

PROBIOTICS FOR FISH CULTURE

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

by

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**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
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Pooja V (39230031)**, who carried out the project entitled "**PROBIOTICS FOR FISH CULTURE**" under our supervision from October 2022 to April 2023.



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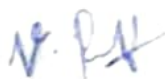
External Examiner

DECLARATION

I, POOJA V (39230031), hereby declare that the Project Report entitled "PROBIOTICS FOR FISH CULTURE" done by me under the guidance of Dr. M. MASILAMANI SELVAM, at the Department of Biotechnology, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 11/05/2023

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

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ABSTRACT

Potential Bifidobacterium strains and other species that are employed for microbial supplementary nutrition of humans and animals are of great interest to food and medical microbiologists. A number of advantages are conferred on the human host by the presence of the native microbiota, ranging from the competitive exclusion of exogenous, frequently pathogenic, microbial species that are pervasive in the environment to the breakdown of complex dietary substances in the gastrointestinal tract.

The current study was completed to identify microscopic organisms from various species that were separated from various sources, which could be identified with the distinct nourishing status of the segregation locals, such as soil from the rhizosphere of a plant, which is beneficial for animal and human use. The bacterial strains enclosed were examined for a few trials like antimicrobial action, antibacterial sensitivity, biochemical testing and siderophore production assays. The results show the characteristics of the segregated strain. Strains from soil samples were identified using the 16S rRNA gene sequence.

In vitro tests were carried out on the bacterial strains. The Bacterial sample is fed to the fish as probiotic feed to monitor their behaviour. The assessment of the potential health benefits of probiotic cultures in vitro generally requires the investigation of the pharmacokinetic outlook (distribution and eradication) of biotherapeutic agents over time after administration to humans and animals. Weight and length are measured as growth parameters. The current study discovered that various isolates from traditional sources, as well as strains isolated from soil, are used as probiotics. Furthermore, some isolated soil strains demonstrated antibacterial and antimicrobial activity against some bacteria. Moreover, an In depth understanding of the bacteria's in vivo physiological properties and impacts on the host, as well as the identification and quantification of these bacterial groups in a variety of food and biological materials, are all being pursued.

Keywords - bacteria, probiotics, microorganisms, antibacterial, antimicrobial

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LIST OF ABBREVIATIONS

SYMBOL	ABBREVIATION
LAB	<i>Lactobacillus</i> species
&	And
Spp	Species
L. Subspecies	<i>Lactobacilli</i>
B. Subspecies	<i>Bifidobacterium</i>
E. Subspecies	<i>Enterococcus</i>
S. Subspecies	<i>Saccharomyces</i>
Ph	Power of hydrogen
μ l	Micro liter
IAA	Indole Acetic Acid
MRS	De Man Rogosa Sharpe
TOS-MUP	Transgalctosylated oligosaccharide agar medium
LB	Luria-Bertani
CAS	chrome azurol S
ml	Milliliter
Hrs	Hours
Min	Minute
°C	Degree Celsius
Gms	Grams
%	Percentage
Ppm	Parts Per Million
DO	Dissolved Oxygen
Cm	Centimeter
TPC	Total Plate Count
S subunit	Svedberg subunit

