

# **PROBIOTICS FOR FISH CULTURE**

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

by

Subhashini R (39230046)



**DEPARTMENT OF BIOTECHNOLOGY  
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

## **SATHYABAMA**

**INSTITUTE OF SCIENCE AND TECHNOLOGY  
(DEEMED TO BE UNIVERSITY)  
Accredited with Grade "A" by NAAC  
JEPPIAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119**

**MAY - 2023**



# SATHYABAMA

INSTITUTE OF SCIENCE AND TECHNOLOGY  
(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119

[www.sathyabama.ac.in](http://www.sathyabama.ac.in)



## DEPARTMENT OF BIOTECHNOLOGY

### BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Subhashini R (39230046)** who carried out the project entitled "**PROBIOTICS FOR FISH CULTURE**" under my supervision from October 2022 to December 2022.

Internal Guide

(Dr.V.RAMESH KUMAR)

External Guide

(Dr.M.MASILAMANI SELVAM)

Head of the Department

(Dr.V.RAMESH KUMAR)

Submitted for Viva voce held on 11/05/2023

Internal Examiner

External Examiner

## DECLARATION

I, SUBHASHINI R (39230046), hereby declare that the Project Report entitled "PROBIOTICS FOR FISH CULTURE" done by me under the guidance of Dr.V.RAMESH KUMAR and Dr.M.MASILAMANI SELVAM at DEPARTMENT OF BIOTECHNOLOGY, SATHYABAMA UNIVERSITY is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 11.05.2023

PLACE: CHENNAI

*R. Subhashini*

SIGNATURE OF THE CANDIDATE

## ACKNOWLEDGEMENT

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

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

I would like to express my sincere and deep sense of gratitude to my project guides **Dr.V.RAMESH KUMAR and Dr.M.MASILAMANI SELVAM** for their valuable guidance, suggestions and constant encouragement paved way for the successful completion of my project work.

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

## **ABSTRACT**

One of the most viable and promising industries for supplying humans with nutrition and food security, especially those living in areas where livestock is relatively scarce, is aquaculture. This is because it can keep up with the growing demand for animal protein. Probiotics have recently gained attention and are being used as an unconventional strategy that has many positive effects in fish and shellfish culture, including improved gastrointestinal microbiota activity, improved immune status, increased disease resistance, increased survival, improved feed utilization, and growth performance. Probiotics are natural items with a lot of promise to improve the productivity and sustainability of aquaculture. Therefore, it is reasonable to do thorough study to thoroughly characterize the intestinal microbiota of well-known fish species, probiotic action mechanisms, and their impacts on the intestinal environment, immunity, fish health, and performance.

In order to refer to species and substances that support gut microbial equilibrium, Parker invented the word "probiotics." Probiotics are live cultures of bacteria and fungi that improve health when they are added to food. Probiotics can be found in many different places, including bacteria (Rengpipat et al. 1998), brown and red algae, terrestrial fungi (Bricknell & Dalmo 2005), and marine yeast (Sajeevan, Philip, & Singh 2006). Gram-negative facultative anaerobes predominate in fish and shellfish, while Gram-positive facultative anaerobes are frequently found in the gastrointestinal tracts of terrestrial mammals. The maximum averages for the traits maximum growth velocity, final viable cell count, and inhibition halo against nine freshwater and marine pathogens, as well as the lowest averages for the traits duplication time and strain resistance to NaCl (1.5 and 3%), pH (6, 8, and 9), and biliary salts (5%), were used to define the ideotype (ideal proposed strain).

The Food and Agriculture Organization (FAO) claims that infections in the fish farming industry are a major problem that lowers output rates and ultimately has an impact on the national economy. The most prevalent illnesses in freshwater fish are those caused by the bacteria *Aeromonas hydrophila* and *Aeromonas salmonicida*. A variety of bacterial, fungal, parasitic, and viral illnesses can affect fish at any time. Additionally, bad water quality, excessive fish stocking densities, and overfeeding

make fish more susceptible to disease. Improvements in feed value, enzymatic digestion assistance, pathogenic microorganism inhibition, anti-mutagenic and anti-carcinogenic action, as well as an increase in immunological response are all advantages of such supplements. These probiotics are beneficial bacteria that aid the host animal and serve to defend it against harmful diseases, either directly or indirectly.

According to the current study, many isolates from conventional sources as well as soil-isolated strains are used as probiotics. Additionally, a few isolated strains of soil demonstrated antibacterial and antimicrobial properties against a few microorganisms. Additionally, a deeper comprehension of these bacterial groups' physiological characteristics and impacts on the host in vivo, as well as their detection and enumeration in a variety of foods and biological materials.

**Keywords:** aquaculture, probiotics, bacteria, physiological characteristics, in vivo studies, pathogens, fish culture

## TABLE OF CONTENTS

|                                  |     |
|----------------------------------|-----|
| ABSTRACT                         | IV  |
| LIST OF CONTENTS                 | VI  |
| LIST OF FIGURES                  | XI  |
| LIST OF TABLES                   | XII |
| LIST OF SYMBOLS AND ABBREVIATION | XIV |

### LIST OF CONTENTS

| SERIAL NUMBER | TOPIC                                  | PAGE NUMBER |
|---------------|----------------------------------------|-------------|
| 1             | INTRODUCTION                           | 1           |
| 1.1           | PROBIOTICS                             | 1           |
| 1.1.1         | HISTORY OF PROBIOTICS                  | 2           |
| 1.2           | CHARACTERIZATION OF PROBIOTICS         | 3           |
| 1.2.1         | PROBIOTIC FOODS                        | 3           |
| 1.3           | APPLICATION OF PROBIOTICS              | 4           |
| 1.3.1         | FOOD APPLICATION OF PROBIOTICS         | 4           |
| 1.3.1.1       | DAIRY-BASED PROBIOTIC FOODS            | 4           |
| 1.3.1.2       | NON-DAIRY BASED PROBIOTIC PRODUCT      | 5           |
| 1.3.2         | AGRICULTURAL APPLICATION OF PROBIOTICS | 5           |
| 1.3.2.1       | ANIMALS                                | 5           |

|          |                                             |           |
|----------|---------------------------------------------|-----------|
| 1.3.2.2  | PLANTS                                      | 5         |
| 1.3.3    | APPLICATION OF PROBIOTICS ON<br>AQUACULTURE | 6         |
| 1.4      | HEALTH BENEFITS OF PROBIOTICS               | 6         |
| <b>2</b> | <b>REVIEW OF LITERATURE</b>                 | <b>8</b>  |
| <b>3</b> | <b>AIM AND OBJECTIVE</b>                    | <b>13</b> |
| 3.1      | AIM                                         | 13        |
| 3.2      | OBJECTIVE                                   | 13        |
| <b>4</b> | <b>MATERIALS AND METHODOLOGY</b>            | <b>14</b> |
| 4.1      | MATERIALS                                   | 14        |
| 4.1.1    | BACTERIAL ISOLATES                          | 14        |
| 4.1.2    | CHEMICALS                                   | 14        |
| 4.1.3    | EQUIPMENTS                                  | 14        |
| 4.2      | METHODOLOGY                                 | 14        |
| 4.2.1    | SAMPLE COLLECTION                           | 14        |
| 4.2.2    | ISOLATION OF BACTERIA                       | 14        |

|           |                                             |    |
|-----------|---------------------------------------------|----|
| 4.2.2.1   | SERIAL DILUTION                             | 15 |
| 4.2.2.2   | POUR PLATE METHOD                           | 15 |
| 4.2.2.3   | ISOLATION OF BACTERIA FROM<br>SOIL SAMPLE   | 16 |
| 4.2.3     | CHARACTERISATION OF<br>BACTERIAL COLONIES   | 16 |
| 4.2.3.1   | CARBOHYDRATE (LACTOES)<br>FERMENTATION TEST | 16 |
| 4.2.3.2   | INDOLE ACETIC ACID PRODUCTION<br>TEST       | 17 |
| 4.2.3.3   | ANTIMICROBIAL ACTIVITY TEST                 | 18 |
| 4.2.3.4   | ANTIBIOTIC SUSCEPTIBILITY TEST              | 18 |
| 4.2.3.5   | SIDEROPHORE PRODUCTION TEST                 | 18 |
| 4.2.4     | IN VIVO TESTING                             | 19 |
| 4.2.4.1   | ANALYSIS OF PHYSIO-CHEMICAL<br>PARAMETERS   | 20 |
| 4.2.4.1.1 | TEMPERATURE                                 | 20 |
| 4.2.4.1.2 | pH                                          | 20 |
| 4.2.4.1.3 | DETERMINATION OF AMMONIA<br>LEVEL           | 20 |
| 4.2.4.1.4 | DETERMINATION OF DISSOLVED<br>OXYGEN LEVEL  | 21 |
| 4.2.4.2   | FISH GROWTH ANALYSIS                        | 21 |

|           |                                        |    |
|-----------|----------------------------------------|----|
| 4.2.4.2.1 | LENGTH                                 | 22 |
| 4.2.4.2.2 | WEIGHT                                 | 22 |
| 4.2.4.3   | MICROBIAL ANALYSIS                     | 22 |
| 4.2.5     | IDENTIFICATION OF THE BACTERIAL STRAIN | 22 |
| 4.2.5     | PRODUCT DEVELOPMENT                    | 24 |
| 4.2.6.1   | POWDER FORM                            | 25 |
| 4.2.6.1   | LIQUID FORM                            | 25 |
| <b>5</b>  | <b>RESULTS AND DISCUSSION</b>          | 26 |
| 5.1       | RESULTS                                | 26 |
| 5.1.1     | ISOLATION OF MICROBES FROM SOIL SAMPL  | 26 |
| 5.1.2     | CHARACTERIZATION OF BACTERIAL COLONIES | 27 |
| 5.1.2.1   | CARBOHYDRATE FERMENTATION TEST         | 27 |
| 5.1.2.2   | ANTIMICROBIAL ACTIVITY TEST            | 28 |
| 5.1.2.3   | ANTIBIOTIC SUSCEPTIBILITY TEST         | 29 |
| 5.1.2.4   | SIDEROPHORE PRODUCTION TEST            | 29 |
| 5.1.3     | IN VIVO TESTING                        | 30 |

|           |                                         |           |
|-----------|-----------------------------------------|-----------|
| 5.1.3.1   | ANALYSIS OF PHYSIO-CHEMICAL PARAMETERS  | 31        |
| 5.1.3.1.1 | TEMPERATURE                             | 31        |
| 5.1.3.1.2 | pH                                      | 32        |
| 5.1.3.1.3 | DETERMINATION OF AMMONIA LEVEL          | 33        |
| 5.1.3.1.4 | DETERMINATION OF DISSOLVED OXYGEN LEVEL | 34        |
| 5.1.3.2   | FISH GROWTH ANALYSIS                    | 34        |
| 5.1.3.2.1 | LENGHT                                  | 35        |
| 5.1.3.2.2 | WEIGHT                                  | 35        |
| 5.1.4     | IDENTIFICATION OF THE BACTERIAL STRAIN  | 36        |
| 5.1.5     | PRODUCT DEVELOPMENT                     | 40        |
| 5.1.5.1   | POWDER FORM                             | 40        |
| 5.1.5.2   | LIQUID FORM                             | 40        |
| 5.2       | DISCUSSION                              | 41        |
| <b>6</b>  | <b>SUMMARY AND CONCLUSION</b>           | <b>43</b> |
| <b>7</b>  | <b>REFERENCE</b>                        | <b>44</b> |

