and pigments which give color to living things. By learning about these molecules, I hope to help others see how important marine life is for medicine, food, and industry. This dissertation is the result of my love for science and my desire to learn more about the sea.

ACKNOWLEDGEMENT

I am pleased to convey my gratitude to all those who supported and helped me during this dissertation work.

First of all, I am extremely grateful to my research guide, Mrs Dviti, for her guidance, supervision, valuable suggestions, willingness to clarify my doubts despite her busy schedule, and constant encouragement & support to me through the journey.

I am also thankful to all my teachers, Prof. Sanjeev Ghadi, Prof. Savita Kerkar, Dr. Meganath Prabhu, Dr. Samantha Fernandes, Ms. Snesha Bhomkar and Ms. Snigdha, for their constant support.

I duly acknowledge the financial support from Goa University and the Department of Biotechnology (DBT, New Delhi), Govt. of India.

My acknowledgement will never be complete without the special mention of our non-teaching staff Mrs Sandhya Singh, Mr Serrao, Mr Ashish, Mr Sameer, and Mrs Jaya, for providing all the amenities in the laboratory.

I would also like to thank my parents, and I am highly indebted to them for their support throughout my life and for believing in me.

Last but not least, I would like to thank all my friends and my fellow classmates for their help, support, and motivation when needed.

LIST OF TABLES:

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LIST OF ABBREVIATIONS USED:

CU	Citrate utilization
CMC	Carboxy methyl cellulose
CFU	Colony forming unit
^{O}C	Degree Celsius
g	Gram
µl	Micro liter
MR	Methyl red
VP	Vogues Proskauer
UV	Ultra violet
ZMA	Zobell marine agar
NA	Nutrient agar
TSA	Tryptic soya agar
hrs	Hours
ml	Milli liter

ABSTRACT

Aquatic ecosystems, rich in biodiversity, offer untapped resources for discovering bioactive compounds. Fish gut microbiota, particularly in species like grey mullet, presents a promising source of diverse enzymes, antimicrobials, and pigments. These molecules play crucial roles in physiological regulation and biochemical pathways, with potential applications in biomedicine, nutrition, and industry, driving advancements in health sciences and sustainable development. The different types of enzymes include amylases, lipases, xylanases, cellulases, proteases and phytases each specialized in catalyzing specific reactions such as the hydrolysis of carbohydrates, lipids, and proteins, thereby enabling processes like digestion, nutrient assimilation, and waste disposal.

This research aims to isolate bacteria from the grey mullet's gut, screen for bioactive compounds like enzymes, pigments, antimicrobials; and biochemically characterize the isolates. The fish was collected from two different sites, the gut was extracted and isolates were obtained after serially diluting the gut homogenate. Then these isolates were used check if they were showing any positive results towards screening different bioactive molecules.

The qualitive enzyme assay for different enzymes of all the 78 isolates from the fish gut of both the ocean and river waters were carried out. Almost 60-70 % of the isolates showed positive results for at least any one of the bioactive molecules. 12 isolates produced different colored pigments ranging from red to orange to yellow. 3 isolates even showed zone of inhibition against the pathogens while studying for antimicrobial activity.

CHAPTER – 1

INTRODUCTION

The vast open oceans and deep seas, covering 70% of the Earth's surface, host around 80% of global plant and animal species. This diverse habitat includes prokaryotic bacteria, marine invertebrates, and complex multicellular organisms such as sharks and whales. (Varijakzhan, D.,et.al. 2021). Marine biotopes, spanning nearly three-quarters of the Earth's surface, are crucial natural resources. They are increasingly vital in biomedical research and development, serving as drugs or foundational structures for bioinspired chemical synthesis. (Debbab, A.,et.al. 2010)

The majority of bioactive compounds originate from microorganisms found on land. While the land is a rich source of bioactive producers, the identification of new metabolites is being reduced. Therefore, it is necessary to investigate novel sources of bioactive molecules. In this way, the vast diversity found in marine environments presents a potentially fruitful resource for discovering novel chemicals with a variety of valuable biological properties. According to the Global Biodiversity Assessment by the United Nations Environment Programme, 178,000 marine species belong to 34 phyla. Because the biodiversity of the oceans makes up 50% of that of the entire planet, marine microbes are a promising and sustainable source of new biologically active substances. (Ameen et al., 2021)

Marine environments are among the most fascinating areas for the isolation of novel metabolites. Because of their distinct and changing physical parameters encourage the producer microorganisms to acquire metabolic and physiological capacities for adjusting to a variety of environments with a broad range of temperature, pressure, salinity, pH, and nutritional levels (Floris et al., 2021a). In fact, microbial compounds with broad-spectrum activity have been identified and produced from various aquatic environments and matrices, including

antimicrobial, antiviral, antioxidant, antitumor, and anti-inflammatory properties. (Floris et al., 2021a) (Harikrishnan et al., 2021) . Non-pathogenic microorganisms, produce bioactive microbial metabolites in small quantities with antibacterial and antimicrobial activities. These compounds, valuable in medicine, biochemistry, and agriculture, defy the earlier belief that no new antibiotics could be discovered after the golden age of antibiotic exploration. (Liaqat, I. 2021)

Furthermore, it is easier to culture marine microorganisms than marine macro-organisms, and they are renewable. Microorganism's use would also prevent harmful collection techniques and overuse of marine resources and marine macro-organisms. (Ameen et al., 2021)

Fishes' digestive tracts include microbial communities, just like those of all other vertebrates and many other invertebrates (Bairagi et al., 2002). Specifically, it was discovered that fish guts provide a unique biological niche where bacteria travel and settle to perform a variety of essential functions (boosting fish immunity, pathogen-defending barriers, fish nutrition, and so on). Fish intestinal microbiota is a reflection of the environment and a range of other characteristics, according to several research conducted to date (genotype, physiological status, fish behavior, feeding habit) (Floris et al., 2021a).

The enzymatic potential represented by the bacterial flora of the gastrointestinal system is enormous and diverse, and it makes sense to believe that the enzymatic mass located in the digestive tract could significantly interfere with a significant portion of the host animal's metabolism (Bairagi et al., 2002). A multitude of studies on the gut microbiota of freshwater and marine fish have established that the gastrointestinal (GI) tract of fish is home to a dense population of microorganisms, and that the diverse range of enzymes produced by these bacteria may be a source of enzymes in fish.(Dutta & Ghosh, 2015).

Grey mullet (*Mugil cephalus*) is the common and most abundant fish species found in coastal waters, estuaries, and brackish water. Grey mullet is even the state animal of Goa. This fish is a significant part of the diet and economy for both the fishing industry and public health. It can even inhabit in a variety of aquatic environments like freshwater, brackish water and coastal waters. As they are omnivorous and often feed on variety of organisms, including algae, detritus, and small vertebrates, results in diverse gut microbiota, potentially producing a wide range of bioactive compounds with various functions.

Encompassing a diverse array of compounds, bioactive molecules play pivotal roles in regulating physiological functions, biochemical pathways, and cellular processes. Among the prominent categories of bioactive molecules are enzymes, antimicrobials, antioxidants, and pigments. Enzymes, the catalysts of biological reactions, facilitate the conversion of substrates into products, driving essential metabolic pathways and biochemical transformations crucial for life. Examples include amylases, lipases, xylanases, cellulases, and proteases, each specialized in catalyzing specific reactions such as the hydrolysis of carbohydrates, lipids, and proteins, thereby enabling processes like digestion, nutrient assimilation, and waste disposal. (A. Pandey et al.,2021) Antimicrobials, on the other hand, exert inhibitory or lethal effects on microorganisms, serving as frontline defenses against infectious diseases and preserving the safety and shelf-life of food products. (N. N. Zhang et al., 2021) Pigments, while primarily contributing to the visual appearance of organisms, also exhibit diverse biological activities and find applications in food coloring, cosmetic formulations, and pharmaceutical preparations (S. K. Kim and A. Karadeniz, 2012). Collectively, these bioactive molecules represent a treasure trove of natural compounds with immense potential for biomedical, nutritional, and industrial

applications, underpinning advancements in health sciences, biotechnology, and sustainable development.

1.1) Applications of different Bioactive molecules:

1.1.1) Amylase enzyme:

Amylase enzymes, vital in carbohydrate metabolism, find extensive applications in various scientific fields. In biotechnology, they serve as crucial catalysts in starch hydrolysis for bioethanol production (Saboury, A. A., & Divsalar, A., 2012). In food processing, amylases facilitate starch liquefaction, improving texture and shelf-life of products (Gopinath, S. C. B., & Anbu, P., 2008). Additionally, in molecular biology, amylases are employed for DNA extraction protocols, aiding in the disruption of plant cell walls (Murray, M. G., & Thompson, W. F., 1980).

1.1.2) Cellulase enzyme:

Cellulase enzymes, pivotal in cellulose degradation, hold versatile applications. They are indispensable in biofuel production from lignocellulosic biomass (Bayer, E. A., et al., 2013). Additionally, they find utility in textile, paper, and detergent industries for efficient degradation of cellulose-based materials (Beg, Q. K., et al., 2001).

1.1.3) Xylanase activity:

Xylanase enzymes, crucial in xylan degradation, have diverse applications. They are essential in pulp and paper industries for bleaching and improving paper quality (Beg, Q. K., et al., 2001). Additionally, they aid in animal feed processing to enhance nutrient availability (Ahmed, S., & Rao, M. B., 2019).