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CHAPTER-1

3. INTRODUCTION

3.1 WHAT IS PROBIOTICS

The original definition of probiotics as organisms and substances contributing to intestinal microbial balance was provided by Parker (1974). Over the last three decades, several definitions of probiotics have been modified and proposed. Fuller (1989) gave a precise definition of probiotics that is still widely referred to: Probiotics are a live microbial feed supplement, which beneficially affects the host by improving its intestinal balance. A probiotic is a mono or mixed culture of live microorganisms to improve the properties of the indigenous microflora (Havenaar, Ten Brink, & Huisin't Veld 1992). Moriarty (1998) proposed to extend the definition of probiotics in aquaculture to microbial "water additives." Simply, probiotics are often defined as live intestinal bacteria that are added to promote the viability of the host (Skjermo & Vadstein 1999). Probiotics can also be defined as 4microbial cells administered through the gastrointestinal tract with the aim of improving health of the hosts (Gatesoupe 1999). Probiotics have also been defined as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO 2002). The intestinal microbiota does not exist as an entity by itself; there is a constant interaction with the environment and the host (Verschuere et al. 2000). The relationship of the intestinal microbiota to the aquatic habitat or food has been investigated (Cahill 1990). The host-microbe interactions are often qualitatively and quantitatively different for aquatic and terrestrial species as in the aquatic environment, where the hosts and microorganisms share the ecosystem (Verschuere et al. 2000). Aquatic animals are surrounded by an environment that supports their pathogens independently of the host animals, and opportunistic pathogens can reach high densities around the animals (Moriarty 1998). Generally in aquaculture, probiotics are live microbial feed supplements in the form of mixed strains that are administered for improving the rearing water quality, enhancing the physiological and immune responses of aquatic animals, and reducing the use of chemicals and antibiotics.

3.2 CHARACTERISTICS OF PROBIOTICS

The following traits of strains are crucial before they are even given a chance to be used as probiotics: resistance to bile acid, bile salt hydrolase activity, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria or fungi, ability to reduce pathogen adhesion to surfaces, resistance to gastric acidity, and resistance to bile acid.

Some of the major health benefits attributed to probiotics include improvement of gastrointestinal microflora, enhancement of immune system, reduction of serum cholesterol, cancer prevention, treatment of irritable bowel-associated diarrhoeas, antihypertensive effects as well as improvement of lactose metabolism ([Saarela et al. 2000](#); [Nagpal et al. 2012](#)).

3.3 POTENTIAL ROLE OF PROBIOTICS

Probiotics are used in a variety of novel functional food products, including increasing the number of dietary ingredients linked to health claims. There are many different types of bacteria in the marine environment that can prove to be efficient and secure probiotics. current state of the science has raised the possibility of their usage in a number of contexts, such as energy drinks for athletes, fortified foods and beverages, and dairy products. such is dairy products with prebiotics, like yoghurt, cheese, ice cream, and milk. The probiotics sector is booming, but it needs a lot of support. depends on additional study, support from science, and biotechnological intervention while investigating the marine environment. The speed of growth Antibiotic resistance is a serious public health issue (Neu, 1992). all throughout the world (Bengmark, 1998). The immunostimulatory properties of probiotics have the potential to increase immunity (Marteau and Rambaud, 1993).the host's resilience to illness, possibly lowering the incidence of using antibiotics. Probiotic use has been suggested by a number of people.a place of antibiotics in animals, according to researchers (Canganella et al., (Underdahl et al., 1982; Gardiner et al., 1999; Fuller, 1989) and poultry (Edens et al., 1997; Gardiner et al., 1999).the creation of novel marine A better substitute for this would be probiotics for pathogen inhibition. increased worry about antibiotic resistance.

Salmonella enteritidis, enterotoxigenic Escherichia coli, and other enteropathogens have been demonstrated to be inhibited by certain probiotic strains. Gonzalez et al. (1993) and Drago et al. (1997; Serratia marcescens, in vitro) may have significant therapeutic potential in this area. According to a paper, Lactobacillus has antagonistic effects on Salmonella typhimurium C5 infection in both in vitro and in vivo settings (Hudault et al., 2016).1997). These investigations offer proof and serve as a foundation for the clinical usage of probiotics in pathogen eradication In addition to the antibacterial action of therapeutic significance stated above, potential uses for probiotic bacteria in clinical settings include the management of eating disorder (Salminen et al, lowering in blood pressure (Hata et al.,1996) and their usage as vehicles for the administration of oral vaccinations (Pouwels et al.,1996).