## LIST OF FIGURES

| SERIAL NUMBER | TOPIC                                                                | PAGE NUMBER |
|---------------|----------------------------------------------------------------------|-------------|
| 4.1           | SERIAL DILUTION METHOD                                               | 15          |
| 4.2           | POUR PLATE METHOD                                                    | 15          |
| 4.3           | SAMPLE COLLECTION                                                    | 16          |
| 4.4           | BLACK TELESCOPE GOLDFISH, KOI FISH, GOLD FISH AAND IRIDESCENT SHARKS | 20          |
| 5.1           | ISOLATION OF BACTERIA                                                | 26          |
| 5.2           | ISOLATION OF PURE BACTERIAL COLONIES                                 | 26          |
| 5.3           | CARBOHYDRATE FERMENTATION TEST                                       | 27          |
| 5.4           | SAMPLE-1 CARBOHYDRATE FERMENTATION TEST                              | 27          |
| 5.5           | SAMPLE-2 CARBOHYDRATE FERMENTSTION TEST                              | 28          |
| 5.6           | ANTIMICROBIAL ACTIVITY TEST OF ESCHERICHA COLI                       | 28          |
| 5.7           | ANTIBIOTIC SUSCEPTIBILITY TEST                                       | 29          |
| 5.8           | SIDEROPHORE PRODUCTION TEST                                          | 30          |
| 5.9           | FISH TANK                                                            | 31          |
| 5.10          | TEMPERATURE (IN °C) ANALYSIS IN FISH TANK                            | 32          |

|      |                          |    |
|------|--------------------------|----|
| 5.11 | Ph ANALYSIS IN FISH TANK | 33 |
| 5.12 | POWDER FORM              | 40 |
| 5.13 | LIQUID FORM              | 41 |

## LIST OF TABLES

| SERIAL NUMBER | TOPIC                                               | PAGE NUMBER |
|---------------|-----------------------------------------------------|-------------|
| 5.1           | ZONE OF INHIBITION                                  | 29          |
| 5.2           | SIDEROPHORE PRODUCTION TEST                         | 30          |
| 5.3           | TEMPERATURE IN CONTROL TANK                         | 31          |
| 5.4           | TEMPERATURE IN SAMPLE TANK                          | 31          |
| 5.5           | PH IN CONTROL TANK                                  | 32          |
| 5.6           | PH IN SAMPLE TANK                                   | 32          |
| 5.7           | AMMONIA LEVEL IN CONTROL TANK                       | 33          |
| 5.8           | AMMONIA LEVEL IN SAMPLE TANK                        | 33          |
| 5.9           | DISSOLVED OXYGEN LEVEL IN CONTROL TANK              | 34          |
| 5.10          | DISSOLVED OXYGEN LEVEL IN SAMPLE TANK               | 34          |
| 5.11          | AVERAGE LENGTH OF FISHES IN CONTROL AND SAMPLE TANK | 35          |
| 5.12          | AVERAGE WEIGHT OF FISHES IN CONTROL AND SAMPLE TANK | 35          |
| 5.13          | LIST OF IDENTIFIED MICROBIAL SPECIES                | 39          |

## LIST OF SYMBOLS AND ABBREVIATION

| SYMBOL        | ABBREVIATION                                   |
|---------------|------------------------------------------------|
| LAB           | Lactic Acid Bacteria                           |
| GI            | Gastrointestinal                               |
| &             | And                                            |
| MH Media      | Muller Hinton media                            |
| Spp           | Species                                        |
| L. Subspecies | <i>Lactobacilli</i>                            |
| B. Subspecies | <i>Bifidobacterium</i>                         |
| CAS           | Chrome Azurol S                                |
| pH            | Power of Hydrogen                              |
| μl            | Micro liter                                    |
| IAA           | Indole Acetic Acid                             |
| TOS-MUP       | Transgalctosylated oligosaccharide agar medium |
| E. Subspecies | <i>Enterococcus</i>                            |

|               |                           |
|---------------|---------------------------|
| S. Subspecies | <i>Saccharomyces</i>      |
| MRS           | De Man Rogosa Sharpe      |
| LB            | Luria-Bertan              |
| TPC           | Total Plate Count         |
| CFU           | Colony Forming Uni        |
| PCR           | Polymerase Chain Reaction |
| DNA           | Deoxyribonucleic Acid     |
| RNA           | Ribonucleic Acid          |
| S subunit     | Svedberg subunit          |
| Cm            | Centimeter                |
| DO            | Dissolved Oxygen          |
| Ppm           | Parts Per Million         |
| %             | Percentage                |
| Gms           | Grams                     |
| °C            | Degree Celsius            |
| Sec           | Seconds                   |

|       |                                                                        |
|-------|------------------------------------------------------------------------|
| Min   | Minute                                                                 |
| Hrs   | Hours                                                                  |
| ml    | Milliliter                                                             |
| NIDDK | National Institute of<br>Diabetes and Digestive and<br>Kidney Diseases |

**1.1 PROBIOTICS**

The term "probiotic" was originally defined as "Organisms and Substances that Contribute to Intestinal Microbial Balance." The wording was then altered to read "live microbial feed supplement that benefits the host animal by enhancing the balance of its intestinal microbes." Aquatic animals interact with their environment far more closely than terrestrial creatures do, and as a result, both their environment and nutrition have a substantial impact on their microbiological health. Therefore, a probiotic for aquatic environments should be viewed as a live microbial supplement that improves the host through altering the microbial community that is either associated with the host or ambient. Recent research defined probiotic as a live, dead, or component of a microbial cell that benefits the host by enhancing disease resistance, growth performance, feed consumption, and health status through the accomplishment of microbial balance in both the host and ambient environments. Probiotics may work in aquaculture in a variety of ways, including (i) as growth promoters, (ii) by producing inhibitory chemicals, (iii) by improving nutrient digestion, (iv) by improving water quality, (v) by enhancing immunological response, and (vi) by competing for nutrients. The bacteria must meet a variety of biosafety and functioning requirements in order to be considered probiotics. A suitable probiotic should have the following qualities: (i) not harming to the host; (ii) ability to survive while being transported to the active site; (iii) capacity for colonising and proliferating within the host; and (iv) absence of virulence or antibiotic resistance genes. The yeast *Saccharomyces cerevisiae* and the bacteria *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bacillus* sp., *Bifidobacterium bifidum*, *Lactococcus lactis* are frequently used as probiotics. Even though Actinobacteria, particularly the genus, are widely recognised as prolific producers of secondary metabolites, little focus has been paid to their use as probiotics in aquaculture. Probiotics from the genus *Streptomyces* have shown good benefits. The purpose of this review is to discuss the potential for employing *Streptomyces* in aquaculture as a probiotic option.

### **1.1.1 HISTORY OF PROBIOTICS**

German chemist Werner Kollath coined the term "probiotic" in 1953 to describe "active substances that are essential for a healthy development of life." It comes from the latin pro and the Greek letter "o," which literally means "for life." According to Lilly and Stillwell in 1965, this phrase was used to refer to "substances secreted by one organism which stimulate the growth of another." Probiotics were more clearly described by Fuller in 1992 as "a live microbial feed supplement that benefits the host animal by enhancing its intestinal microbial balance."

The modern history of probiotics started at the beginning of 1900s with the pioneering investigations of the future Nobel winner Elie Metchnikoff, a Russian chemist working at the Pasteur Institute in Paris. Metchnikoff originally tried to determine whether these germs would have any impact on human health before Louis Pasteur identified the microorganisms in charge of the fermentation process. He connected regular use of fermented dairy products like yoghurt to the longer life expectancy of rural Bulgarians. He connected this to the Bulgarian bacillus, which was found by Stamen Grigorov, a 27-year-old doctor from Bulgaria, and he later proposed that lactobacilli would combat the putrefactive effects of gastrointestinal metabolism that aided in sickness and ageing. Hippocrates, who lived 2000 years ago, also said that "bad digestion is the root of all evil" and that "death sits in the bowels." Although they are now known as proteolytic clostridia, he referred to these bacteria as putrefying bacteria. Metchnikoff also noted that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes." The "probiotic concept" is clearly explained in this statement. Metchnikoff thought of the lactobacilli as probiotics since they may improve health and delay ageing. In accordance with Metchnikoff's scientific theory, the usage of fermented milk derived from *Bacillus bulgaricus* contributed to the establishment and growth of France's dairy sector, the first in all of Europe. Probiotics are live bacteria that, when given in sufficient quantities, boost the host's health. In 2013, an expert consensus paper on the topic was produced. Additionally, modern science has prioritised breeds that provide fermented milk with superior organoleptic and nutritional

properties. According to a consensus document, yoghurt and other products created from fermented milk can be regarded as the first functional foods.

## **1.2 CHARACTERIZATION OF PROBIOTICS**

Numerous probiotics are culturable microbiota elements that have been utilised for their beneficial properties even before the name "probiotic" was created. The most widely utilised probiotic strains are lactic acid bacteria (LAB), Gram-positive microorganisms that have been employed for centuries in the production of foods including yogurt, cheese, and pickles. In the human ileum, jejunum, and colon at more modest densities, members of the LAB include *Lactococcus* and *Streptococcus* play significant roles in the endogenous microbiota. Many LAB are suitable for usage as probiotics since they have been given permission to be used in food, they don't contain lipopolysaccharides (LPS), and they don't release any proteases. Recombinant proteins can also be secreted by these Gram-positive organisms without becoming entrapped in the periplasm, which makes LAB species suitable manufacturing platforms for proteins and enzymes that are safe for consumption.

In order to distribute cytokines, *Lactococcus lactis* is the most popular LAB species. Because it is only momentarily present in the human gut, unlike other *Lactococcus* species, this bacteria is categorised as a noncommensal. This probiotic strain can be used as a delivery system for DNA and, more recently, protein vaccines thanks to the advantage of its brief colonisation. A prolonged colonisation with a strain in these circumstances would be undesirable since it might result in overstimulation of the intended route. The capacity to transport proteins directly to the mucosa creates new therapeutic treatment options where conventional drug routes fall short, for instance, when the therapeutic substance is not readily available in the area.

### **1.2.1 PROBIOTIC FOODS**

On March 11, 2020, the World Health Organization (WHO) proclaimed COVID-19, a virus-infected illness brought on by the SARS-CoV-2, a global pandemic. This virus doesn't yet have a cure or effective treatments. Alternative tactics that concentrate on pathophysiological pathways need to

be developed because there isn't a known and effective treatment for COVID-19. One such strategy is the creation of probiotic-infused meals in the future. Traditional fermented meals that include probiotic bacteria have a great chance of preventing COVID-19. In healthy newborns, kids, adults, and seniors, probiotic fermented milk consumption has been shown to prevent upper respiratory tract infections. Consuming *Lactobacillus casei shirota* (LcS), a popular strain used in Yakult, decreased plasma cytomegalovirus and epstein-barr virus antibody titres in extremely active individuals. The impact of healthy volunteers consuming *Lactobacillus rhamnosus* GG for six months on rhinovirus resistance was assessed. It was found that rhinovirus-infected participants had lower cold frequency, infection rates, and cold severity than the control group.

### **1.3 APPLICATION OF PROBIOTICS**

#### **1.3.1 FOOD APPLICATION OF PROBIOTICS**

The development of foods with health advantages beyond basic nutrition has been facilitated by a growing understanding of functional foods. Consumer interest in functional foods, especially those that include probiotics, has grown over the past 20 years. The inclusion of probiotics in processed foods has been linked to a number of health advantages. As a result, businesses are concentrating on various ways to incorporate probiotics into food products and developing a new line of "probiotic health" foods.

##### **1.3.1.1 DAIRY-BASED PROBIOTIC FOODS**

Due to its natural characteristics and the fact that the majority of milk and milk products are maintained at refrigeration temperatures, milk and its products make effective probiotic strain delivery vehicles. Probiotics can be found in a wide range of commercial dairy goods, such as fresh and sour milk, yogurt, cheese, etc. Since dairy products offer probiotic bacteria a good habitat for growth and survival, they play a significant role in providing probiotic bacteria to humans. When introducing probiotics to dairy products, a number of difficulties must be taken into consideration, including the viability of the probiotics in the dairy, the physical, chemical, and organoleptic aspects of the

finished goods, the probiotic's effects on health, and legal and labelling issues.