1.1.4) Protease activity:

Protease enzymes, pivotal in protein degradation, find extensive applications. They are crucial in detergent formulations for stain removal and textile industries for fabric softening (Bornscheuer, U. T., & Kazlauskas, R. J., 2006). Additionally, they play a vital role in food processing, enhancing flavor and texture (Sumantha, A., et al., 2005).

1.1.5) Lipase activity:

Lipase enzymes, pivotal in lipid hydrolysis, have versatile applications. They are essential in detergent formulations for removing lipid-based stains (Hasan, F., et al., 2006). Additionally, they find utility in biodiesel production from vegetable oils, contributing to sustainable energy solutions (Sharma, R., et al., 2001).

1.1.6) Phytase enzyme:

Phytase enzymes play a crucial role in various applications within the food and feed industry. They are utilized to improve phosphorus availability in animal diets, leading to enhanced growth performance and reduced phosphorus excretion (Coban & Demirci, n.d.). Additionally, phytase enzymes aid in increasing mineral bioavailability, thereby contributing to overall animal health and nutrition.

1.1.7) Pigments:

Pigments, essential for coloration in various applications, have wide-ranging uses. They are crucial in the food industry for enhancing visual appeal and consumer acceptance of products (Jung, J., & Zhao, Y., 2015). Moreover, pigments find applications in cosmetics, where they

contribute to product aesthetics and consumer preferences, highlighting their significance in diverse industries.

1.2) Aim and Objectives:

The overarching aim of this research study is to screen for bioactive molecules from the gut bacteria of Goan fish: Grey mullet (*Mugil cephalus*).

The objectives include:

1) Isolation of bacteria from the gut of grey mullet
2) Screening for bioactive molecules
3) Biochemical characterization of bacterial isolates

CHAPTER – 2

LITERATURE REVIEW

Enzymatic potential of Isolates:

National status:

Understanding the diversity and enzymatic profile of bacterial flora in the gut of fish species is essential for comprehending their role in digestion and overall gut health. Datta, A. A., Sharma, A. K., et al., focused on elucidating the bacterial diversity and enzymatic profile in the gut of an estuarine fish, *Mugil jerdoni*. (Datta, A. A., et.al. 2017)The research began by characterizing the diversity of bacterial flora in the gut of *Mugil jerdoni*. This likely involved molecular techniques such as 16S rRNA gene sequencing to identify and classify the various bacterial taxa present in the gut microbiota. Following the assessment of bacterial diversity, the study investigated the enzymatic profile of the gut microbiota. Enzymes involved in digestion, such as proteases, lipases, and carbohydrase's, were likely analyzed to understand the fish's digestive capabilities and the role of gut bacteria in nutrient metabolism (Datta, A. A., et.al. 2017).

The research may have explored environmental factors and host-specific factors influencing the composition and activity of the gut bacterial flora in *Mugil jerdoni*. Understanding these factors can provide insights into the adaptability of fish gut microbiota to different ecological niches and dietary habits. By elucidating the diversity and enzymatic profile of bacterial flora in the gut of *Mugil jerdoni*, this study contributes to our understanding of fish gut microbiology and its implications for fish health and nutrition. Insights gained from this research may inform strategies for improving aquaculture practices and enhancing the digestive efficiency of farmed fish species. (Datta, A. A., et.al. 2017).

State status:

Nagvenkar, G. S., et al. investigated microbial diversity and enzyme production in the mullet species *Mugil cephalus* L. along the west coast of India, specifically in the region of Goa. Understanding microbial diversity and enzyme production in fish species is crucial for comprehending their ecological roles and potential applications in various fields. The research likely delves into characterizing the microbial diversity present in the mullet *Mugil cephalus* L. gut and surrounding environment along the Goa coast. This exploration involves molecular techniques such as high-throughput sequencing to identify and classify the diverse microbial taxa inhabiting the fish and its habitat. (Nagvenkar, G. S., et.al. 2006)

Enzyme production, particularly those involved in digestion and nutrient metabolism, is another focus of the study. The research investigates the types and quantities of enzymes produced by the microbial communities associated with *Mugil cephalus* L., shedding light on their digestive capabilities and potential applications in biotechnology or aquaculture. Moreover, the study carried out by Nagvenkar, G. S., et.al. finds biotechnological applications, such as the discovery of novel enzymes with industrial or medical relevance. (Nagvenkar, G. S., et.al. 2006)

Enzymes from gut Bacteria:

The study by Dutta and Ghosh focuses on screening extracellular enzyme-producing gut bacteria with pathogen inhibitory properties in *mrigal, Cirrhinus mrigala,* aiming to identify potential probiotics for aquaculture application. The research begins by isolating gut bacteria from mrigal specimens and evaluating their ability to produce key extracellular enzymes essential for various physiological processes and nutrient digestion in fish. Enzymatic screening likely involved assays to detect the presence of enzymes such as amylase, protease, lipase, cellulase, phytase,

and xylanase, critical for carbohydrate, protein, lipid, and plant material digestion and nutrient utilization in fish. Subsequently, the isolated bacteria were assessed for their ability to inhibit pathogenic bacteria commonly associated with aquaculture, thereby potentially enhancing fish health and reducing disease prevalence. This screening process involved evaluating the antibacterial activity of gut bacteria through agar well diffusion assays or similar methods. Moreover, the research contributes to the understanding of the gut microbiota composition in mrigal and underscores the importance of probiotic supplementation in sustainable aquaculture practices. (Dutta & Ghosh, 2015)

The review by Bairagi et al. highlights the importance of selecting appropriate media for screening enzyme-producing bacterial flora isolated from fish digestive tracts. Media selection plays a crucial role in optimizing enzyme production and activity. For screening amylase-producing bacteria, starch agar or nutrient agar supplemented with starch can be effective, while casein agar or skim milk agar are suitable for protease screening. Lipase-producing bacteria can be screened using tributyrin agar or spirit blue agar. Cellulase production can be assessed on carboxymethyl cellulose (CMC) agar, while phytase production may be evaluated on phytase assay agar. Xylanase-producing bacteria can be screened using Xylan agar or beechwood Xylan agar. By selecting appropriate media tailored to each enzyme's substrate, researchers can enhance the efficiency and accuracy of screening enzyme-producing bacterial flora from fish digestive tracts (Bairagi et al., 2002).

Antimicrobial activity of Isolates:

The gut microbiota of fish is known to harbor diverse bacterial species with potential antimicrobial properties. Floris, R., Sanna, G., et al. focused on isolating and identifying bacteria with surface and antibacterial activity from the gut of Mediterranean grey mullets. The bacteria

from the gut of Mediterranean grey mullets were isolated using standard microbiological techniques. This step involved culturing bacterial strains from gut samples to obtain pure cultures for further analysis. The isolated bacterial strains were then screened for surface and antibacterial activity. This screening likely involved assessing the ability of bacterial isolates to inhibit the growth of pathogenic bacteria through agar-based assays and other appropriate methods. (Floris, R., et.al. 2021a)

Bacterial strains exhibiting surface and antibacterial activity were further characterized to identify their taxonomic classification and potential mechanisms of antibacterial action. This characterization included biochemical tests, molecular techniques, and microscopic analysis. The study likely evaluated the antibacterial potential of the isolated strains against relevant fish pathogens or human pathogens. This assessment could provide insights into the suitability of these bacteria for use as biocontrol agents in aquaculture or as sources of novel antimicrobial compounds. (Floris, R., et.al. 2021a)

The isolation and identification of bacteria with surface and antibacterial activity from the gut of Mediterranean grey mullets offer valuable insights into the potential role of fish gut microbiota in antimicrobial defense. This research contributes to the exploration of natural antimicrobial agents for use in aquaculture and other relevant fields (Floris, R., et.al. 2021a).

Production of Pigments from Isolates:

Agarwal et al. provides a comprehensive overview of bacterial pigments and their diverse applications in contemporary biotechnology and pharmacology.

The paper discusses the wide-ranging biotechnological applications of bacterial pigments, including their use as natural dyes, food colorants, antioxidants, and antimicrobial agents. The

review delves into the pharmacological potential of bacterial pigments, focusing on their antioxidant, anti-inflammatory, anticancer, and neuroprotective properties. (Agarwal et al. 2023)

Prokaryotes synthesize various pigments with distinct colors, serving specialized functions. Autotrophic prokaryotes utilize pigments like chlorophyll, carotene, and xanthenes, with hues ranging from yellow to deep pink, orange, and red, aiding in photosynthesis and UV protection. Heterotrophic prokaryotes produce accessory pigments crucial for survival in extreme environments, such as xanthomodin, which exhibits photoprotective properties. These pigments play key roles in taxonomic characterization and genetic relatedness assessment among bacterial species. In pharmaceutical applications, bacterial pigments find extensive use. Carotenoids like canthaxanthin, astaxanthin, zeaxanthin, and β-carotene offer antioxidant, photoprotective, anticancer, and anti-inflammatory effects. Pigments like prodigiosin and un-decylprodigiosin, displaying red hues, show potential in anticancer and antimicrobial activities. Phycobiliproteins contribute cytotoxicity and proinflammatory effects, notably pycocyanin, appearing blue. Oxyindoles like violacein, lending a purple hue, demonstrate antifungal, antibacterial, and anticancer properties. This diversity underscores the multifaceted roles of bacterial pigments in both biological and pharmaceutical realms. (Agarwal et al. 2023)

CHAPTER – 3

MATERIAL & METHODS

3.1) Sampling:

Three Flat-headed grey mullets, *Mugil cephalus*, were caught on December 21 from site 1(oceanic) Caranzalem, Goa (Lat: 15.475717, Long: 15.475717). Another three mullets were caught on February 1 from site 2 (riverine) Chorao island. The fish were identified based on their morphological characteristics.