An efficient procedure for research on food-grade genetic the following would be markers (Allison and Klaenhammer, 1996; Lin et al., 1996): Probiotic formulations against antibiotic-resistant genes are greatly benefited. Probiotics not only help keep living things healthy but may also ward against bacterial infections in the urogenital tract (Reid and Bruce, Reid et al., 1994, 1998; 2006). Some probiotic strains have also been demonstrated to lessen the chance of infections brought on by using medical equipment. The creation of is yet another possible use for probiotic microorganisms. goods made from fermented food that are packed with healthy ingredients, like as linoleic acid conjugated (CLA). CLA has anti-arcinogenic and anti-atherogenic properties, as well as the capacity to lower the immunological stimulation's catabolic consequences and capacity to promote growth promotion, as well as the capacity to lower body fat. foods made with animal products CLA is abundant in fat, which can be found in beef, lamb, milk, and dairy products. (Stanton and others, 1997) Several Propionobacteria strains, frequently utilised as It has been demonstrated that dairy starter cultures can change free linoleic acid into CLA (Jiang, 1998), indicating that fermentation may make it possible to produce fermented food products enriched in CLA. Probiotics may hold a number of other potential applications which are still uncovered due to lack in technological interventions in this field.

3.4 PROBIOTIC BACTERIA IN USE

The probiotic conception is open to different operations in a large variety of fields applicable for mortal and beast health. Probiotic products correspond of different enzymes, vitamins, capsules or tablets and some fermented foods contain microorganisms which have salutary goods on the health of host. They can contain one or several species of probiotic bacteria. The first step in the selection of microbial strains for probiotic use is it must be representative of microorganisms that are Generally Regarded as safe-deposit box. It includes some of the lactic acid directors like *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus* and *Leuconostoc* species. Among these *Lactobacillus* and *Bifidobacterium* are the two prominent groups which are constantly employed in foods.

Selection of Probiotics

A common way to screen the seeker probiotics is to perform in vitro enmity test, since bacterial antago nism is a common miracle in nature. thus, sev eral investigators have used this miracle to elect campaigners by in vitro tests, in which pathogens are ex posed to the seeker probiotics or their extracellular products in liquid and/ or solid medium. nonetheless, it has been reported that in vitro exertion using liquid and/ or solid medium can't be used to prognosticate a possible effect in vivo. For illustration, in vitro enmity of *Pseudomonas fluorescents*(strain AH2) against *A. salmonicida* did not confer protection in Atlantic salmon against furuncu losis, but it conferred protection against vibriosis(Gram et al., 2001). thus, it's necessary to know the origin of the stock(rather to use strains insulated from the host in study) and its security(non-pathogenic). Some bacteria used as seeker probiotics have antiviral goods. Although the exact medium by which these bacteria do this isn't known, laboratory tests indicate that the inactivation of contagions can do by chemical and natural substances, similar as excerpts from marine algae and extracellular agents of bacteria. It has been reported that strains of *Pseudomonas* sp., *Vibrio* sp., *Aeromonas* sp., and groups of coryneforms insulated from salmonid hatcheries, showed antiviral exertion against contagious hematopoietic necrosis contagion(IHNV) with further than 50 shrine reduction(Kamei et al., 1988). Girones et al.(1989) reported that a marine bacterium, tentatively classified in the rubric *Moraxella*, showed antiviral capacity, with high

particularity for poliovirus. Direkbusarakom et al. (1998) isolated two strains of *Vibrio* spp. NICA 1030 and NICA 1031 from a black barracuda shrimp hatchery. These isolates displayed antiviral activities against IHNV and *Oncorhynchus masou* contagion (OMV), with probabilities of shrimp reduction between 80 and 99, independently.