#### **1.3.1.2 NON-DAIRY BASED PROBIOTIC PRODUCT**

Dairy products are the primary sources of probiotic bacteria for humans since they offer the organisms a favourable environment for growth and survival. However, there is a demand for vegetarian probiotic products as consumer vegetarianism spreads throughout affluent nations. Vegetarians and consumers with lactose sensitivity have expressed a strong interest in nondairy probiotic products. The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) of the United States National Institutes of Health estimates that roughly 75% of people worldwide are lactose intolerant. It is quite difficult to create new non-dairy probiotic food products that satisfy customer expectations for health benefits. Granato and others provide an outline of the evolution of functional foods, focusing on non-dairy items that include probiotic bacteria strains.

#### **1.3.2 AGRICULTURAL APPLICATION OF PROBIOTICS**

Probiotics now have a wide range of agricultural applications in addition to their human uses. Animals and plants are used in agriculture.

##### **1.3.2.1 ANIMALS**

In terms of animal uses, probiotics are live microbial feed supplements that help the host animal's gut flora balance [26]. They have been given the go-ahead to offer numerous advantages to the host animal and the creation of animal goods. They are applied in chicken, ruminant, pig, and aquaculture as examples of how they are utilised as animal feed to enhance animal health and food safety.

##### **1.3.2.2 PLANTS**

Beneficial microorganisms that live on plants can interact directly with them, promoting plant growth, or they can indirectly inhibit plant pathogen growth by acting as antagonists. Model organisms for promoting plant development and thwarting plant diseases include the bacterial genera *Azospirillum*,

Rhizobium, Serratia, Bacillus, Pseudomonas, Stenotrophomonas, and Streptomyces as well as the fungi genera Ampelomyces, Coniothyrium, and Trichoderma.

### **1.3.3 APPLICATION OF PROBIOTICS ON AQUACULTURE**

Aquaculture uses for probiotics typically depend on their capacity to bind to intestinal mucus and produce antibacterial metabolites. Common fish pathogens include *Aeromonas hydrophila* and *Vibrio alginolyticus*, however adding probiotic strains (derived from the clownfish, *Amphiprion percula*) was found to be effective in preventing these microorganisms from adhering to fish intestinal mucus and in competing with the pathogens. Shrimp suffering from *Vibrio parahaemolyticus* sickness can be decreased by feeding them probiotics, according to research. The use of probiotics for the prevention of bacterial infections in aquaculture has been examined by Balcazar and others.

### **1.4 HEALTH BENEFITS OF PROBIOTICS**

The compatibility of various species has been a criterion for choosing probiotics. According to the general principle, an isolated probiotic from one animal does less well in another. Probiotics are often used between species since many of them, however, have an unknown origin. The ability to survive transit through the gastrointestinal tract, adhesion to the intestinal epithelial cell lining, production of antimicrobial substances against pathogens, capacity to stabilise the intestinal microflora, antigenotoxic properties, a short generation time, a good shelf life in food or powdered preparations, and non-pathogenic properties are some of the other main selection criteria that have been applied for probiotics. A putative probiotic's capacity to endure in an environment with a low pH level and in the presence of bile have both been utilised as screening criteria for the ability to survive in the gastrointestinal system. Based on historical data demonstrating a lack of known medical issues for the genera to which that probiotic belongs, many commonly utilised probiotics have been believed to be safe. *Lactobacillus* and *Bifidobacterium* have served as examples in the past. It is always a problem that should be taken into account when choosing probiotics that there can be species

differences in pathogenicity. Studies employing acceptable amounts in animal feeding can be useful in this area. In contrast, there are some microbes that have been utilised for a long time as food additives and over-the-counter health products.

- Evaluation of beneficial bacteria from mangrove soil. This study examines a few bacteria that can potentially aid in the growth of mangrove seedlings. They were discovered on the roots of mangrove trees. A type of mangrove called *Rhizophora mucronata* had 48 different bacterial strains in its rhizosphere soil. We examined the strains' capacities to fix nitrogen, solubilize phosphate, create ammonia, and synthesise the plant growth hormone (IAA). We also examined the impact of the strains on the growth of *R. mucronata* shoots. *Azotobacter vinelandii* and *Bacillus megaterium* were found to be the two strains that caused mangrove seedling growth to double. The idea of enhancing the growth of mangrove seedlings by utilising the bacterial species is now more likely. Kandasamy Kathiresan *et al.*, (2006)
- Marine Actinomycetes, New Sources of Biotechnological Products. The Actinomycetales order has a high degree of genetic and functional variety, as well as variation in the production of secondary metabolites, which have benefits in industrial, medicinal, and environmental restoration. Actinomycete species' secondary metabolites are a rich source of antibiotics, anticancer drugs, anthelmintics, and antifungals. Actinomycetes are being researched as potential sources of novel chemicals to fight multidrug resistance in pathogenic bacteria in addition to the actinomycete-derived therapies that are now in use. Another area of recent research focuses on the potential of actinomycetes to address environmental issues, particularly their use in the bioremediation of pesticides, toxic metals, radioactive wastes, and biofouling. Sveta V *et al.*, (2021)
- Isolation and identification of bioluminescent photobacterium *Photobacterium leiognathi* from marine squid by genomic dna and restriction site analysis. The bioluminescent bacteria *Photobacterium leiognathi* was isolated and identified in the current work from marine squid by biochemical analysis. A single luminous bacterium and nine non-luminescent isolates were both found in the 30 samples of marine squid that were collected. Genomic DNA and restriction site analyses of the isolated bacteria *Photobacterium leiognathi* were performed, and the results were interpreted. Vishnu Kiran

Manam *et al.*, (2022).

- **A Review of Probiotics in Shrimp Aquaculture.**Environmental deterioration and a rise in infectious illnesses have been linked to the development of prawn farming.Giving probiotics to the raising system is an efficient substitute for the chemicals and antibiotics used to prevent these issues. In prawn aquaculture, three bacterial genera—*Bacillus*, *Vibrio*, and *Pseudomonas*—are frequently given as probiotics. Candidate probiotics must undergo both in vitro and in vivo testing to determine whether they are effective for a certain species. In comparison to direct application into animal rearing systems, supplementing probiotics into feed is more effective at delivering them to animals. Immunosuppression can be brought on by probiotic overdose or extended use. It is generally advised to maintain a cell density of 10<sup>5</sup> colony-forming units (CFU) per millilitre. The host benefits more from a probiotic combination than from a single probiotic. Probiotics reduce harmful bacteria while enhancing water quality. Shrimps' improved physiological and immunological responses demonstrate the beneficial effects of probiotics. Probiotics are becoming more prevalent and significant in all organic prawn farming. NGO VAN HAI *et al.*, (2015)
- **Health benefits of probiotics.** This essay examines the evidence supporting the usage of probiotics as a means of improving one's health. The types of probiotics now being utilized, their historical background, and the selection criteria for probiotics are all covered. Probiotics can increase the nutritional value and bioavailability of nutrients, and there is scientific evidence to support their value in reducing lactose intolerance symptoms and promoting growth development. The remainder of the review focuses on research on *Lactobacillus GG*, a unique probiotic that has been thoroughly examined for its potential health advantages in both humans and animals. These research provide as an example of the probiotics' possible advantages. It is discussed how *Lactobacillus GG* can treat or prevent diarrheal illness, act as a vaccine adjuvant, stop rotavirus-induced diarrhea, stop milk-based allergy reactions, prevent alcohol-induced liver damage, and prevent colon cancer. The review is concluded with a discussion of the evidence for probiotics' safety. Barry R. Goldin. (1998).
- **Probiotics: History and Evolution.** Over the past two to three decades,

research on probiotics' usefulness in treating various gastrointestinal and non-gastrointestinal illnesses has been progressively advancing. To obtain reliable findings, we require sizable, randomized, placebo-controlled single strain studies with standardised formulation, dose, and treatment duration in a range of disorders. Since the preparation and dosing may not be readily available commercially, it is currently difficult to recommend a specific probiotic for a given condition. One of the key areas of biological research is the relationship between the gut microbiota and its host and how these two entities are regulated. More study is necessary to determine their significance in human disorders. Vijaya K Gogineni *et al.*, (2013).

- **Probiotics History.** The gut microbiota encourages the host's good health and guards against sickness. According to their definition, probiotics are "live microorganisms that, when administered in sufficient amounts, confer a health benefit on the host." Beginning in the early 1900s, Louis Pasteur identified the bacteria in charge of the fermentation process, while E. Metchnikoff attributed the longer life expectancy of Bulgarian farmers to their regular use of fermented dairy products like yoghurt. He hypothesised that lactobacilli could prevent the putrefactive effects of gastrointestinal metabolism, which he believed contributed to disease and ageing. A 2000-year-old Greek physician named Hippocrates said that "death sits in the bowels." Probiotics (also known as "probios," which are beneficial to the host's survival as opposed to antibiotics) were regarded by Metchnikoff to be lactobacilli; Probiotics may improve health and delay the ageing process. The domestication of animals took place during the neolithic period of the age of the stone, when man started consuming fermented food. Most likely, accidental contaminations in favourable environments played a significant influence. A fourth-century Chinese manual for treating food poisoning or acute diarrhoea first mentions the transplantation of faeces' microbiota. Faecal transplant has proven to be more effective than vancomycin at treating *Clostridium difficile* infections and preventing recurrence. Gasbarrini *et al.*, (2016).
- **Probiotics in aquaculture.** The sector of food production with the quickest rate of growth is aquaculture. However, due to viral illnesses, fish culture is currently experiencing significant losses. Resistant bacterial strains have

developed as a result of the usage of antimicrobial medications, insecticides, and disinfectants in aquaculture to prevent disease and promote development. Consequently, as there is a growing desire for environmentally friendly, sustainable aquaculture, so too is study into the use of probiotics in aquaculture. These supplements offer advantages like higher feed value, enzymatic digestion assistance, pathogenic microorganism inhibition, anti-mutagenic and anti-carcinogenic activity, and boosted immunological response. These probiotics are healthy bacteria that support the host animal's wellbeing and aid to defend it against dangerous bacterial diseases, either directly or indirectly. Because the gastrointestinal microbiota of aquatic creatures has not been well characterised and because their effects have not been thoroughly explored, probiotics have only recently been used in aquaculture. In this review, basic information on the probiotic organism, how probiotics work in fish culture, and how safe probiotics are in aquaculture are summarised and evaluated. Priyadarshini Pandiyan *et al.*, (2013).

- “Review: Developments of Lactic Acid Bacteria as Probiotic for Bacterial Diseases control in Aquaculture.” Lactic acid bacteria (LAB) in aquatic organisms have emerged as one of the most fascinating study topics in recent years because of the numerous strains that present in fish microbiota. Additionally, LABs are occasionally prevalent in the intestine of several fish species. Numerous recent studies found that a number of LAB strains are safe and have been linked to positive benefits on fish health. Converging information about LAB's potential as a probiotic in aquaculture further encouraged further investigation and discovery. This page gives a general review of the evolution of this species as probiotics and the variation of LAB in fish's gastrointestinal (GI) tract. LAB has antagonistic activity against various fish infections and is known to be able to colonise the gut. The host animals' development, illness resistance, and nutritional absorption may all be enhanced by this non-pathogenic bacteriocin-producing strain. Additionally, these strains may lessen the requirement for antibiotic use in the aquaculture sector in the future. Masduki *et al.*, (2018).
- “The use of probiotics in aquaculture.” Aquaculture is increasingly using probiotics as an efficient, ecologically friendly method of disease prevention. Probiotics are live microorganisms or their derivatives that benefit the host.

Gram-positive and Gram-negative bacteria, microalgae (Tetraselmis), yeasts (Phaffia and Saccharomyces), and photorhodobacterium (Aeromonas, Alteromonas, Photorhodobacterium, Pseudomonas, Roseobacter, and Vibrio) have all been considered as candidates. The probiotic actively prevents potential pathogens from colonising the body by altering microbial metabolism, stimulating host immunity, or competing for nutrients and/or mucosal space, according to experience from agriculture and human medicine. This is accomplished through antibiosis, competition for nutrients and/or mucosal space, and other mechanisms. Probiotics may also increase nutrition and increase hunger by producing vitamins, detoxifying chemicals from the meal, dissolving indigestible diet components, or supplying exogenous enzymes. Irianto *et al.*, (2000).