3.2) Isolation of bacteria:

The fish caught were euthanized by slow ice immersion method using 1: 1 ratio of ice and water.(STANDARD OPERATING PROCEDURES DIVISION OF COMPARATIVE MEDICINE UNIVERSITY OF SOUTH FLORIDA, n.d.) The entire intestine was aseptically removed washed in 0.85% saline by dipping twice to get rid of surface bacteria. (Floris et al., 2021b) The gut was homogenized and serially diluted from 10^{-1} to 10^{-9} dilutions (dilutions used for plating were 10^{-3}, 10^{-5}, $10^{-7}$and 10^{-9}). 100 µl from each dilution was spread plated on Nutrient Agar, Zobell Marine Agar, Zobell Marine Agar+ Artificial Sea Water /River Water, and Tryptone Soya Agar and incubated at 28 ºC (room temperature). After 48-96 hours of incubation, the colonies were counted and CFU/ml was calculated. The well-separated colonies with apparently different morphological appearances were picked and sub-cultured to obtain pure cultures. The pure cultures were further used to screen different bioactive molecules (enzymes, pigments, antimicrobials, and antioxidants).

3.3) Morphological characterization of Isolates:

3.3.1) Gram staining:

Gram staining of the bacteria was done using. (Tripathi N et.al 2023) The smear of isolated colony was made on a clear slide cleaned with ethanol. They were stained with crystal violet (primary stain), then rinsed; now the grams iodine was added (the mordant), then rinsed; then the decolorizer was used, and again rinsed; and finally counter stained with saffranine, and final rinse was given. The slide was examined using the 100 X oil immersion objective.

3.4) Screening of Isolates for Bioactive molecules:

3.4.1) Screening of isolates for extra-cellular qualitative enzyme production:

Isolates were screened for extra-cellular amylase, cellulase, lipase, xylanase, protease, and phytase production.

3.4.1.a) Amylase activity:

For screening of amylase-producing strains, the isolates were spot inoculated onto starch agar media and stained with Lugol's iodine after 48 hours of incubation.(Bairagi et al., 2002)

3.4.1.b) Cellulase activity:

For screening of cellulase producing strains, the isolates were spotted on Carboxymethylcellulose agar media and kept for 48 hours of incubation. Later the zones were observed by staining with 0.5% Congo red dye and 5% ethanol and made up to 100

ml with distilled water. 1M NaCl is used to wash and destain the plates. (Bairagi et al., 2002)

3.4.1.c) Lipase activity:

For screening of lipase producing strains, the isolates were spot inoculated on to Tributyrin agar plates and incubated for 48 hours to observe clear zones.(Bairagi et al., 2002)

3.4.1.d) Xylanase activity:

The isolates were spotted on Xylan agar medium plates for screening of xylanase-producing strains. After 48 hours incubation, plates were stained with Congo red dye. (Ninawe et al., 2006)

3.4.1.e) Protease activity:

For screening of protease producing strains, the isolates were spotted on to Skim milk agar plates. After 24 hours of incubation, plates were observed for clear zones. (Mushtaq et al., 2023)

3.4.1.f) Phytase activity:

The isolates were spotted against Modified phytase screening media for screening of phytase-producing strains and incubated at 37 ^{o}C for 72 hrs. After incubation plates were observed for clear zones. (Dan & Ray, 2014)

3.4.2) Screening of isolates for production of pigments: (Mushtaq et al., 2023)

3.4.2.a) Production of microbial pigments:

Pigment-producing isolates were grown in respective broth of isolates. A loop full of inoculum of the culture was picked and inoculated into the respective freshly prepared

broth of 150 ml. The broth was kept on the shaker at room temperature for 3 days and visually observed for pigment production.

3.4.2.b) Extraction of microbial pigments:

After 3 days of growth the culture broth was centrifuged at 8000 rpm for 15 mins at 4^{o}C. After centrifugation supernatant was checked if it was colored. As the supernatant was not colored hence it was discarded and the cell pellet was saved. To the cell pellet 5ml of methanol: acetone in 2:1 ratio was added and vortexed vigorously for 1 hour or sonicated for 30 mins (to those cultures which didn't produce pigments upon vortexing). The model of sonicator used was 'Ultrasonic Processor [SONIC-650WT-V2]'. Now the vortexed isolate with solvent added in it was centrifuged again at 8000 rpm for 15 mins at 4^{o}C. The supernatant was collected, filtered and stored for further use.

3.4.2.c) UV- Visible spectrophotometric analysis:

The pigment extract was analyzed by scanning in UV- Visible spectrophotometer to detect the absorption maxima. The scanning range was 200-800 nm. The instrument used was 'UV-Vis Spectrophotometer [TS-2080]'.

3.4.3) Screening of isolates for antimicrobials:

3.4.3.a) Inoculum preparation:

To screen for antimicrobial production, the culture isolates were inoculated in 20 ml of freshly prepared respective broth and kept in a shaker at room temperature for more than 48 hrs. The broth was centrifuged at 10000rpm for 15 mins at 4^{o}C.

3.4.3.b) Agar well diffusion assay:

The agar well diffusion method was performed. The pathogens used were *Escherichia coli, Klebsiella pneumoniae, Candida albicans, Pseudomonas aeruginosa*. The pathogens were spread plated on MHA plates. 50 µl of supernatant of each isolate's culture broth was diffused into wells against all 4 pathogens. Zone of inhibition of isolates were measured. (Mukherjee et. al, 2012)

3.5) Biochemical tests:

3.5.1) Catalase test:

Isolated bacterial colony from each culture plate was picked and a smear was made on glass slide. Now 2-3 drops of 3% H_2O_2 on to the smear was added. Appearance of bubbles within 5-10 seconds after addition of H_2O_2 is positive result.(Catalase Test Protocol, 2010)

3.5.2) Oxidase test:

HiMedia oxidase discs were used. Isolated colony was picked using a toothpick and spotted/smeared on to the disc. A change in color to dark purple within 5-10 seconds shows positive result. Stainless steel or nichrome inoculating wires gives false positive results. [HiMedia Oxidase disc DD018]

3.5.3) MR-VP test:

MRVP broth was inoculated with test culture organism. Cultures were incubated at room temperature. After 48 hours of growth 5 drops of methyl red was added for MR test. Add 1ml 0f 40% KOH (Barret's A reagent) and 3ml of 5% alpha- naphthol (Barret's B reagent) for VP test. (Methyl Red and Voges-Proskauer Test Protocols, 2009)

3.5.4) Citrate utilization test:

The isolates were streaked on Simmon's Citrate Agar slants. Tubes were incubated for 48hours. The development of blue color on the slants indicated a positive result. (Citrate Test Protocol, 2009)

3.5.5) SIM Deep tests:

A stab of SIM medium was made. Using a nichrome loop culture were picked and stabbed to 2/3 of the way down in to each tube. Incubated for 48 hours. (*SIM-Test*, n.d.) After 48 hours of incubation tubes were checked for motility and H_2S production. For Indole test now 10 drops of Kovacs reagent are added and observed for color change.

CHAPTER- 04

RESULTS & DISCUSSION

The primary objective of this dissertation was to **assess the production of bioactive molecules by bacteria from fish gut & comparing the bacteria from fish of two different sites (oceanic & riverine).** The gut was homogenized, serially diluted and plated on 4 different kinds of growth media Nutrient agar, Zobell Marine Agar, Tryptic Soya broth and Zobell Marine Agar with river water/ artificial sea water.

Figure 4.1: Sampling site 1: Caranzalem beach(oceanic)

Figure 4.2: Sampling site 2: Chorao Island (riverine)

4.1) Isolation of bacteria:

4.1.1) Measurements of Fish:

4.1.1.a) Site 1 (Caranzalem):

The fish from the Site 1weighted average of 60.6 g and the length was measured to be an average of 18.5 cm as mentioned below in the table 4.1.

Table 4.1: Average weight and average length of fish from Site 1

Fish	Size	average size	Weight	average weight
Fish 1	16.5 cm	18.5 cm	41 g	60.6 g
Fish 2	19.5 cm		65 g	
Fish 3	19.5 cm		76g	

4.1.1.a) <u>Site 2 (Chorao Island):</u>

The fish from the Site 2 weighted average of 24.87 g and the length was measured to be an average of 17.3 cm as mentioned below in the table 4.2.

Table 4.2: Average weight and average length of fish from Site 2

Fish	Size	average size	Weight	average weight
Fish 1	17 cm	17.3 cm	25.35 g	24.87 g
Fish 2	17 cm		23.26 g	
Fish 3	18 cm		26 g	

4.1.2) <u>Viable count</u>:

The viable count of the fish gut sample was determined by spread-plating the dilutions in three sets. The number of colonies obtained was recorded after 48-96 hours of growth. The sample was plated on four different kinds of media, i.e., Nutrient Agar, Zobell Marine Agar, Zobell Marine Agar+ Artificial Sea Water /River Water, and Tryptone Soya Agar.