3.4.1 Lactobacilli species

The genus *Lactobacillus* is a taxonomically complex and is composed of over 170 species that can not be fluently discerned phenotypically and frequently bear molecular identification. Although they're part of the normal mortal gastrointestinal and vaginal flora, they can also be occasional mortal pathogens. They're considerably used in a variety of marketable products including probiotics. Their antimicrobial vulnerability are inadequately defined in part because of their taxonomic complexity and are compounded by the different styles recommended by Clinical Laboratory norms Institute and transnational Dairy Foundation. Their use as probiotics for forestallment of *Clostridium difficile* infection is current among consumers worldwide but raises the question of will the use of any concurrent antibiotic effect their capability to survive. *Lactobacillus* species are generally acid resistant and are suitable to survive ingestion. They're generally resistant to metronidazole, aminoglycosides and ciprofloxacin with *L. acidophilus* being susceptible to penicillin and vancomycin, whereas *L. rhamnosus* and *L. casei* are resistant to metronidazole and vancomycin.

3.4.2 Saccharomyces Species

Saccharomyces boulardii is a probiotic incentive frequently used for the treatment of GI tract diseases similar as diarrhea symptoms. It's genetically close to the model incentive *Saccharomyces cerevisiae* and its bracket as a distinct species or a *S. cerevisiae* variant has long been bandied. we review the main inheritable divergencies between *S. boulardii* and *S. cerevisiae* as a strategy to uncover the capability to acclimatize to the host physiological conditions by the probiotic. *S. boulardii* does retain perceptible phenotypic traits and physiological parcels that uphold its success as probiotic, similar as optimal growth temperature, resistance

to the gastric terrain and viability at low pH. Its probiotic exertion has been illustrated as a confluence of multiple pathways, ranging from enhancement of gut hedge function, pathogen competitive rejection, product of antimicrobial peptides, vulnerable modulation, and trophic goods. This review summarizes the participation of *S. boulardii* in these mechanisms and the multifactorial nature by which this incentive modulates the host microbiome and intestinal function.

3.4.3 Bifidobacterium Species

Bifidobacterium is a non-spore-forming, Gram-positive, anaerobic probiotic actinobacterium and generally set up in the gut of babies and the uterine region of pregnant mothers. Like all probiotics, *Bifidobacteria* confer health benefits on the host when administered in acceptable quantities, showing multifaceted probiotic goods. exemplifications include *B. bifidum*, *B. breve*, and *B. longum*, common *Bifidobacterium* strains employed to help and treat gastrointestinal diseases, including intestinal infections and cancers. Herein, we review the rearmost development in probiotic *Bifidobacteria* exploration, including studies on the remedial impact of *Bifidobacterial* species on mortal health and recent sweats in engineering *Bifidobacterium*. This review composition would give compendiums with a wholesome understanding of *Bifidobacteria* and its capabilities to ameliorate mortal health.

3.5 APPLICATIONS OF PROBITICS ON AQUACULTURE

The need for sustainable monoculture has promoted exploration into the use of probiotics on submarine organisms. The original interest was concentrated on their use as growth promoters and to ameliorate the health of creatures; still, new areas have been Setup, similar as their effect on reduplication or stress forbearance, although this requires a more scientific development.

Growth Promoter

Probiotics have been used in aquaculture to increase the growth of cultivated species, in reality it is not known whether these products increase the appetite, or if, by their nature, ameliorate insipidity. Some people are inclined to suppose that it could be both factors; likewise, it would be important to determine whether probiotics actually taste good for monoculture species. According to Balcazar et al., probiotic microorganisms are suitable to populate gastrointestinal tract when administered over a long period of time because they've a advanced addition rate than the rate of expatriation, so as probiotics constantly added to fish societies, they cleave to the intestinal mucosa of them, developing and exercising their multiple benefits. This also depends on factors similar as hydrobionts species, body temperature, enzyme situations, inheritable resistance, and water quality. The effect of probiotics has been tested on phytoplankton (microalgae), which forms the base of submarine food chains, due to its nutrient- producing photosynthetic machinery.