## **CHAPTER-3**

## **AIM AND OBJECTIVE**

### **3.1 AIM**

- To encourage the growth of aquatic organisms, whether directly by assisting in their absorption or by providing them with nutrients.
- Functioning as sources of nutrients, giving digestive enzymes, regulating the immunological system, and boosting the immune response to harmful microorganisms.

### **3.2 OBJECTIVE**

- Obtaining probiotic organisms through isolation from numerous sources.
- Screening of probiotic species for their efficiency
  - a. Lactose fermentation
  - b. Antimicrobial activity
  - c. Antibiotic Sensitivity test
  - d. Siderophore production
- In vivo testing.
- Identification of the bacterial strain.
- Product development for human and animal use.

## **CHAPTER-4 MATERIALS AND METHODOLOGY**

### **4.1 MATERIALS**

The materials used for this process are:

#### **4.1.1 BACTERIAL ISOLATES**

The bacterial isolates used in this research study were isolated from rhizosphere soil sample which is taken from sea shore.

#### **4.1.2 CHEMICALS**

The chemicals used are Pikovskaya's agar, Tri-calcium phosphate, glucose, ammonium sulphate, sodium chloride, magnesium sulphate, yeast extract, agar powder, Antibiotic discs (Streptomycin, Chloramphenicol, Ampicillin, Tetracycline), Pathogens (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*), Tryptophan broth, Kovac's reagent, CAS reagent, glycerol, Muller Hinton agar, LB broth, Ethanol, distilled water, sea water

#### **4.1.3 EQUIPMENTS**

The equipments used are test tubes, petriplates, conical flask, glass beakers, micropipette, Weighing balance, Autoclave, incubator, pH paper, micro-oven, inoculation loop, spirit lamp, Hot air oven, Durham tube, parafilm, cotton, Non-absorbent cotton, shaker, laminar air flow, measuring cylinder, cork borer, sterile cotton swabs.

### **4.2 METHODOLOGY**

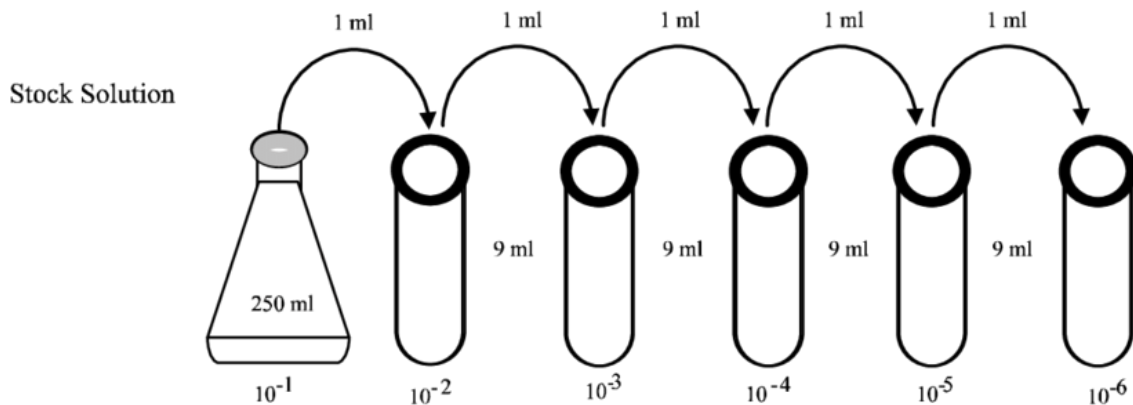
#### **4.2.1 SAMPLE COLLECTION**

The sample used in the process are soil sample which is collected from the rhizosphere of the plant which is taken from beach.

## 4.2.2 ISOLATION OF MICROBES

### 4.2.2.1 SERIAL DILUTION METHOD

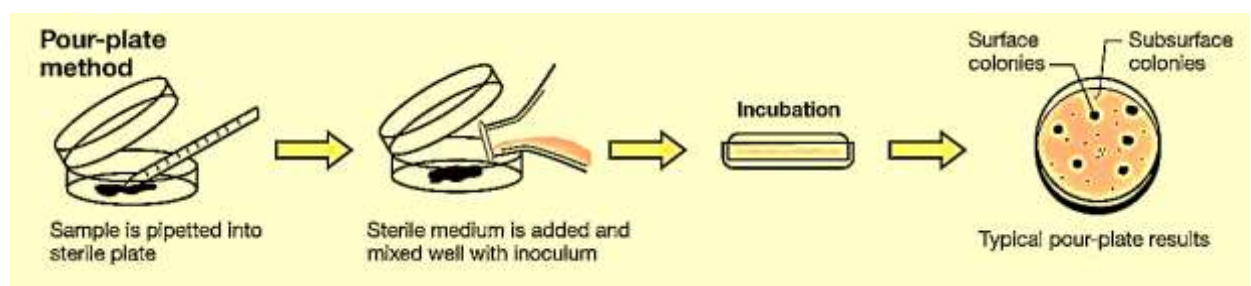
A solution sample is created by combining the sample (1 gramm) with 10 ml of sterile, clean water. This solution sample has undergone multiple dilutions up to  $10^{-5}$ . A sample is diluted numerous times using a procedure called serial dilution.



**Figure 4.1: Serial Dilution Method**

The five test tubes are then filled with 9ml of sterile, distilled water. One microlitre of the sample solution is pipetted into a  $10^{-1}$  micropipette. The test tube thoroughly mixed. Maintain aseptic method dilution for test  $10^{-2}$  till  $10^{-6}$ .

### 4.2.2.2 POUR PLATE METHOD



**Figure 4.2: Pour Plate Method**

Then, using a micropipette, each serial dilution is put into a petri dish. Pikovskaya's Agar is added to the plate, the sample is diluted, and 1ml of a properly diluted culture is added. After that, the plate is incubated until colonies form.

#### 4.2.2.3 ISOLATION OF MICROBES FROM SOIL SAMPLE



**Figure: 4.3 Sample collection**

The soil sample is mixed with 10ml of sterile distilled water. Six test tubes are taken with 9ml sterile distilled water. Pikovskaya's agar is prepared for isolation of bacterial colonies from soil sample. 1000 $\mu$ l of sample is taken and mixed to  $10^{-1}$  test tube and it is diluted to  $10^{-2}$  test tube and it goes on till  $10^{-6}$  dilution. 1ml of sample is taken from each test tube and transferred into labelled petri plates. Pikovskaya's agar is poured and mixed thoroughly with the sample and kept for solidification. The plates are incubated for 24-48 hrs.

#### 4.2.3 CHARACTERISATION OF BACTERIAL COLONIES

##### Screening tests

The bacterial isolates were characterized biochemically by various tests like Indole test, carbohydrate (lactose) fermentation test and bactericidal activity of bacterial isolates, Siderophore production test.

##### 4.2.3.1 CARBOHYDRATE (LACTOSE) FERMENTATION TEST

- To find out whether a bacterium can utilize a certain carbohydrate or not, apply the carbohydrate fermentation test.
- It checks for the presence of acid and/or gas generated when one carbohydrate ferments.

## **Procedure**

1. Put trypticase, sodium chloride, and phenol red into conical flasks after weighing and dissolving them in 100 ml of distilled water.
2. Each flask should contain 0.5 to 1 percent of the desired carbohydrate.
3. Place inverted Durham tubes into each tube; the Durham tubes must have broth inside of them all.
4. Sterilize for 15 minutes at 115 °C in an autoclave.
5. Inoculate each test tube with micro organisms.
6. Incubate the tubes at 18-24 hours at 37°C
7. Examine the tube for acid and gas production.

### **4.2.3.2 INDOLE ACETIC ACID PRODUCTION TEST**

This experiment shows that specific bacteria can convert the medium-accumulating ammonia acid tryptophan to indole. The test for indole synthesis is crucial for identifying enterobacteria.

## **Principle**

An organism's capacity to break down the amino acid tryptophan to produce the chemical indole is assessed using the indole test. Tryptophanase hydrolyzes tryptophan to produce three end products, indole being one of them. By using Kovac's reagent or Ehrlich's reagent, which both include 4 (p)-dimethylamino benzaldehyde, to react with indole to form a red-colored molecule, it is possible to identify the presence of indole.

## **Procedure**

1. Take a sterilized tube containing 4 ml of tryptophan broth.
2. Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.
3. Incubate the tube at 37°C for 24 - 28 hrs.
4. Add 0.5 ml of Kovac's reagent to the broth.
5. Observe the presence or absence of the ring.

#### **4.2.3.3 ANTIMICROBIAL ACTIVITY TEST**

##### **Preparation of Inoculum**

Stock cultures were maintained at 4°C on slant of nutrient agar. Active cultures for experiments were prepared by transferring a loop full of cells from the stock cultures to test tubes of nutrient broth for bacteria that were incubated at 24hrs at 37° C. The Assay was performed by agar well diffusion method.

##### **Procedure**

1. By using the well diffusion method on Muller Hinton agar (MHA) medium, the antibacterial activity of the sample was assessed.
2. Muller Hinton agar medium, weighing 3.8 grammes, was dissolved in 100 ml of distilled water before being mixed with 1 gram of agar.
3. The medium is then maintained to undergo sterilization.
4. The media was placed into sterile petri plates after sterilization, and they were given one hour to harden.
5. The inoculums were applied to the solid plates with sterile swabs wet with the bacterial suspension after the medium had hardened.
6. Cork borer was used to create wells.
7. Pathogens (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Escherichia coli*).
8. 50 µl and 100 µl of samples of the corresponding concentrations were loaded into the corresponding wells.
9. At 37°C, these plates were incubated for 24 hours
10. The diameter of the zone of inhibition was then measured to assess the rate of microbial growth.

#### **4.2.3.4 ANTIBIOTIC SUSCEPTIBILITY TEST**

##### **Procedure**

1. By using the disc diffusion method on Muller Hinton agar (MHA) medium, antibiotic sensitivity was evaluated.
2. 100ml of distilled water and 1g of agar were added to the 3.8g of Muller Hinton Agar medium that had been previously dissolved.

3. The media is then preserved for sterilisation.
4. After sterilisation, the media was added to sterile petri dishes and let to set for an hour.
5. The inoculums were dispersed on the solid plates with sterile swabs wet with the bacterial suspension after the medium had hardened, and antibiotic discs (Streptomycin, Chloramphenicol, Ampicillin, and Tetracycline) were added.
6. These plates were incubated at 37 °C for 24 hours.
7. After that, the diameter of the zone of inhibition was used to calculate the rate of microbial growth.

#### **4.2.3.5 SIDEROPHORE PRODUCTION TEST**

CAS assay is based on the observation that when a strong chelator (Eg. a Siderophore) removes iron from CAS /iron / detergent complex, the release of free dye is accompanied by change in color from blue to orange.

##### **Principle**

The CAS/HDTMA complexes tightly with ferric iron to produce a blue color. When a strong iron chelator such as a siderophore removes iron from the dye complex, the color changes from blue to orange.

##### **Procedure**

1. 10 ml of the CAS reagent was combined with 90 ml of sterilised LB agar medium to create the CAS agar plates.
2. On each plate, bacterial strains were spot-inoculated.
3. Plates were incubated at 28 °C for 72 hours following inoculation, and the development of an orange zone around the bacterial colonies was monitored.

#### **4.2.4 IN VIVO TESTING**

In vivo refers to a phenomenon in which experiments are performed using a whole, living organism.

##### **Probiotic sampling in fishes**

The black moor fish, koi fish, gold fish and iridescent sharks were collected. The fishes were visually looked out for their active conditions and were stocked in

plastic container for two days for adaption to laboratory conditions.



**Figure: 4.4 Black telescope goldfish, koi fish, gold fish and iridescent sharks**

For each fresh water tank with aerator, six fish were examined. Two tanks are used, with one serving as the control tank and the other as the sample tank. The experiment was carried out in a 40-litre glass tank for 30 days. By examining the sample culture's growth analysis and water quality, fish are being tested on it. Every two days, 5 ml of culture was added to the sample tank. Every morning, water exchanges were done for 20% of the culture water. 10% of the body weight of the animal was fed once every day.