The average CFU/ml is ranging from 10^7-10^9 from gut of site 1 fish. And the average CFU/ml is ranging from 10^4-10^6**.** This shows that the fish from oceanic zone bears comparatively higher amount of viable count of bacteria than the fish from river waters.

Table 4.3: Viable count of bacteria from site 1 fish

Sr.No	Media	Dilution	Fish	Number of colonies	Viable count	Average (CFU/ ml)
1	ZMA	10^{-3}	I	Matt	-	-
				Matt	-	
		10^{-5}		27	$2.7x\ 10^7$	$2.3x\ 10^7$
				19	$1.9x\ 10^7$	
		10^{-7}		4	$4x\ 10^8$	$5x\ 10^8$
				6	$6x\ 10^8$	
		10^{-3}	II	Matt	-	-
				Matt	-	
		10^{-5}		40	$4x\ 10^7$	$6.55x\ 10^7$
				~91	$9.1x\ 10^7$	
		10^{-7}		3	$3x\ 10^8$	$4x\ 10^8$
				5	$5x\ 10^8$	
		10^{-3}	III	Matt	-	-
				Matt	-	
		10^{-5}		~210	$2.1x\ 10^8$	$2.025x\ 10^8$
				~195	$1.95x\ 10^8$	
		10^{-7}		23	$2.3x\ 10^9$	$2.75x\ 10^9$
				32	$3.2x\ 10^9$	
2	TSA	10^{-5}	I	29	$2.9x\ 10^7$	$2.1x\ 10^7$
				13	$1.3x\ 10^7$	
		10^{-3}	II	Matt	-	-
		10^{-5}		43	$4.3x\ 10^7$	$4.3x\ 10^7$
		10^{-3}	III	Matt	-	-
3	NA	10^{-5}	I	17	$1.7x\ 10^7$	$1.7x\ 10^7$
			II	36	$3.6x\ 10^7$	$3.6x\ 10^7$
4	ZMA+ ASW	10^{-3}	I	Matt	-	-
		10^{-3}	II	Matt	-	-
		10^{-7}		13	$1.3x\ 10^9$	$1.3x\ 10^9$
		10^{-5}	III	~230	$2.3x\ 10^8$	$2.3x\ 10^8$
				Matt	-	-
		10^{-7}	III	35	$3.5x\ 10^9$	$3.5x\ 10^9$

Table 4.4: Viable count of bacteria from site 2 fish

Sr.No	**Media**	**Dilution**	**Fish**	**Number of colonies**	**Viable count (CFU/ ml)**
1	NA	10^{-3}	I	15	$1.5x\ 10^5$
			II	25	$2.5x\ 10^5$
			III	185	$1.85x\ 10^6$
2	ZMA	10^{-3}	I	38	$3.8x\ 10^5$
			II	78	$7.8x\ 10^5$
			III	168	$1.68x\ 10^6$

		10^{-5}	III	4	$4x\ 10^6$
3	ZMA+ RW	10^{-3}	I	5	$5x\ 10^4$
			II	27	$2.7x\ 10^5$
			III	133	$1.33x\ 10^6$
4	TSA	10^{-3}	I	7	$7x\ 10^4$
			II	85	$8.5x\ 10^5$
			III	175	$1.75x\ 10^6$
		10^{-5}	III	4	$4x\ 10^6$

Catla catla (1.50×10^6 bacterial cells/g digestive tract), followed by silver carp, *Hypophthalmichthys molitix* (0.80×10^6 bacterial cells/g digestive tract), and minimum in murrel, *Channa punctatus* (0.06×10^6 bacterial cells/g digestive tract). (Bairagi et.al. 2002) Bacterial counts on MA medium showed values of heterotrophic marine bacteria from $10x10^3$ to $10x10^4$ colony forming units (CFU) in autumn and from $12x10^4$ to 40x104 CFU in winter. (Floris.R. et.al, 2021)

4.1.3) <u>Gram's staining</u>:

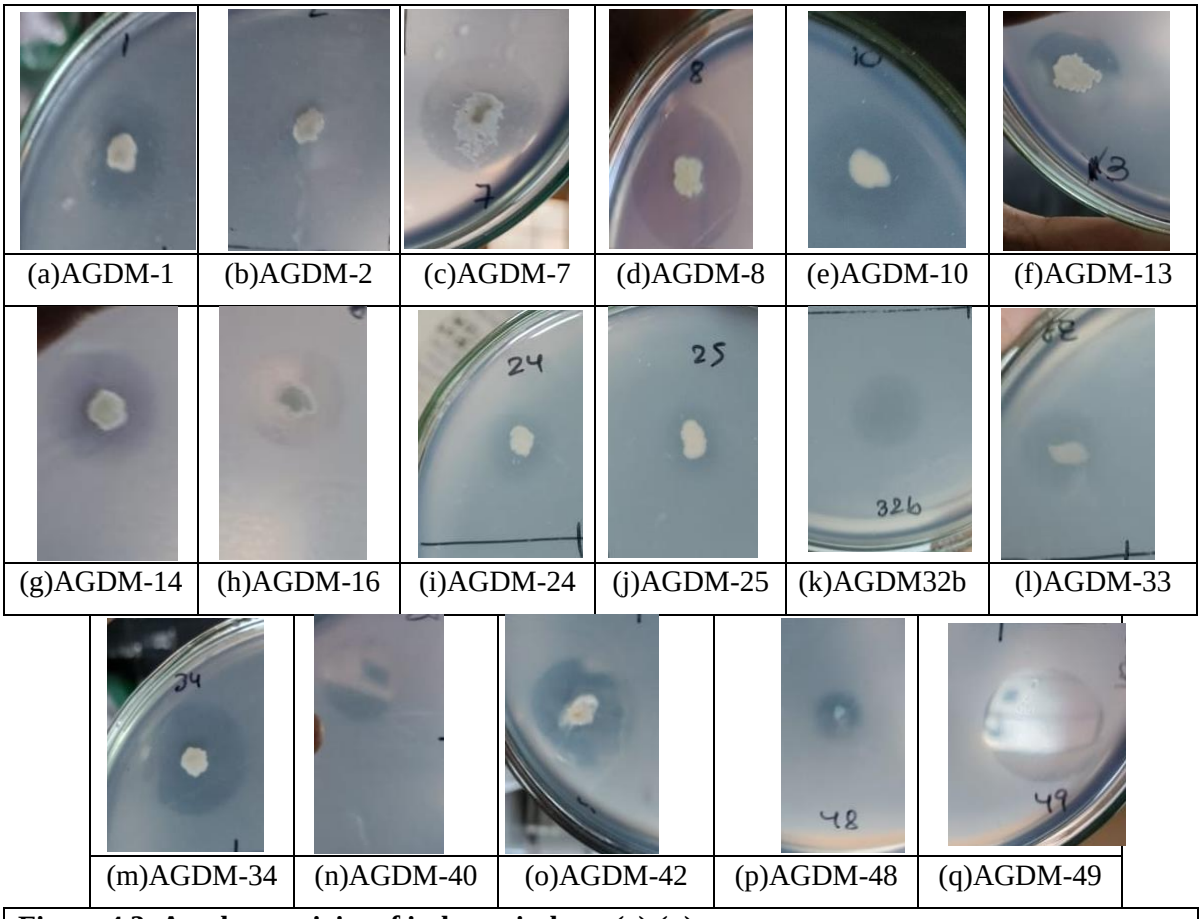
54 cultures from the total of 78 cultures have shown gram negative characteristics, whereas remaining 24 cultures have shown gram negative characteristics. This shows that Marine bacteria, develop a lipopolysaccharide (LPS) layer to withstand harsh environments, hence almost 70% of the bacteria were gram negative. This adaptation is evident in their survival strategies. (Anwar, M. A., & Choi, S. et.al 2014).

4.2) <u>Enzyme activity of Isolates:</u>

The isolated colonies were tested for 7 different enzyme production using the plate assay technique. Almost all the isolates produced at least one enzyme from the seven different enzymes. The results of enzyme screening are summarized in Table 4.7.

4.2.1) <u>Amylase activity:</u>

Total of 17 isolates from both the sites have shown amylase activity (i.e. 13 isolates from fish gut of Site 1 and 4 isolates from fish gut of Site 2). The plates were stained with 1% Lugol's Iodine and the zones were visualized. The diameter of the zone of inhibition of isolates from Site 1 ranged between 2.46 cm- 0.8 cm. The diameter of the zone of inhibition of isolates from Site 2 ranged between 2.03 cm-0.7 cm. The zone of clearance of all the positive isolates is shown in the below figure 4.3. Positive results shown by AGDM-1, AGDM-2, AGDM-7, AGDM-8, AGDM-10, AGDM-13, AGDM-14, AGDM-16, AGDM-24, AGDM-25, AGDM-32b, AGDM-33, AGDM-34, AGDM-40, AGDM-42, AGDM-48 and AGDM-49.