Inhibition of Pathogens

Antibiotics were used for a long time in monoculture to help conditions in the crop. still, this caused colorful problems similar as the presence of antibiotic remainders in beast apkins, the generation of bacterial resistance mechanisms, as well as an imbalance in the gastrointestinal microbiota of submarine species, which affected their health. In fact, the European Union has regulated the use of antibiotics in organisms for mortal consumption moment, consumers demand natural products, free of Complements similar as antibiotics; also, there's a tendency for precluding conditions rather than treating them. therefore, the use of probiotics is a feasible volition for the inhibition of pathogens and complaint control in monoculture species.

Improvement in Nutrient Digestion

A study has suggested that probiotics have a salutary effect on the digestive processes of submarine creatures because probiotic strains synthesize extracellular enzymes similar as proteases, amylases, and lipases as well as give growth factors similar as vitamins, adipose acids, and amino acids. thus, nutrients are absorbed more efficiently when the feed is supplemented with probiotics, probiotics have been used in comestible fishes, as in the case of naiads of European bass(*Dicentrarchus labrax*). It has been reported that the probiotic incentive *Debaryomyces hansenii* HF1 has the capability to produce spermine and spermidine, two polyamines involved in the isolation and development of the gastrointestinal tract in mammals. In addition this incentive secretes amylase and trypsin, enzymes that aid digestion in ocean bass naiads . Studies in juvenile common Dentex showed that when diet is supplemented with 0.5 g of *Bacillus cereus* strain E kg ⁻¹ of food, increased fish growth due to more effective use of the food. In the case of rainbow trout, analogous results were attained using *B. subtilis*, *B. licheniformis*, and *Enterococcus faecium*, when these probiotics were handed for 10 weeks on with the diet of fish.

Improvement of Water Quality

Water quality was recorded during the addition of probiotic strains especially of the gram-positive rubric *Bacillus*. presumably since this bacterial group is more effective than gram-negative in transubstantiating organic matter to CO₂. It's suggested that maintaining high situations of probiotics in product ponds, fish growers can minimize the accumulation of dissolved and particulate organic carbon during the growing season. In addition, this can balance the product of phytoplankton.

Stress Tolerance

Monoculture practices demand intensive products in shorter times, causing stress in crop species. For illustration, it has been reported that habitual stress in zebra fish, *Danio rerio*, induces a general depression on the conflation of muscle protein. As a result, it was sought to increase stress forbearance by using probiotics. One of the firsts formal reports on this field studied the supplementation of *Lactobacillus delbrueckii* sp in the diet of European ocean bass (*Dicentrarchus labrax*), at time intervals of 25 to 59 days. In addition to assessing the growth enhancement, hormone cortisol was quantified in fish towel as stress marker, since it's directly involved in the beast's response to stress. Cortisol situations attained in the treated fishes were significantly lower than those in the control $3.6 \pm 0.36 \text{ ng g}^{-1}$.

Effect on Reproduction of Aquatic Species

According to Izquierdo et al, breeding monoculture species have high nutritive conditions, therefore reproductive capacity depends on applicable attention of lipids, proteins, Adipose acids, vitamins C and E, and carotenoids. likewise, the relationship of these factors influences reproduction in colorful processes similar as fertility, fertilization, birth and development of naiads. At present, for utmost dressed fish species, there are commercially available "brood stock diets" that just are larger- sized diets. In practice, numerous fish hatcheries ameliorate the nutrition of their brood stock by feeding them solely on fresh fish derivations or in combination with marketable diets. The most common fresh organisms used to feed brood stock fishes include squid, cuttlefish, mussels, krill, and small crustaceans. The use of these undressed fish products frequently don't give acceptable situations of nutrients demanded by brood stock fishes, adding the threat of pathogens transmission to the parents and seed, including spongers, bacteria, and contagions. thus, probiotics added to food or water were used in order to help infections and to explore their effect on reduplication.