#### **4.2.4.1 ANALYSIS OF PHYSI-CHEMICAL PARAMETERS**

##### **4.2.4.1.1 TEMPERATURE**

Temperature (°C) of the water was measured daily using a Celsius thermometer.

##### **4.2.4.1.2 pH**

pH of the water was measured daily using the pH meter or pH paper.

##### **4.2.4.1.3 DETERMINATION OF AMMONIA LEVEL**

The fish excrete ammonia into the water and release it through their gills,

urine, and faeces. Ammonia weakens the immune system, harms gill membranes, and hinders breathing. The amount of ammonia level present in the water sample is determined using ammonia kit analysis.

### **Method**

1. 5ml of the water being tested should be put into a clean test tube.
2. Holding the dropper bottle upside-down and totally vertically, add 8 drops of Ammonia Test Solution 1 to ensure uniform drop placement.
3. To ensure consistency of droplets, add 8 drops of Ammonia Test Solution 2 while holding the dropper bottle upside-down and vertically.
4. Shake the test tube ferociously for 5 seconds with the cap on.
5. Observe how the colour changes.
6. By contrasting the colour of the solution with the corresponding ammonia colour card, you can read the test results. The closest match represents the ammonia concentration in ppm (mg/l) in the water sample.

#### **4.2.4.1.4 DETERMINATION OF DISSOLVED OXYGEN LEVEL**

Dissolved oxygen (DO) is one of the most important parameters for growing fish and is also critical to the beneficial nitrifying bacteria that convert fish waste into nutrients plants can use. Dissolved oxygen kit analyses define the oxygen levels in the water sample.

### **Method**

1. 5ml of the water being tested should be put into a clean test tube.
2. To ensure consistency of droplets, add 4 drops of Dissolved Oxygen Test Solution 1 while holding the dropper bottle upside-down and vertically.
3. Holding the dropper bottle upside-down and totally vertically, add 4 drops of the Dissolved Oxygen Test Solution 2 to ensure uniform drop placement.
4. Shake the test tube ferociously for 5 seconds with the cap on.
5. Observe how the colour changes.
6. By contrasting the colour of the solution with the corresponding dissolved oxygen colour card, you can read the test results. The closest match shows how much dissolved oxygen is present in the water sample (mg/l).

#### **4.2.4.2 FISH GROWTH ANALYSIS**

The following parameters were used to evaluate the growth of fish such as length gain (cm), weight gain (gram).

##### **4.2.4.2.1 LENGTH**

Total length was measured at the time intervals of (10 days, 20 days, and 30 days) with the help of a steel graduated scale.

##### **4.2.4.2.2 WEIGHT**

Weight was taken by weighing the animal in live condition in an electronic balance at the time intervals of (10 days, 20 days, and 30 days) weight was recorded.

#### **4.2.4.3 MICROBIAL ANALYSIS**

Checks were made for bacterial growth in the water samples taken from the sample and control fish tanks. The TPC (Total plate count) method is used to determine the rate of bacterial growth. Pour plate method is used to count the number of colonies that have grown after the samples are serially diluted. We can find the colony forming unit per millilitre by adding up all the plates.

#### **4.2.5 IDENTIFICATION OF THE BACTERIAL STRAIN**

The isolated bacterial strain is identified using the 16S rRNA sequencing technique. All living cells include the intricate ribosome, which serves as the machinery for protein synthesis. The ribosome essentially consists of two subunits, each of which is made up of protein and ribosomal RNA (rRNA), a form of RNA. In prokaryotic ribosomes, S stands for Svedberg unit non-SI unit for sedimentation rate, and the 30S subunit (small subunit) and 50S subunit (big sub unit) combine to form the entire 70S ribosome. While the 50S subunit is made up of two rRNA species, the 5S and 23S rRNAs, the 30S subunit is made up of 16S ribosomal RNA and 21 polynucleotide chains. The 16S rRNA gene has hyper variable sections that offer a species-specific signature sequence that aids in bacterial identification. The identification of the diversity in prokaryotic organisms as well as other creatures, as well as the study of the phylogenetic links between them, are all accomplished

through the use of 16S ribosomal RNA sequencing in microbiology investigations.

### **DNA extraction**

DNA was extracted from bacterial culture using the QIAmp DNA Minikit (Qiagen) according to the appropriate protocols in the manufacturer's instructions; with a final elution volume of 50 µl. Extracted DNA was stored at 4°C until required for PCR.

### **PCR Amplification**

The highly sensitive PCR method enables quick DNA amplification of a particular section. Using visual methods based on size and charge, PCR may detect and identify gene sequences by producing billions of copies of a certain DNA fragment or gene. Since its discovery, the polymerase chain reaction (PCR) has had a profound impact on biological science. It made it possible to produce and specifically detect enormous quantities of DNA for the first time. PCR-based techniques have fueled significant scientific initiatives like the Human Genome Project. Clinicians and academics alike frequently utilise the method nowadays to quickly and sensitively diagnose illnesses, clone and sequence genes, and conduct complex quantitative and genomic analyses. Pathogen detection is one of the most crucial medical uses for the traditional PCR technique. The PCR technique is additionally utilised in forensic medicine to identify criminals. Due to its extensive use, it is crucial to comprehend the fundamentals of PCR and how its application can be changed to enable complex analyses of genes and the genome.

The 1 µL of bacterial DNA extract and controls were amplified with 1 µL of 10Mm primers, 3.5 µL of GoTaq Green Mastermix (Promega), 3.5 µL of nuclease free water. Amplification conditions for the PCR assay was as follows: 5 min at 94°C to denature the DNA, followed by 30 cycles of denaturation at 94°C for 30 sec, primer annealing at 55°C for 10 second strand extension at 72°C for 30 sec on a Eppendorf thermal cycler. PCR products were separated on a 2% agarose gel and DNA bands were visualized with ethidium bromide. Primers and excess nucleotides were removed from the amplified DNA using a PCR clean-up kit (Qiagen). The amount of DNA in the cleaned-up product was quantified by comparing the intensity of the band to bands of known intensity in a Hyper Ladder marker (Bioline). Spiked samples were included with each PCR to determine whether or not the sample was inhibited. Spiked samples contained 1 CFU of E. coli culture.

### **PCR Sequencing and Sequence Analysis**

Purified PCR products were sequenced in the forward and reverse direction in separate reactions. Each reaction contained 1µL of template DNA, 1µL of the appropriate PCR primer (10µM), 1µL of nuclease water and 2µL of Big Dye Terminator v3.1 Ready Reaction Mix (Applied Bio systems). Each reaction was heated to 96°C for 1 min, followed by 35 cycles at 96°C for 10 sec, 55 °C for 5 sec and 60 °C for 1 min. The sequencing products were purified using an ethanol precipitation method to remove unincorporated reagents and ensure a neutral charge. To the sequencing reaction 0.5µL of 0.5M EDTA was added and vortexed briefly for homogenization of the reaction mixture. The homogenized reaction mixture was further washed by adding 21µL of ethanol precipitation mix (1µL of 3M sodium acetate and 20µL of absolute ethanol) and centrifuging at 4000 RPM for 15 min. The washing step was repeated twice. The pelleted DNA was dried by incubating in concentrator for 5 min at room temperature under vacuum condition. The dried DNA pellet was reconstituted in 10µL of formamide and denatured by heating the samples at 95°C and then snap cooling over ice. The denatured samples were loaded onto a 3730xl Genetic Analyzer Capillary Array for detection (Applied Bio systems). One forward and one reverse sequences for each sample were aligned using BioEdit to obtain a composite sequence. The quality of each sequence trace was visually assessed and poor quality sequence was edited and removed. Organisms were identified for each assay by comparing consensus sequences to a database library of known 16S rRNA gene sequences in GenBank (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) by multiple sequence alignment. The bacterial source of the sequence was identified by matching it with a sequence with the highest maximum identity score from the GenBank database. Where more than one bacterial species had the same highest score, the species identification of the test organism was deduced based upon the taxonomy hits.

#### **4.2.6 Product development**

The sample efficiency is checked and the product is being developed from the selected culture. Product is developed in two forms powder and liquid form. Whereas, glycerol and calcium phosphate are used as substrate because they act

as inert material and doesn't affect the bacteria present in it.

#### **4.2.6.1 Powder form**

The broth culture which is incubated at room temperature is mixed with the sterile calcium tri phosphate to form powder and it is dried until it losses the moisture and stored in a dark place. The product is checked for their efficiency by testing it in MRS broth for its efficiency.

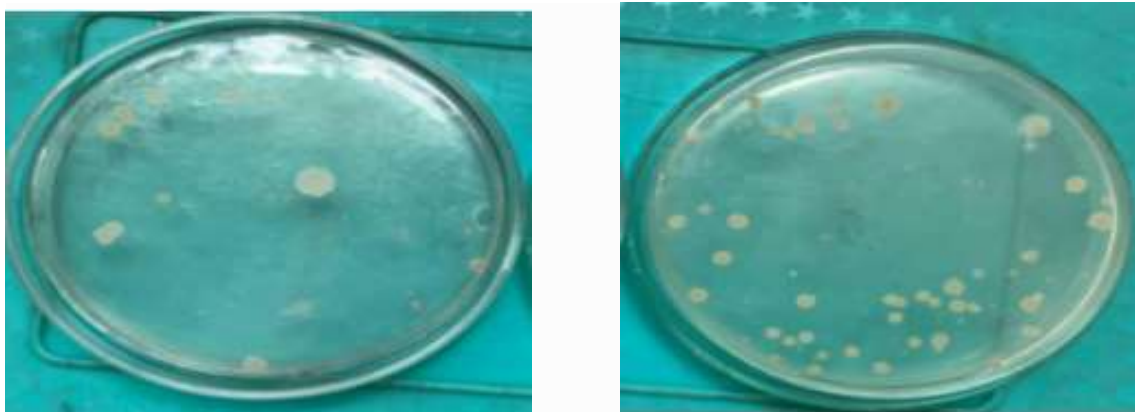
#### **4.2.6.2 Liquid form**

The broth culture which is incubated at room temperature in the shaker is poured into the petri plates and kept for drying at 45°C in hot air oven for 24 hrs. After drying the sterile glycerol is mixed with the petri plates to form the liquid product. The liquid product is being serially diluted and cultured in MRS broth for checking its efficiency.

## 5.1 RESULTS

### 5.1.1 ISOLATION OF BACTERIA FROM SOIL SAMPLE

In the petri plates, a number of unique colonies were developed, and the dilutions  $10^{-2}$  and  $10^{-5}$  show the clear colony that inhibited the neighbouring bacterial colonies.



**Figure: 5.1 Isolation of bacteria**

To create pure colonies, the colonies from these dilutions were continually streaked onto a plate of pikovskaya agar media. The microbial loop is collected, streaked on the slants, and then preserved for further use.



**Figure: 5.2 Isolation of pure bacterial colonies**

## 5.1.2 CHARACTERIZATION OF BACTERIAL COLONIES

### 1.1.2.4 CARBOHYDRATE FERMENTATION TEST



**Figure: 5.3 Carbohydrate fermentation test**

The liquid present in the sample test tube-1 turns yellow and there is no gas production in the test tube. It indicates that there is drop in the pH because of the production of the acid by the fermentation of the carbohydrate (sugar) present in the media



**Figure: 5.4 Carbohydrate fermentation test**

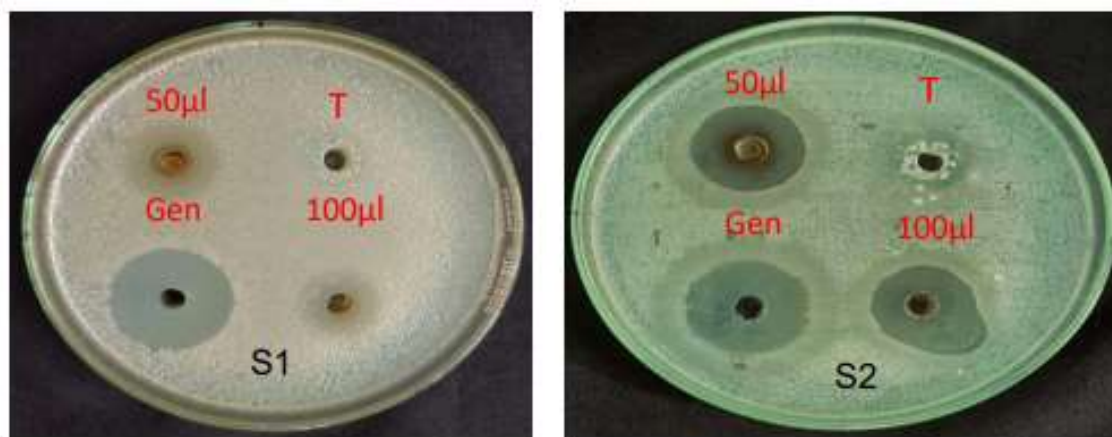
The liquid present in the sample test tube-2 turns yellow and there is no gas production in the test tube. It indicates that there is drop in the pH because of the production of the acid by the fermentation of the carbohydrate present in the media.