(a)AGDM-1 (b)AGDM-2 (c)AGDM-7 (d)AGDM-8 (e)AGDM-10 (f)AGDM-13

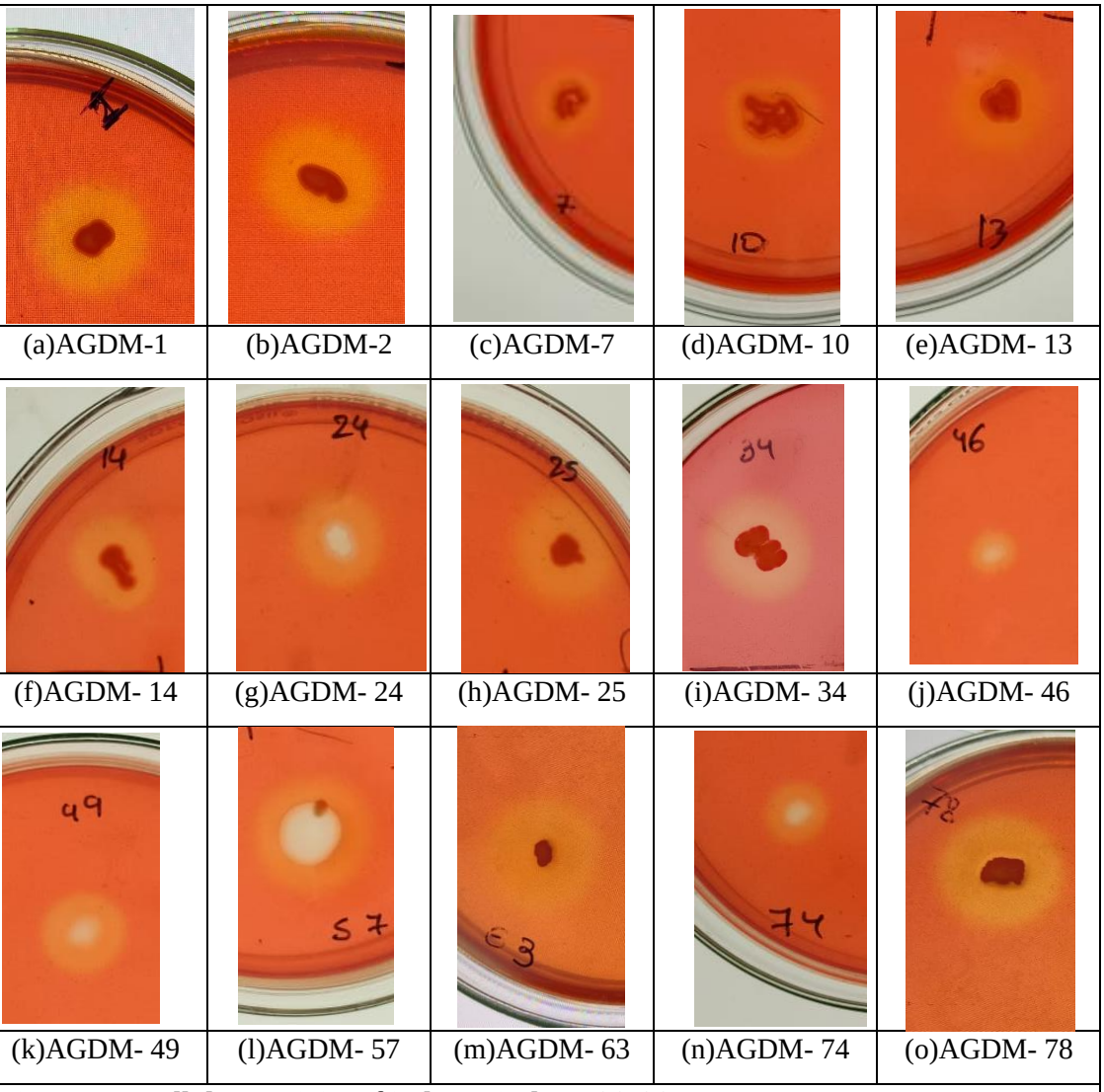
(g)AGDM-14 (h)AGDM-16 (i)AGDM-24 (j)AGDM-25 (k)AGDM32b (l)AGDM-33

(m)AGDM-34 (n)AGDM-40 (o)AGDM-42 (p)AGDM-48 (q)AGDM-49

Figure 4.3: Amylase activity of isolates: isolates (a)-(q)

4.2.2) <u>Cellulase activity</u>:

Total of 15 isolates from both the sites have shown cellulase activity (i.e. 9 isolates from fish gut of Site 1 and 6 isolates from fish gut of Site 2) after staining with 0.5 % Congo red dye dissolved in 5% ethanol. Then the plates were washed with 1M NaCl and zones were visualized. The diameter of the zone of inhibition of isolates from Site 1 ranged between 1.9 cm- 0.8 cm. The diameter of the zone of inhibition of isolates from Site 2 ranged between 2.1 cm-0.8 cm. The zone of clearance of all the positive isolates is shown in the below figure 4.4. Positive results shown by AGDM-1, AGDM-2, AGDM-7, AGDM-10, AGDM-13, AGDM-14, AGDM-24, AGDM-25, AGDM-34, AGDM-46, AGDM-49, AGDM-57, AGDM-63, AGDM-74 and AGDM-78.



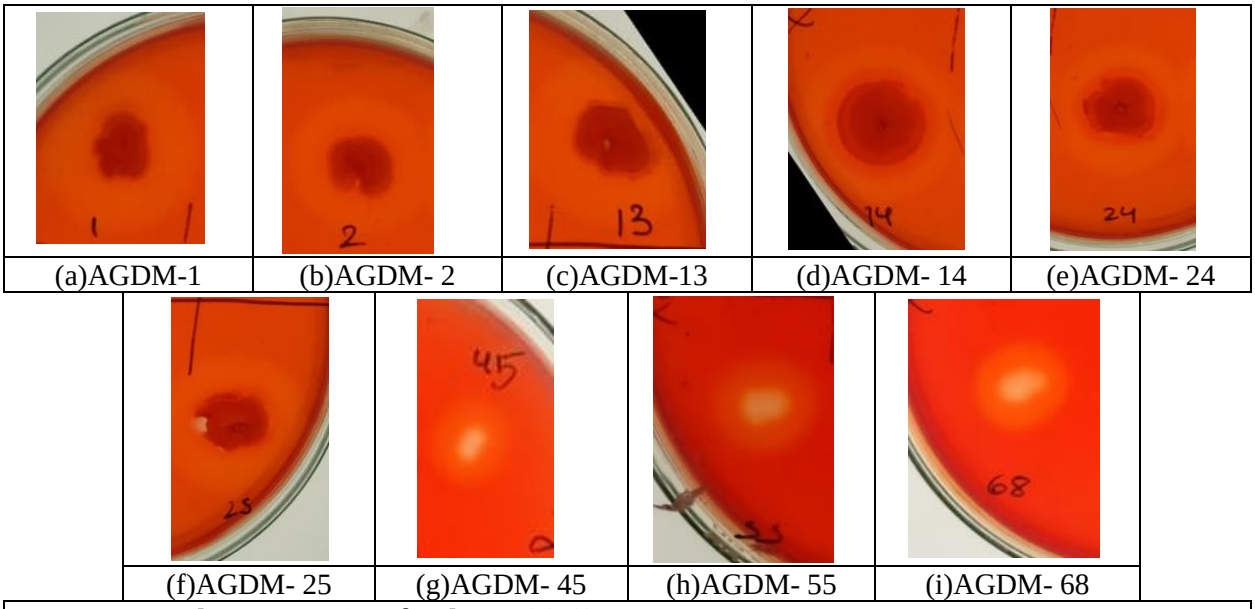
(a)AGDM-1	(b)AGDM-2	(c)AGDM-7	(d)AGDM- 10	(e)AGDM- 13
(f)AGDM- 14	(g)AGDM- 24	(h)AGDM- 25	(i)AGDM- 34	(j)AGDM- 46
(k)AGDM- 49	(l)AGDM- 57	(m)AGDM- 63	(n)AGDM- 74	(o)AGDM- 78

Figure 4.4: Cellulase activity of isolates: isolates (a)-(o)

4.2.3) <u>Xylanase activity</u>:

Total of 9 isolates from both the sites have shown xylanase activity (i.e. 6 isolates from fish gut of Site 1 and 3 isolates from fish gut of Site 2) after staining with 0.5 % Congo red dye dissolved in 5% ethanol. Then the plates were washed with 1M NaCl and zones were visualized. The diameter of the zone of inhibition of isolates from Site 1 ranged between 2.43 cm- 1.5 cm. The diameter of the zone of inhibition of isolates from Site 2 ranged between 1.93 cm-1.46 cm. The zone of clearance of all the positive isolates is shown in the below figure 4.5. Positive results

shown by AGDM- 1, AGDM-2, AGDM-13, AGDM-14, AGDM-24, AGDM-25, AGDM-45, AGDM-55 and AGDM-68.