**Figure: 5.5 Carbohydrate fermentation test**

#### **2.1.2.4 ANTIMICROBIAL ACTIVITY TEST**

Agar well diffusion susceptibility method was used to examine the antibacterial test of samples 1 and 2. *Escherichia coli* and *Staphylococcus aureus* were the two bacterial species used in the antibacterial bioassay. The bacterial species were added to the wells at concentrations of 50 $\mu$ l and 100 $\mu$ l and left to incubate for 24 hours. The results show that there is a zone of inhibition against *Escherichia coli* for samples 1 and 2. The zone of inhibition for samples 1 is 13 mm, while the zone of inhibition for samples 2 is 18 mm for 100 $\mu$ l, 11 mm, and 15 mm for 50 $\mu$ l concentration.



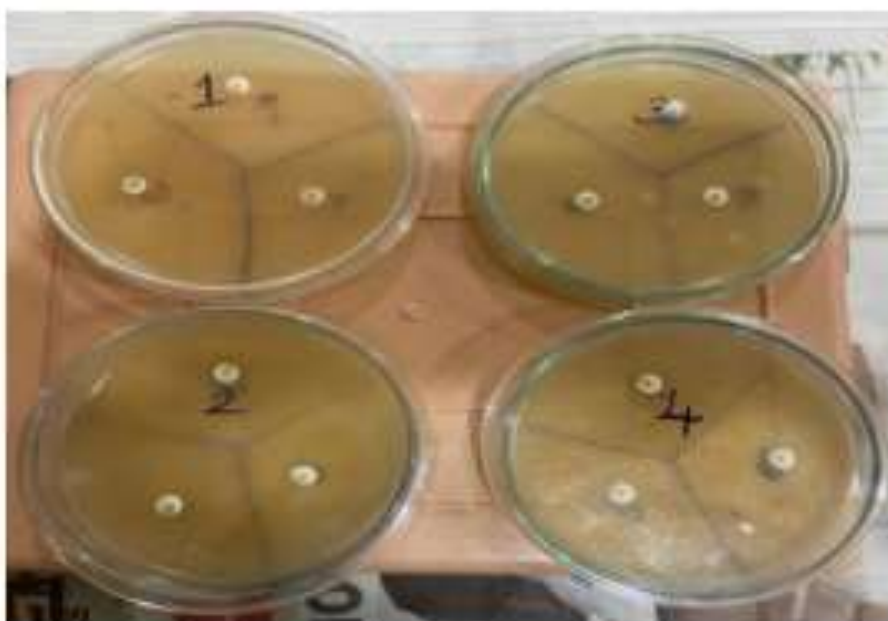
**Figure: 5.6 Antimicrobial activity test of *Escherichia coli***

**Table: 5.1 Zone of inhibition**

| Microorganisms               | Zone of Inhibition (in mm) |           |           |           |
|------------------------------|----------------------------|-----------|-----------|-----------|
|                              | Sample-1                   |           | Sample-2  |           |
|                              | 50µl                       | 100µl     | 50µl      | 100µl     |
| <b>Staphylococcus aureus</b> | -                          | -         | -         | -         |
| <b>Escherichia coli</b>      | <b>11</b>                  | <b>13</b> | <b>15</b> | <b>18</b> |

#### 5.1.2.3 ANTIBIOTIC SUSCEPTIBILITY TEST

The bacterial isolates after incubation found that there is no zone of inhibition against the antibiotics like Streptomycin, Chloramphenicol, Ampicillin, and Tetracycline. Hence, the bacterial isolates are resistant to the antibiotics.



**Figure: 5.7 Antibiotic susceptibility test**

#### 5.1.2.4 SIDEROPHORE PRODUCTION TEST

The detection of Siderophore was confirmed by plating the bacterial isolates in the CAS agar. The sample-1 bacterial isolate was showing distinct zone with the appearance of orange color indicating the production of Siderophore. Orange zone

appearance clearly demonstrates Siderophore production. The sample-2 bacterial isolate shows negative result for Siderophore production.



**Figure: 5.8 Siderophore production test**

**Table: 5.2 Siderophore production test**

| SAMPLES | RESULTS |
|---------|---------|
| 1       | +       |
| 2       | -       |

### **5.1.3 *IN VIVO* TESTING**

Two tanks are set up in which one is control and the other one is for testing the sample-1, as it showed best results in seed germination. Sample is being given to fishes along with feed. The fishes used in testing are Gold, Black moor and iridescent shark fishes.



**Figure:5.9 Fish tank**

### **5.1.3.1 ANALYSIS OF PHYSIO-CHEMICAL PARAMTERS**

#### **5.1.3.1.1 TEMPERATURE**

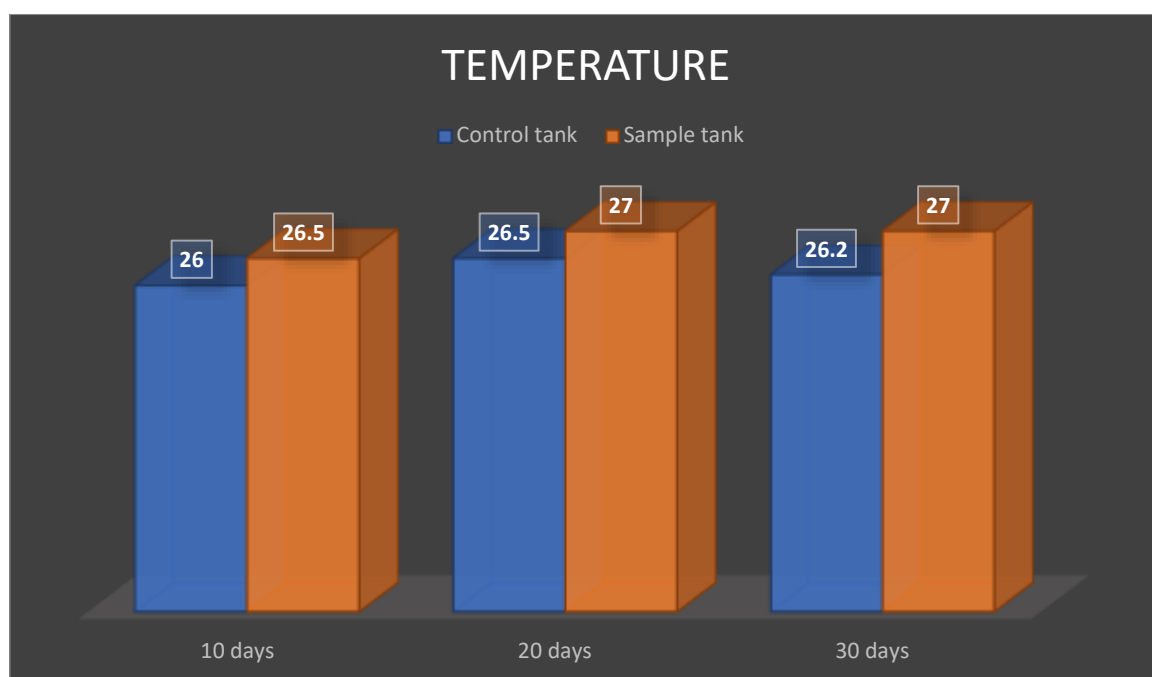
The temperature in control tank is at 26°C for 10 days and there is change in temperature to 26.5°C for 20 days and it is maintained till 30th day. The temperature in sample tank is 26.5°C at 10th day and there is change in temperature to 27°C at 20th day and it is maintained the same till 30th day. The temperature should be maintained at 25°C - 28°C, which is an optimum temperature.

**Table: 5.3 Temperature in control tank**

| <b>Number of Days</b> | <b>Temperature (°C)</b> |
|-----------------------|-------------------------|
| 10                    | 26                      |
| 20                    | 26.5                    |
| 30                    | 26.5                    |

**Table: 5.4 Temperature in sample tank**

| <b>Number of Days</b> | <b>Temperature °C</b> |
|-----------------------|-----------------------|
| 10                    | 26.5                  |
| 20                    | 27                    |
| 30                    | 27                    |



**Figure: 5.10 Temperature (in °C) Analysis in fish tank**

#### 5.1.3.1.2 pH

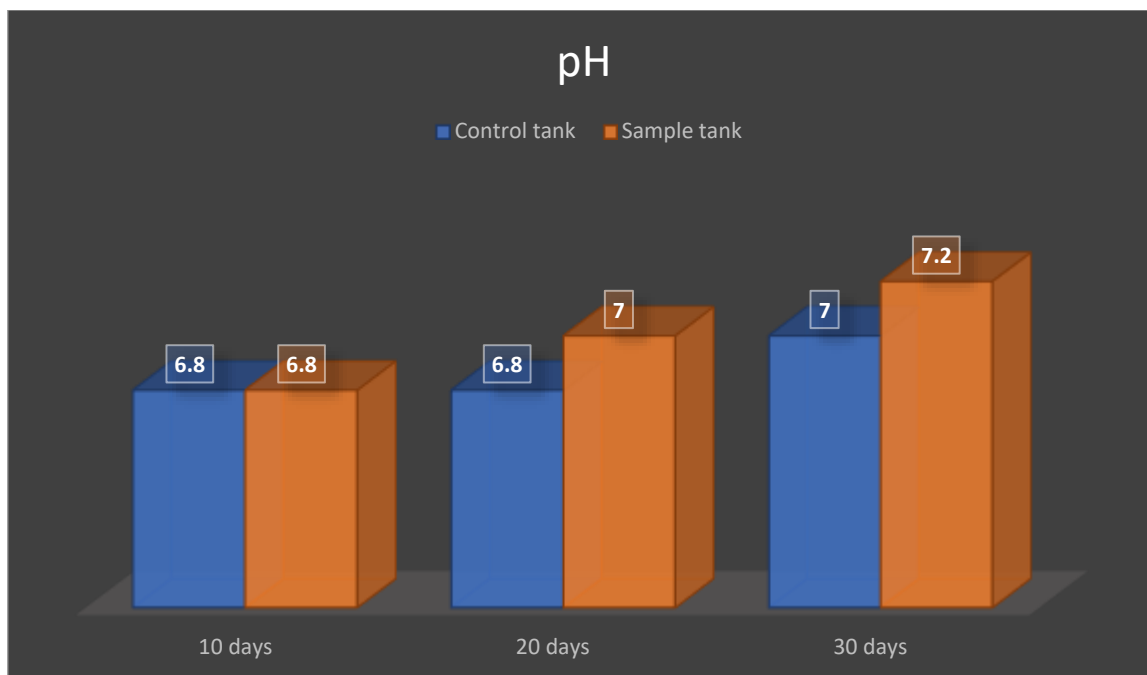
The initial pH is 6.8 in control & sample tank for 10 days. There is change in pH to 7 in sample tank and it is maintained 6.8 in control tank on 20th day. There is change in pH to 7.2 in sample tank and 7 in control tank on 30th day. The rise in ammonia raises the pH level. The optimum pH to be maintained in fresh water aquarium is 6.8 - 7.6.

**Table: 5.5 pH in control tank**

| No. of Days | pH  |
|-------------|-----|
| 10          | 6.8 |
| 20          | 6.8 |
| 30          | 7.0 |

**Table: 5.6 pH in sample tank**

| No. of Days | pH  |
|-------------|-----|
| 10          | 6.8 |
| 20          | 7.0 |
| 30          | 7.2 |



**Figure: 5.11 pH Analysis in fish tank**

#### 5.1.3.1.3 DETERMINATION OF AMMONIA LEVEL

The ammonia level in the control tank and sample tank is 0.1ppm for 10 days. The ammonia level is increased to 0.15 ppm and 0.2 ppm in the control and sample tank on 20th day. The increase in ammonia level indicates the ammonia content from the fish excreta and the bacterial sample. The ammonia is 0.2 ppm in both the tanks on the 30th day. The increase in ammonia also increases the pH.

**Table: 5.7 Ammonia level in Control tank**

| No. of Days | Ammonia level (in ppm) |
|-------------|------------------------|
| 10          | 0.1                    |
| 20          | 0.1                    |
| 30          | 0.2                    |

**Table: 5.8 Ammonia level in Sample tank**

| No. of Days | Ammonia level (in ppm) |
|-------------|------------------------|
| 10          | 0.15                   |
| 20          | 0.2                    |
| 30          | 0.2                    |

#### **5.1.3.1.4 DETERMINATION OF DISSOLVED OXYGEN LEVEL**

The dissolved oxygen is maintained at 6 ppm in sample and control tank for 10 days. There is increase in dissolved oxygen to 6.5 on 20th day and in sample tank the DO is maintained at 6 ppm. The dissolved oxygen is maintained at 6.5 ppm in both the tanks on 30th day. The optimum dissolved oxygen level is 6 – 8 ppm.

**Table: 5.9 Dissolved Oxygen level in control tank**

| <b>No. of Days</b> | <b>Dissolved Oxygen level (in ppm)</b> |
|--------------------|----------------------------------------|
| 10                 | 5                                      |
| 20                 | 5.5                                    |
| 30                 | 5.5                                    |

**Table: 5.10 Dissolved Oxygen level in sample tank**

| <b>No. of Days</b> | <b>Dissolved Oxygen level (in ppm)</b> |
|--------------------|----------------------------------------|
| 10                 | 6                                      |
| 20                 | 6.5                                    |
| 30                 | 6.5                                    |

#### **5.1.3.2 FISH GROWTH ANALYSIS**

The parameters that are used to evaluate fish growth are length (cm) and weight (grams).

##### **5.1.3.2.1 LENGTH**

The average length of the fishes in control tank for 10 days was 1.82 cm and the final average length was 2.17 cm. The average length of the fishes measured in sample tank for 10 days are 1.88 cm and the final average length is 2.40 cm.

**Table 5.11: Average length of fishes in control and sample tank**

| No. of days | Average length |             |
|-------------|----------------|-------------|
|             | Control tank   | Sample tank |
| 10          | 10.54          | 10.62       |
| 20          | 10.63          | 10.64       |
| 30          | 10.64          | 10.68       |

#### **5.1.3.2.2 WEIGHT**

The average weight of the fishes in control tank for 10 days was measured to be 11.51 grams and the final average weight measured was 12.34 grams on 30th day. The average weight of the fishes in sample tank for 10 days was measured to be 11.62 grams and the final average weight measured was 12.51 grams on 30th day.

**Table 5.12: Average weight of fishes in control and sample tank**

| No. of days | Average weight |             |
|-------------|----------------|-------------|
|             | Control tank   | Sample tank |
| 10          | 15.63          | 15.71       |
| 20          | 15.64          | 15.65       |
| 30          | 15.76          | 15.82       |

#### **5.1.4 IDENTIFICATION OF THE BACTERIAL STRAIN**

The phenotypic characteristics of the traditional identification of bacteria are not as accurate as genotypic identification. The comparison of the bacterial 16s rRNA sequence was done by preferred genetic technique. To estimate the relationship between bacteria (phylogeny), sequence of the 16s rRNA was widely used as a molecular clock but it became more important as a means to identify an unknown

genus or species. To study the bacterial phylogeny and taxonomy the 16s rRNA gene sequencing used and as far as the most common genetic marker. To amplify the 16srRNA gene as a template, the extracted DNA was used. The use of 16S rRNA gene sequence to characterize the bacterial isolated was found to be *Enterobacter hormaechei* strain F2 chromosome, complete genome CP047570.1. *Enterobacter hormaechei* strain EB\_P6\_L3\_02.19 chromosome, complete genome: CP043853.1. The simple and effective identification of strains method is done of genotyping by using 16s rRNA gene sequencing.

#### DNA Quantification:

| S.No | Sample ID | DNA (ng/μl) |
|------|-----------|-------------|
| 1.   | Sriram    | 181         |

#### PCR Conditions

##### PCR Amplification of 16S Gene:

181 ng of Extracted DNA is used for amplification along with 10pM of each primer

##### Composition of TAQ Master MIX:

- 1) High-Fidelity DNA Polymerase
- 2) 0.5mM dNTPs
- 3) 3.2mM MgCl<sub>2</sub>
- 4) PCR Enzyme Buffer

| Cycling Conditions   |                   |           |
|----------------------|-------------------|-----------|
| Initial Denaturation | 3 minutes at 94°C | 30 Cycles |
| Denaturation         | 1 minutes at 94°C |           |
| Annealing            | 1 minutes 50°C    |           |
| Extension            | 2 minutes at 72°C |           |
| Final Extension      | 7 minutes at 72°C |           |

| PCR Amplification conditions        | Volume |
|-------------------------------------|--------|
| DNA                                 | 1 ul   |
| 16s Forward Primer                  | 2 ul   |
| 16s Reverse Primer                  | 2 ul   |
| dNTPs (2.5mM each)                  | 4 ul   |
| 10X Taq DNA polymerase Assay Buffer | 10 ul  |
| Taq DNA Polymerase Enzyme (3U/ ml)  | 1 ul   |
| Water                               | 30 ul  |
| Total reaction volume               | 50 ul  |

#### Primer Details - The PCR product size ~1.5 kb

| No. | Oligo Name  | Sequence (5' à 3')  | Tm (°C) | GC- Content |
|-----|-------------|---------------------|---------|-------------|
| 1   | 16s Forward | GGATGAGCCCGCGGCTA   | 57      | 72.22%      |
| 2   | 16s Reverse | CGGTGTGTACAAGGCCCGG | 58      | 68.42%      |

- The Microbe was found to be *Pseudomonas aeruginosa* strain DSM 50071

16S ribosomal RNA

- Sequence ID: NR\_117678.1
- The next closest homologue was found to be *Pseudomonas aeruginosa* strain NBRC 12689 16S ribosomal RNA
- Sequence ID: NR\_113599.1

**Table: 5.13 List of identified microbial species**

| SI.NO | ORGANISM NAME                                                     | ACCESSION NO. | % MATCH |
|-------|-------------------------------------------------------------------|---------------|---------|
| 1     | <i>Pseudomonas aeruginosa</i> strain DSM 50071 16S ribosomal RNA  | NR_117678.1   | 97.32%  |
| 2     | <i>Pseudomonas aeruginosa</i> strain NBRC 12689 16S ribosomal RNA | NR_113599.1   | 97.32%  |
| 3     | <i>Pseudomonas aeruginosa</i> strain ATCC 10145 16S ribosomal RNA | NR_114471.1   | 97.32%  |
| 4     | <i>Pseudomonas lalkuanensis</i> strain PE08 16S ribosomal RNA     | NR_179771.1   | 96.60%  |

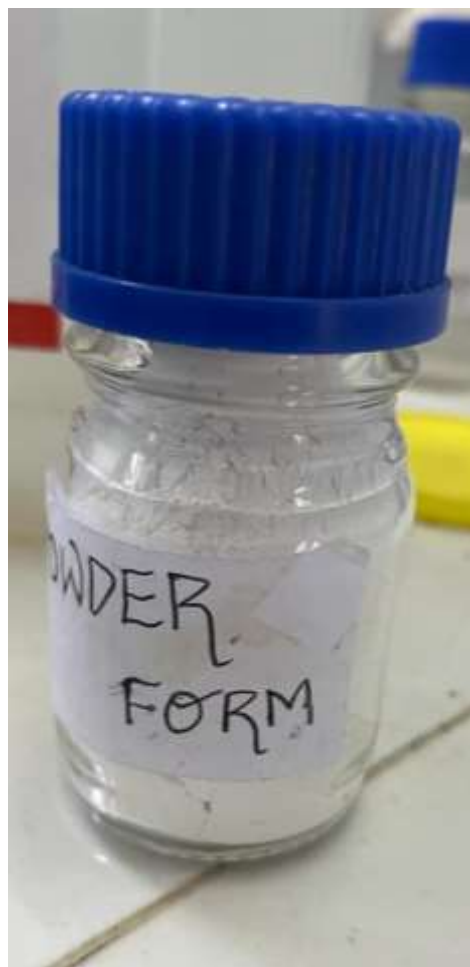
|    |                                                                    |             |        |
|----|--------------------------------------------------------------------|-------------|--------|
| 5  | [Pseudomonas]<br>nosocomialis strain A31/70<br>16S ribosomal RNA   | NR_179563.1 | 96.37% |
| 6  | Pseudomonas otitidis<br>strain MCC10330 16S<br>ribosomal RNA       | NR_043289.1 | 96.30% |
| 7  | Pseudomonas guezenei<br>strain RA26 16S ribosomal<br>RNA           | NR_114957.1 | 96.30% |
| 8  | Pseudomonas<br>resinovorans strain ATCC<br>14235 16S ribosomal RNA | NR_112062.1 | 96.13% |
| 9  | Pseudomonas aeruginosa<br>strain DSM 50071 16S<br>ribosomal RNA    | NR_026078.1 | 96.02% |
| 10 | Pseudomonas glareae<br>strain KMM 9500 16S<br>ribosomal RNA        | NR_145562.1 | 95.97% |

## **5.1.5 PRODUCT DEVELOPMENT**

### **5.1.5.1 POWDER FORM**

The cultured MRS broth is mixed with sterile calcium tri phosphate, and formed into thick slurry. The slurry is spread evenly on a tray and dried at room temperature. The powder is formed.

The powder is being tested for checking the bacteria present in it. The powder is serially diluted up to 10 dilutions and cultured in MRS broth for checking the microbial load. The results show that there is bacterial growth up to 10-10 dilutions in MRS broth. Therefore, the bacterium present is highly efficient in powder form.



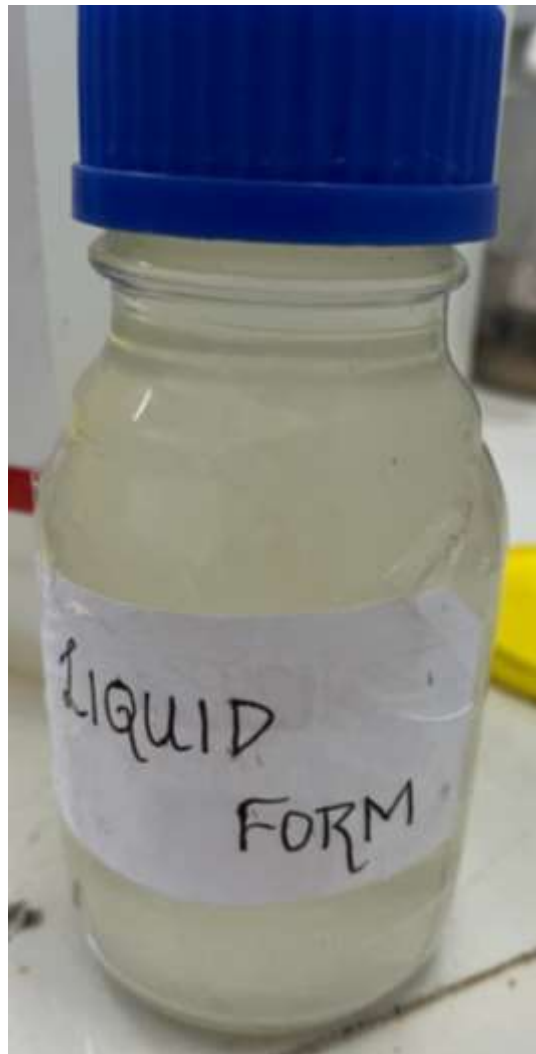
**Figure 5.12 Powder form**

### **5.1.5.2 LIQUID FORM**

The cultured MRS broth is dried at 45°C in hot air oven. The dried sample is mixed with the sterile glycerol, which act as substrate to the bacterial media. The liquid

product is formed.