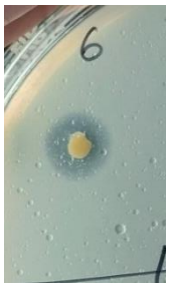
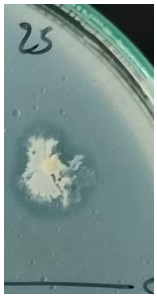
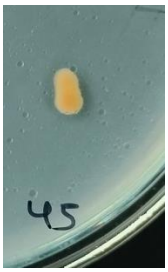
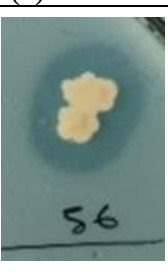
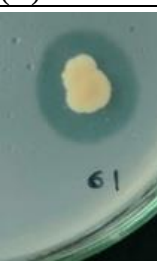
(a)AGDM-1 (b)AGDM- 2 (c)AGDM-13 (d)AGDM- 14 (e)AGDM- 24

(f)AGDM- 25 (g)AGDM- 45 (h)AGDM- 55 (i)AGDM- 68

Figure 4.5: Xylanase activity of isolates: (a)-(i)

4.2.4) <u>Lipase activity</u>:

Total of 34 isolates from both the sites have shown lipase activity (i.e. 15 isolates from fish gut of Site 1 and 19 isolates from fish gut of Site 2). The diameter of the zone of inhibition of isolates from Site 1 ranged between 1.53 cm- 0.53 cm. The diameter of the zone of inhibition of isolates from Site 2 ranged between 2.06 cm-0.43 cm. The zone of clearance of all the positive isolates is shown in the below figure 4.6. Positive results shown by AGDM- 2, AGDM- 3, AGDM- 5, AGDM- 8, AGDM- 13, AGDM- 21, AGDM- 23, AGDM- 24, AGDM- 25, AGDM- 30, AGDM- 32a, AGDM- 32b, AGDM- 34, AGDM- 37, AGDM- 39, AGDM- 40, AGDM- 43, AGDM- 45, AGDM- 48, AGDM- 49, AGDM- 50, AGDM- 51, AGDM- 53, AGDM- 54, AGDM- 55, AGDM- 56, AGDM- 60, AGDM- 61, AGDM- 63, AGDM- 64, AGDM- 65, AGDM- 69 and AGDM- 70.

					
(a)AGDM-2	(b)AGDM- 3	(c)AGDM- 5	(d)AGDM- 6	(e)AGDM- 8	(f)AGDM- 13
					
(g)AGDM- 21	(h)AGDM- 23	(i)AGDM- 24	(j)AGDM-25	(k)AGDM-30	(l)AGDM- 32a
					
(m)AGD32b	(n)AGDM-34	(o) AGDM-37	(p)AGDM-39	(q)AGDM-40	(r)AGDM-43
					
(s)AGDM-45	(t)AGDM-48	(u)AGDM-49	(v)AGDM-50	(w)AGDM-51	(x)AGDM-53
					
(y)AGDM-54	(z)AGDM-55	(a.a)AGDM56	(a.b)AGDM60	(a.c)AGDM61	(a.d)AGDM63

(a.c)AGDM64

(a.d)AGDM65

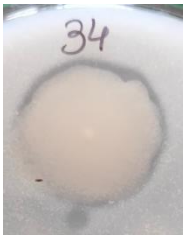
(a.e)AGDM69

(a.f) AGDM70

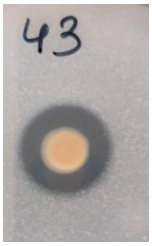
Figure 4.6. Lipase activity of different isolates: isolates from (a) to (a.f)

4.2.5) Protease activity:

Total of 2 isolates from both the sites have shown protease activity (i.e. 1 isolate from fish gut of Site 1 and 1 isolate from fish gut of Site 2). The diameter of the zone of inhibition of isolates were 2.5 cm & 1.8 cm of 34 & 43 respectively. The zone of clearance of all the positive isolates is shown in the below figure 4.7. Positive results shown by AGDM- 34 and AGDM 43.



(a) AGDM- 34



(b) AGDM- 43

Figure 4.7: Protease activity of different isolates: (a); (b)

4.2.6) Phytase activity:

No zones of clearance were observed. No isolate is being able to utilize the phytate present in the media.

4.3) Screening for Pigments from Isolates:

4.3.1) Production of pigments:

The pigments were extracted using methanol and acetone in 2:1 ratio as solvent. Twelve pigment producing isolates showed better pigment extraction from nineteen colored isolates. The remaining produced no pigment. The different shades of extracted pigments are shown in the below figure 4.7. Pigments are given by the isolates (a) AGDM-78; (b) AGDM-75; (c) AGDM-36; (d) AGDM-32b; (e) AGDM-74; (f) AGDM-54; (g) AGDM-69; (h) AGDM-64; (i) AGDM-63; (j) AGDM-5; (k) AGDM-55; (l) AGDM-35.

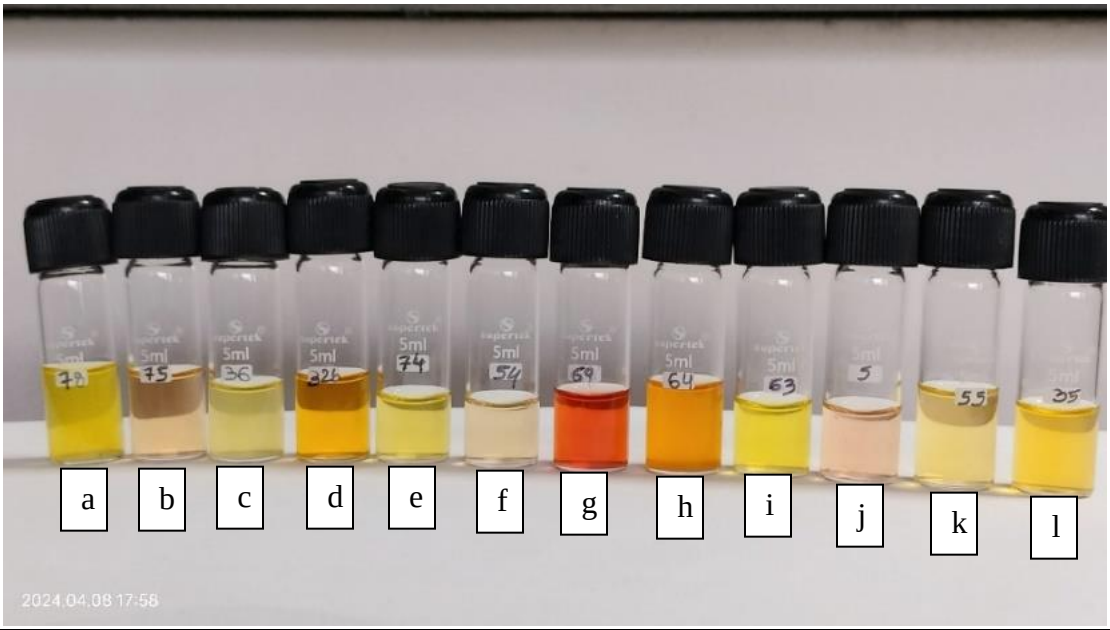


Figure 4.8. Different pigments from bacterial isolates: (a) – (l)

4.3.2) UV- visible spectrophotometric analysis:

Possible pigments were determined using Munsell color system for bacterial colorants. The observed and expected λ max of all the pigments extracted are mentioned below in the table 4.6.

The observed λ max of almost all pigments are falling near expected λ max, this depicts what the possible pigments might be, which have been extracted. Even the graph showing the peaks of all the pigments is shown in different colors in figure 4.9.

Table 4.5: Different pigment producing isolates and their observed and expected λ max values**.**

Sr. no	Media	Isolate	Pigment color	Possible pigment	Observed λ max (nm)	Expected λ max (nm)	Reference
1	Zobell marine broth	AGDM-5	Peach	Astaxanthin	488	472-475	Edge et al., 1997
2		AGDM-32b	Orange	β carotein,	465	450-470	Britton, 1995; Edge et al., 1997
				Flexirubin,		-	
				Canthaxanthin		475	Britton, 1995
3		AGDM-35	Golden yellow	Staphyloxanthin	468	445	Singh et al., 2010
4		AGDM-36	Pale yellow	Carotenoids,	439	400-500	-
				Staphyloxanthin,		445	Singh et al., 2010
				xanthomonadin,		445	Engelhardt et al., 1990
				Zeaxanthin		445	Edge et al., 1997
5		AGDM-74	Yellow	Carotenoids,	468	400-500	-
				Staphyloxanthin,		445	Singh et al., 2010
				xanthomonadin,		445	Engelhardt et al., 1990
				zeaxanthin,		445	Edge et al., 1997
				Riboflavin		445	Edge et al., 1997
6	Nutrient broth	AGDM-54	Cream	Zeaxanthin	400	445	Edge et al., 1997
7		AGDM-55	Pale yellow	Carotenoids,	468	400-500	-
				Staphyloxanthin,		445	Singh et al., 2010
				xanthomonadin,		445	Engelhardt et al., 1990
				Zeaxanthin		445	Edge et al., 1997
8		AGDM-63	Yellow	Carotenoids,	448	400-500	-
				Staphyloxanthin,		445	Singh et al., 2010

				xanthomonadin,		445	Engelhardt et al., 1990
				Zeaxanthin		445	Edge et al., 1997
9		AGDM-64	Orange	Beta carotein,	470	450-470	Britton, 1995; Edge et al., 1997
				Flexirubin,		-	
				Canthaxanthin		475	Britton, 1995
10		AGDM-78	Yellow	Carotenoids,	446	400-500	-
				Staphyloxanthin,		445	Singh et al., 2010
				Zeaxanthin		445	Edge et al., 1997
				xanthomonadin,		445	Engelhardt et al., 1990
11	Tryptic soya broth	AGDM-69	Red	Astaxanthin,	535	472-475	Edge et al., 1997
				Prodigiosin,		535	Williamson et al., 2006
				Canthaxanthin,		475	Britton, 1995
				anthraquinone		-	-
12		AGDM-75	Pale orange	Beta carotein,	473	450-470	Britton, 1995; Edge et al., 1997
				Flexirubin,		-	-
				Canthaxanthin		475	Britton, 1995

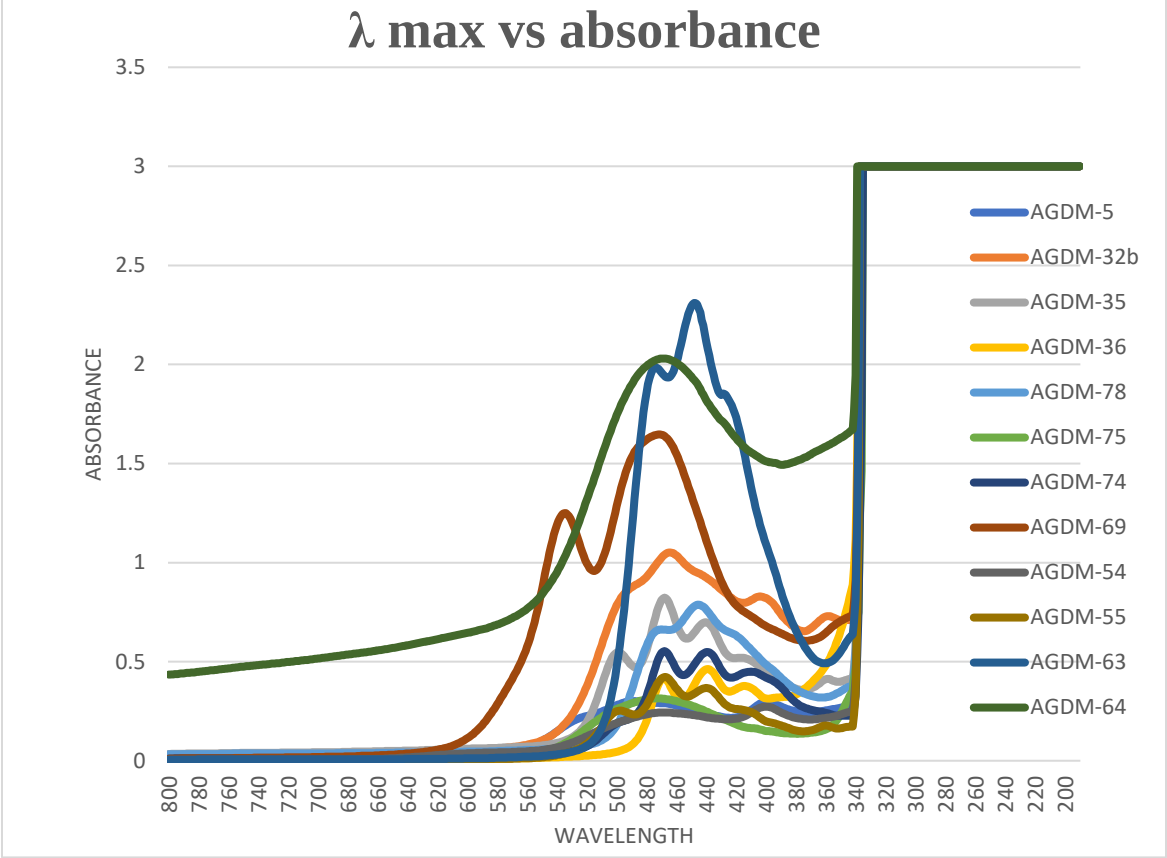


Figure 4.9: Graph showing λ max vs Absorbance graph of all the 12 pigments

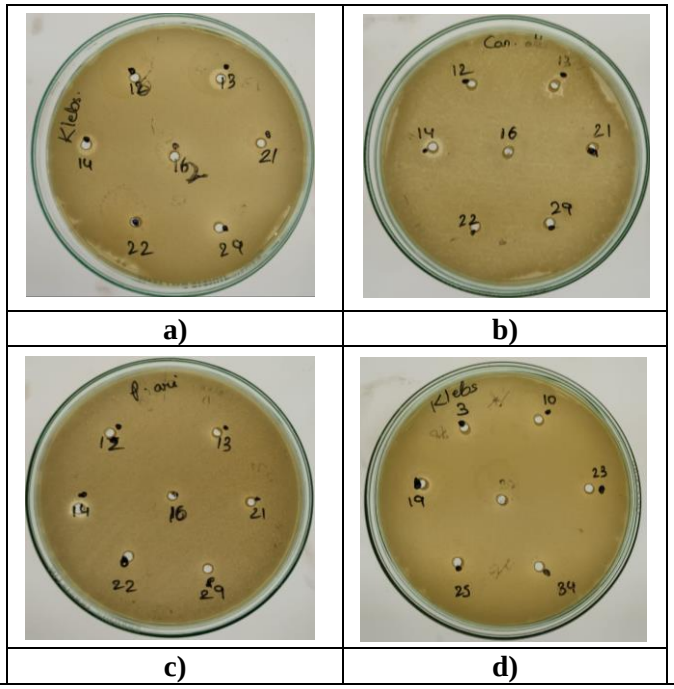
4.4) Screening of Isolates for Antimicrobials:

Only three isolates gave positive results for antimicrobial screening. The zones obtained were slightly bigger than the zone formed due to negative control ethanol. The diameter of zone of negative control on plate with *Klebsiella pneumoniae* was 0.6 cm including the well dimension, zone of negative control on plate with *Candida albicans* was 0.6 cm including the well dimension and zone of negative control on plate with *Pseudomonas aeruginosa* was 0.6 cm including the well dimension.

On MHA plate with *Klebsiella* the diameter of zone of inhibition given by AGDM-13 was 0.8cm, AGDM-14 was 0.75cm and AGDM-19 was 0.6 cm. On MHA plate with *Candida* the diameter of zone of inhibition given by AGDM-13 was 0.8cm (the zone had patches of growth of pathogen) and AGDM-14 was 0.75cm. On MHA plate with *Pseudomonas* the diameter of zone of inhibition given by AGDM- 14 was 0.8 cm.

Table 4.6: Antimicrobial activity on MHA plates

Isolate	**Antimicrobial activity**		
	Klebsiella pneumoniae	***Candida albicans***	***Pseudomonas aeruginosa***
AGDM-13	+	+	_
AGDM-14	+	+	+
AGDM-19	+	_	_



a) **b)**

c) **d)**

Figure 4.10: Antimicrobial activity: Zone of inhibition shown by isolates AGDM-13, AGDM- 14 on plate (a) with *Klebsiella pneumoniae*, Zone of inhibition shown by isolates AGDM-13, AGDM- 14 on plate (b) *with Candida albicans.* Zone of inhibition shown by isolate AGDM-14 on plate (c) with *Pseudomonas aeruginosa.* Zone of inhibition shown by isolate AGDM-19 on plate (d) with *Klebsiella pneumoniae* .

Table 4.7: Summary of Bioactive molecules produced by isolates.

Isolate	Amylase activity	Cellulase Activity	Xylanase activity	Lipase Activity	Protease Activity	pigments	Antimicrobial activity			
							E. coli	*K. pne*	*C. alb*	*P. aer*
AGDM-1	+	+	+	-	-	-	-	-	-	-
AGDM-2	+	+	+	+	-	-	-	-	-	-
AGDM-3	-	-	-	+	-	-	-	-	-	-
AGDM-4	-	-	-	-	-	-	-	-	-	-
AGDM-5	-	-	-	+	-	+	-	-	-	-
AGDM-6	-	-	-	-	-	-	-	-	-	-
AGDM-7	+	+	-	-	-	-	-	-	-	-
AGDM-8	+	-	-	+	-	-	-	-	-	-
AGDM-9	-	-	-	-	-	-	-	-	-	-
AGDM-10	+	+	-	-	-	-	-	-	-	-
AGDM-11	-	-	-	-	-	-	-	-	-	-
AGDM-12	-	-	-	-	-	-	-	-	-	-
AGDM-13	+	+	+	+	-	-	-	+	+	-
AGDM-14	+	+	+	-	-	-	-	+	+	+
AGDM-16	+	-	-	-	-	-	-	-	-	-
AGDM-18	-	-	-	-	-	-	-	-	-	-
AGDM-19	-	-	-	-	-	-	-	+	-	-
AGDM-20	-	-	-	-	-	-	-	-	-	-
AGDM-21	-	-	-	+	-	-	-	-	-	-
AGDM-22	-	-	-	-	-	-	-	-	-	-
AGDM-23	-	-	-	+	-	-	-	-	-	-
AGDM-24	+	+	+	+	-	-	-	-	-	-
AGDM-25	+	+	+	+	-	-	-	-	-	-
AGDM-26	-	-	-	-	-	-	-	-	-	-
AGDM-27	-	-	-	-	-	-	-	-	-	-
AGDM-29	-	-	-	-	-	-	-	-	-	-
AGDM-30	-	-	-	+	-	-	-	-	-	-
AGDM-31	-	-	-	-	-	-	-	-	-	-
AGDM-32a	-	-	-	+	-	-	-	-	-	+
AGDM-32b	+	-	-	+	-	+	-	-	-	-
AGDM-33	+	-	-	-	-	-	-	-	-	-
AGDM-34	+	+	-	+	+	-	-	-	-	-
AGDM-35	-	-	-	-	-	+	-	-	-	-
AGDM-36	-	-	-	-	-	+	-	-	-	-