The liquid is serially diluted up to 10 dilutions and cultured in MRS broth for checking the microbial load. The results show that there is bacterial growth up to  $10^{-5}$  dilutions in MRS broth. Therefore, the bacterium present is not efficient in liquid form.



**Figure 5.13 Liquid form**

## **5.2 DISCUSSION**

In general, water probiotic treated fishes showed better growth response than the feed application. The present study was carried out to isolate and identify the bacteria from different species were isolated from different sources, which could be related to the different nutritional status of the isolation sites, e.g. the nutrition in the rhizosphere of medicinal plant, which is useful for animal and human use. Studies

have shown that probiotics can improve the growth performance and survival rates of various fish species, including common carp, tilapia, and salmon. Furthermore, probiotics have been shown to be effective in preventing and controlling bacterial and fungal infections in fish, potentially reducing the need for antibiotics and other medications.

However, it is important to note that the effectiveness of probiotics can be influenced by a number of factors, including the quality of the probiotic, the dose and frequency of administration, and the environmental conditions in which the fish are cultured. Additionally, there is a need for further research to optimize the use of probiotics in different fish species and production systems.

In summary, the use of probiotics in fish culture has the potential to improve fish health and growth, and reduce the reliance on antibiotics and other medications. However, careful consideration and monitoring are required to ensure the effectiveness and safety of probiotics in different fish species and culture systems. There have been practically no variations within the levels of temperature among control and experimental tanks. The oxygen dissolved in the subculture medium is an critical factor no longer simplest for the respiration of aquatic organisms, however also to preserve beneficial chemical and hygienic environment within the water-body. When the oxygen stage may be very low, anaerobic condition exists; nitrate is decreased through denitrifying microbes into ammonia which is toxic. This additionally increases the pH. Therefore, continuous aeration became done in the course of the prevailing study and oxygen stage did no longer vary substantially between the control and experimental tanks.

In the present study the activity & growth parameters of the fishes are being checked by giving bacterial sample as feed to the fishes. The growth parameters like weight, height are checked. The present study revealed different isolates to those from traditional sources, and the strains isolated from soil. Further, some soil isolated strains showed antibacterial and antimicrobial activities against some bacterias.

## **CHAPTER-6            SUMMARY AND CONCLUSION**

The plant's rhizosphere soil and fermented rice water are where the bacterial samples are obtained. The pure colonies from the isolated bacterial samples are kept in slants and broth medium for further examination. Additional research was planned for the bacterial strain's characteristics. These isolates' phenotypic characteristics were determined using gramme staining, various biochemical tests, and testing for carbohydrates.

In addition to other biochemical testing, lactose and indole acetic acid synthesis tests were conducted. Using 16s rRNA sequencing, morphological, biochemical, and other factors, the chosen strain was classified as an *Enterobacter* species. The tank received bacterial culture for a month in a lab setting. Ammonia and dissolved oxygen, two indicators of water quality, were recorded; the On the first, fifth, tenth, fifteenth, twenty-fifth, and twenty-fifth day of culture, separate bacterial cultures were inoculated in the culture tanks. On the 31st day of culture, the experiment was over. The fish's total length and wet weight were measured. Application of probiotic microorganisms along with feed had generally shown positive outcomes. The application of water also greatly lowered the ammonia levels. Overall, the probiotic water treatment demonstrated a stronger growth response than the probiotic feed administration. By promoting fish growth and water quality, the bacterial strain *Enterobacter* species had a positive impact on the molly fish culture tank.

## REFERENCE

1. Ahmadova A, Todorov SD, Hadji-Sfaxi I, Choiset Y, Rabesona H, Messaoudi S, Kuliyeu A, de Melo Franco BD, Chobert JM, Haertlé T. Antimicrobial and antifungal activities of *Lactobacillus curvatus* strain isolated from homemade Azerbaijani cheese. *Anaerobe*. 2013. vol. 20, 42-9.
2. Asmaa Omar Mohamed Mohamed., Experimental Studies on the Effect of some Probiotics on the Immune Response and Resistance of Tilapia Fish. 2018.
3. Augustine, Deepthi & Jacob, Jimly & Philip, Rosamma. Exclusion of *Vibrio* spp. by an antagonistic marine actinomycete *Streptomyces rubrolavendulae* M56. *Aquaculture Research*. (2015)
4. Goldin, Barry R. "Health Benefits of Probiotics." *British Journal of Nutrition*, 1998 vol. 80, no. S2, pp. S203–S207.
5. Butler, Mark S. "Natural products to drugs: natural product-derived compounds in clinical trials." *Natural product reports* 25.3 (2008): 475-516.
6. Charalampopoulos, D et al. "Application of cereals and cereal components in functional foods: a review." *International journal of food microbiology*, 2002, vol. 79,1-2: 131-41.
7. Merenstein, D et al. "Use of a fermented dairy probiotic drink containing *Lactobacillus casei* (DN-114 001) to decrease the rate of illness in kids: the DRINK study. A patient-oriented, double-blind, cluster-randomized, placebo-controlled, clinical trial." *European journal of clinical nutrition* (2010). vol. 64,7: 669-77.
8. Das, S., Ward, L. R., and Burke, C. Screening of marine *Streptomyces* spp. for potential use as probiotics in aquaculture. *Aquaculture* 2010 Vol.305 No.1/4 pp.32-41
9. Defoirdt, Tom et al. "Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example." *Trends in biotechnology* 2007 vol. 25,10: 472-9.
10. "Scientific concepts of functional foods in Europe. Consensus document." *The British journal of nutrition* (1999) vol. 81 Suppl 1: S1-27.
11. Dubey JP, Choudhary S, Kwok OC, Ferreira LR, Oliveira S, Verma SK, Marks DR, Pedersen K, Mickley RM, Randall AR, Arsnøe D, Su C. Isolation and

- genetic characterization of *Toxoplasma gondii* from mute swan (*Cygnus olor*) from the USA. *Vet Parasitol.* 2013 Jul
12. Felipe do Nascimento Vieira, Adolfo Jatobá, José Luiz Pedreira Mouriño, Eduardo Alano Vieira, Mariana Soares, Bruno Corrêa da Silva, Walter Quadros Seiffert, Maurício Laterça Martins and Luis Alejandro Vinatea., In vitro selection of bacteria with potential for use as probiotics in marine shrimp culture. 2013
  13. Fuller, R. "Probiotics in man and animals." *The Journal of applied bacteriology* (1989) vol. 66,5: 365-78.
  14. Garibyan, Lilit, and Nidhi Avashia. "Polymerase chain reaction." *The Journal of investigative dermatology* (2013) vol. 133,3: 1-4.
  15. Granato, Daniel et al. "Functional Foods and Nondairy Probiotic Food Development: Trends, Concepts, and Products." *Comprehensive reviews in food science and food safety* (2010) vol. 9,3: 292-302.
  16. Hai, N V. "The use of probiotics in aquaculture." *Journal of applied microbiology* (2015) vol. 119,4: 917-35.
  17. Schuhegger, Regina et al. "Induction of systemic resistance in tomato by N-acetyl-L-homoserine lactone-producing rhizosphere bacteria." *Plant, cell & environment* (2006) vol. 29,5: 909-18.
  18. Swanson, Kelly S et al. "The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics." *Nature reviews. Gastroenterology & hepatology* (2020) vol. 17,11: 687-701.
  19. Ibrahim, Fandi et al. "Probiotics and immunosenescence: cheese as a carrier." *FEMS immunology and medical microbiology* (2010) vol. 59,1: 53-9.
  20. Shida, Kan et al. "Daily intake of fermented milk with *Lactobacillus casei* strain Shirota reduces the incidence and duration of upper respiratory tract infections in healthy middle-aged office workers." *European journal of nutrition* (2017) vol. 56,1: 45-53.
  21. Gleeson, Michael et al. "Effects of *Lactobacillus casei* Shirota ingestion on common cold infection and herpes virus antibodies in endurance athletes: a placebo-controlled, randomized trial." *European journal of applied physiology* (2016) vol. 116,8: 1555-63.
  22. Kumpu, M et al. "Effect of live and inactivated *Lactobacillus rhamnosus* GG

- on experimentally induced rhinovirus colds: randomised, double blind, placebo-controlled pilot trial." *Beneficial microbes* (2015) vol. 6,5: 631-9.
23. McFarland, Lynne V. "From yaks to yogurt: the history, development, and current use of probiotics." *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* (2015) vol. 60 Suppl 2: S85-90.
  24. Mullis, K B. "The unusual origin of the polymerase chain reaction." *Scientific American* (1990) vol. 262,4: 56-61, 64-5.
  25. Muñoz, Camilo et al. "Use of cellulolytic marine bacteria for enzymatic pretreatment in microalgal biogas production." *Applied and environmental microbiology* (2014) vol. 80,14: 4199-206.
  26. Ngo van Hai & Ravi Fotedar (2010): A Review of Probiotics in Shrimp Aquaculture, *Journal of Applied Aquaculture*, 22:3, 251-266
  27. Chau, Khanh Minh et al. "Molecular Identification and Characterization of Probiotic *Bacillus* Species with the Ability to Control *Vibrio* spp. in Wild Fish Intestines and Sponges from the Vietnam Sea." *Microorganisms* (2021) vol. 9,9 1927.
  28. Kanauchi Osamu \*, Andoh Akira , AbuBakar Sazaly and Yamamoto Naoki, Probiotics and Paraprobiotics in Viral Infection: Clinical Application and Effects on the Innate and Acquired Immune Systems, *Current Pharmaceutical Design* 2018; 24(6)
  29. Popova, M et al. "Beneficial effects of probiotics in upper respiratory tract infections and their mechanical actions to antagonize pathogens." *Journal of applied microbiology* vol. 113,6 (2012): 1305-18.
  30. Priyadarshini Pandiyan, Deivasigamani Balaraman, Rajasekar Thirunavukkarasu, Edward Gnana Jothi George, Kumaran Subaramaniyan, Sakthivel Manikkam, Balamurugan Sadayappan, Probiotics in aquaculture, *Drug Invention Today*, (2013) Vol 5, 55-59.
  31. Parker, R.B. Probiotics, the Other Half of Antibiotic Story. *Animal Nutrition & Health*, (1974), vol 29, 4-8.
  32. Perrig, D et al. "Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation." *Applied microbiology and biotechnology* (2007) vol. 75,5: 1143-50.

33. Makino, Seiya et al. "Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1." *The British journal of nutrition* vol. 104,7 (2010): 998-1006.
34. Sahadeva, R.P.K. & Leong, S.F. & Chua, K.H. & Tan, C.H. & Chan, H.Y. & Tong, E.V. & Wong, Stephenie & Chan, H.K. Survival of commercial probiotic strains to pH and bile. *International Food Research Journal*. (2011). 18. 1515-1522.
35. Salamoura, Chrysoula & Kontogianni, Artemis & Katsipi, Despoina & Kandyli, Panagiotis & Varzakas, Theo. Probiotic fermented milks made of cow's milk, goat's milk and their mixture. *Journal of Biotechnology*. (2014). Vol 185.
36. Stanton, Catherine & Fitzgerald, Gerald & Ross, R & Desmond, Colette & Coakley, Mairead & Collins, J. Challenges Facing Development of Probiotic-Containing Functional Foods. (2003)
37. Verschuere, L et al. "Probiotic bacteria as biological control agents in aquaculture." *Microbiology and molecular biology reviews : MMBR* vol. 64,4 (2000): 655-71.