AGDM-37	-	-	-	+	-	-	-	-	-	-
AGDM-39	-	-	-	+	-	-	-	-	-	-
AGDM-40	+	-	-	+	-	-	-	-	-	-
AGDM-42	+	-	-	-	-	-	-	-	-	-
AGDM-43	-	-	-	+	+	-	-	-	-	-
AGDM-44	-	-	-	-	-	-	-	-	-	-
AGDM-45	-	-	+	+	-	-	-	-	-	-
AGDM-46	-	+	-	-	-	-	-	-	-	-
AGDM-47	-	-	-	-	-	-	-	-	-	-
AGDM-48	+	-	-	+	-	-	-	-	-	-
AGDM-49	+	+	-	+	-	-	-	-	-	-
AGDM-50	-	-	-	+	-	-	-	-	-	-
AGDM-51	-	-	-	+	-	-	-	-	-	-
AGDM-52	-	-	-	-	-	-	-	-	-	-
AGDM-53	-	-	-	+	-	-	-	-	-	-
AGDM-54	-	-	-	+	-	+	-	-	-	-
AGDM-55	-	-	+	+	-	+	-	-	-	-
AGDM-56	-	-	-	+	-	-	-	-	-	-
AGDM-57	-	+	-	-	-	-	-	-	-	-
AGDM-59	-	-	-	-	-	-	-	-	-	-
AGDM-60	-	-	-	+	-	-	-	-	-	-
AGDM-61	-	-	-	+	-	-	-	-	-	-
AGDM-62	-	-	-	-	-	-	-	-	-	-
AGDM-63	-	+	-	+	-	+	-	-	-	-
AGDM-64	-	-	-	+	-	+	-	-	-	-
AGDM-65	-	-	-	+	-	-	-	-	-	-
AGDM-66	-	-	-	-	-	-	-	-	-	-
AGDM-67	-	-	-	-	-	-	-	-	-	-
AGDM-68	-	-	+	-	-	-	-	-	-	+
AGDM-69	-	-	-	+	-	+	-	-	-	-
AGDM-70	-	-	-	+	-	-	-	-	-	-
AGDM-71	-	-	-	-	-	-	-	-	-	-
AGDM-72	-	-	-	-	-	-	-	-	-	-
AGDM-73	-	-	-	-	-	-	-	-	-	-
AGDM-74	-	+	-	-	-	+	-	-	-	-
AGDM-75	-	-	-	-	-	+	-	-	-	-
AGDM-76	-	-	-	-	-	-	-	-	-	-

AGDM-77	-	-	-	-	-	-	-	-	-	-
AGDM-78	-	+	-	-	-	+	-	-	-	-

4.5) <u>Biochemical tests:</u>

Biochemical tests of those cultures which gave positive results for different bioactive molecules were carried out. The biochemical tests performed were catalase, oxidase, motility, indole, methyl red, Voges Proskauer and citrate utilization tests. The data obtained is as shown in the table 4.8.

Table 4.8: Biochemical characteristics of selected isolates

Culture	cat	Oxi	Mot	Ind	MR	VP	Cit	culture	cat	Oxi	mot	Ind	MR	VP	Cit
AGDM-1	+	+	+	-	+	-	-	**AGDM-36**	+	+	-	-	-	-	-
AGDM-2	-	+	-	-	+	-	-	**AGDM-40**	+	+	+	-	-	-	-
AGDM-3	+	+	+	-	-	+	-	**AGDM-43**	+	+	+	-	+	-	-
AGDM-5	+	-	-	-	+	-	-	**AGDM-49**	-	+	-	-	-	-	-
AGDM-8	+	+	+	-	-	-	+	**AGDM-54**	+	-	-	-	-	-	-
AGDM-10	+	+	+	-	+	-	-	**AGDM-55**	+	-	-	-	+	+	-
AGDM-12	+	-	+	-	+	-	-	**AGDM-56**	-	+	-	-	+	-	-
AGDM-13	+	+	+	-	+	-	-	**AGDM-57**	+	-	-	+	+	-	+
AGDM-23	+	-	+	-	-	-	+	**AGDM-63**	+	-	+	-	-	-	-
AGDM-24	-	+	+	-	+	-	-	**AGDM-64**	+	+	-	-	+	+	-
AGDM-25	-	+	-	-	+	-	-	**AGDM-69**	+	-	-	-	+	-	-
AGDM-32a	+	-	+	-	-	-	-	**AGDM-70**	+	-	-	+	+	+	+
AGDM-32b	+	-	+	-	+	-	-	**AGDM-71**	+	-	+	+	+	+	+
AGDM-34	-	+	+	-	+	-	-	**AGDM-74**	-	-	-	-	-	-	-
AGDM-35	+	-	-	-	-	-	-	**AGDM-75**	+	-	+	-	+	-	-
								AGDM-78	+	-	-	-	+	+	-

SUMMARY

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CONCLUSION

Goa is a state situated in coastal region of India. Grey Mullet is state fish of Goa, locally called as ‘Shevto’. Grey mullet being able to thrive in different kind of waters harbors multiple kinds of microorganisms in different parts of the body especially gut. There is a study conducted in Goa where they tried to screen just enzymes as bioactive molecules from gut of grey mullet.

The present dissertation thesis focuses on the few various bioactive molecules produced from bacteria isolated from fish gut of grey mullet. The result and conclusion are that the fish gut is a rich source of microflora capable of producing various Bioactive molecules. These fish gut bacteria are capable of producing various industrially important enzymes such as amylase, cellulase, xylanase, protease, phytase and lipase reported in this study. The isolated organisms also produced pigments. Antimicrobial assays have revealed the potential of the isolates to serve as anti- microbial agents.

AGDM-13, AGDM-14, AGDM-24, AGDM-25, AGDM-34 and AGDM-57 has produced the greatest number of Bioactive molecules.

FUTURE PROSPECTS

The following suggestions can be investigated in the future:

- The 16s rDNA sequencing of the isolates can be carried out.
- The quantitative assay of the enzymes can be carried out.
- The applications of the Bioactive molecules can be done.
- The unculturable approach can be carried out by performing whole genome sequencing of the gut bacteria.
- The pigments extracted can be identified by HPLC.
- SEM analysis of isolates to identify the bacteria can be done.
- Other fish can be used as a source to isolate bacteria or else different organs from same bacteria can be used as a source to isolate bacteria.

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APPENDIX

Media composition:

1) **Starch- Agar medium:{4}**

Chemicals	**gl^{-1}**
Starch	10 gl^{-1}
KH2PO4	4 gl^{-1}
Na2HPO4	4 gl^{-1}
Tryptone	2 gl^{-1}
MgSO4.7H2O	0.2 gl^{-1}
CaCl2	0.001 gl^{-1}
FeSO4.7H2O	0.004 gl^{-1}
Agar	15 gl^{-1}
pH	7.0 ± 0.2

2) **Carboxymethylcellulose- Agar medium:**

Chemicals	**gl^{-1}**
Starch	10 gl^{-1}
KH2PO4	4 gl^{-1}
Na2HPO4	4 gl^{-1}
Tryptone	2 gl^{-1}
MgSO4.7H2O	0.2 gl^{-1}
CaCl2	0.001 gl^{-1}
FeSO4.7H2O	0.004 gl^{-1}
Agar	15 gl^{-1}

pH	7.0 ± 0.2

3) Tributyrin- Agar medium:

Chemicals	**gl^{-1}**
Tributyrin	10 gl^{-1}
Peptone	5 gl^{-1}
Agar	15 gl^{-1}
pH	7.0 ± 0.2

4) Xylan- Agar medium: {46}

chemicals	**gl^{-1}**
Nutrient agar	28 gl^{-1}
Xylan	2.5 gl^{-1}
pH	7.0 ± 0.2

5) Skimmed milk Agar medium:{57}

Chemicals	**gl^{-1}**
Agar	15 gl^{-1}
Dextrose	1 gl^{-1}
skim milk powder	28 gl^{-1}
Tryptone	5 gl^{-1}
yeast-extract	2.5 gl^{-1}
pH	7.0 ± 0.2

6) Modified Phytase Agar medium:{58}

Chemicals	**gl^{-1}**
Glucose	10 gl^{-1}
$(NH_4)_2 SO_4$	1 gl^{-1}
Urea	10 gl^{-1}
Citric acid	3 gl^{-1}
Sodium citrate	2 gl^{-1}
$MgSO_4. 7H_2O$	1 gl^{-1}
Sodium phytate	3 gl^{-1}
$FeSO_4. 7H_2O$	1 gl^{-1}
Agar	20 gl^{-1}
1M tris buffer	100 ml l^{-1}
Biotin	50 mgl^{-1}
Thiamine	20 mgl^{-1}
	7.0 ± 0.2

Reagents and solutions:

- **Gram's Staining Kit:**
  - Crystal violet (primary stain)
  - Gram's iodine
  - Decolourizer (95% ethanol or 1:1 acetone with ethanol)
  - Safranin
- **Congo red:**
  - 0.5 % of congo red in 5% ethanol
- **Lugol's iodine**
- **Kovac's reagent**
- **Vogues- Proskauer test:**

- **Barrit's A reagent:** 6 g of α- napthol in 95% ethanol
- **Barrit's B reagent:** 40% KOH

- **Methyl red reagent:** 0.1 g of methyl red in 300 ml ethanol (95%). Add 200 ml of distilled water to make it to 500 ml.

Instruments:

- Cooling centrifuge
- UV- Visible spectrophotometer
- Sonicator