CHAPTER-2

4. LITERATURE SURVEY

- Arunachalam.K et al.,[2000], their work was based on determining the effects of dietary consumption of Bifidobacterium lactis (strain HN019,DR10TM) on natural immunity
- Bharti Mahajan and Vinodh Singh, [2014], their work illustrates the health benefits of probiotics in various diseases and their possible mechanism and also highlights current challenges for future studies.
- Carlos Ricardo Soccel et al.,[2010], their review is based on determining which in vitro, animal or human assessments meet the minimum criteria for being considered a probiotic.
- Carlos Ricardo Soccol et al.,[2010],their review is based on the history of probiotics, their application in the health and food areas and new trends in probiotic products and processes are presented.
- Hemaiswarya S et al., [2013], their objective of the study was to provide a deep insight into the effect of probiotics against the diseases, their applications and proposed mechanism of action.
- Joel Ogbonna et al., [2010], their review work describes the cause of the loss of life of fishes, so they decided on physicochemical and microbiological parameters were used to determine the concentration which led to deadly effect on the fishes.
- Jose Luis Balcazar et al., [2009], their work describes the state of the art of probiotic research in preventing and controlling infectious diseases in aquaculture, with the mechanisms of actions involved in it.
- Lagos.L et al., [2015], their review work provides an overview of the current knowledge on the study of bacterial community in the rhizosphere by using modern molecular techniques, describing the bias of classical molecular techniques, next generation sequencing platforms and post-genomics techniques.
- Maria Kechagia et al., [2012], their work was based on the concept and the possible beneficial properties of probiotic bacteria, focusing on those available in foods.
- Mercenier.A et al.,[2008], their review is based on determining which in vitro, animal or human assessments meet the minimum criteria for being considered a probiotic.

- Mohapatra.S et al., [2012], their work describes the desirable traits of probiotics, their mode of action & their useful effects in fishes and also highlights the role of probiotics in helping the fishes to combat against the different physical, chemical and biological stress.
- Nayak.S.K, [2010], their review work is based on, aiming to highlight the immunomodulatory activity of probiotics and also to evaluate the factors that regulate for the optimum induction of immune responses in fish.
- Patricia Martinez Cruz,[2012], The aim of this paper is to present an updated concept of probiotics, current uses in aquaculture, commercial presentations, and a final approach to prospect applications in this area.
- Westerik.N et al., [2016], their article describes the production protocol that enables the production of nutritious and probiotic fermented milk and other probiotic fermented foods with the help of a seed culture that proven health benefits.
- Gadhiya. D et al., (2015), their work is based on recent advances in the technologies aimed at incorporating probiotics into chocolate and related products, the ways to enhance or sustain their viability in the presence of stressed surroundings throughout the manufacturing process, and their market potential with future prospects.
- Abul Kalam Azad.Md et al., [2018], their work was based on the review of several *Lactobacillus* spp., *Bifidobacterium* spp., and other coliform bacteria as the most promising probiotic species and their role in the prevention of degenerative diseases.
- Andrew Pradeep M et al., [2017], their review aims to evaluate the antimicrobial effect of probiotic organism and to compare the safety therapeutic index over the commercially available antibiotics.
- Chiron.C et al., [2017], their work was based on applying flow cytometry methods to probiotic enumeration and viability assessment in the multistrain products.
- Huu-Thanh Nguyen et al., [2016], their review is based on the different technical tools that can be used in order to define the proper condition for improving viability of probiotic bacteria and their implementation at the industrial scale.
- Julio Plaza-Diaz et al., [2019], their review work was based on the main mechanisms of actions of probiotics, including colonization and normalization of perturbed intestinal microbial communities in children and adults; competitive exclusion of

pathogens and bacteriocin production; modulation of fecal enzymatic activities associated with the metabolism of bile salts and inactivation of carcinogens and other xenobiotics; production of short-chain and branched-chain fatty acids, which, in turn, have wide effects not only in the intestine but also in the peripheral tissues via interactions with short-chain fatty acids receptors, modulating mainly tissue insulin sensitivity; cell adhesion and mucin production; modulation of the immune system, which results mainly in the differentiation of Tregulatory cells and up regulation of anti-inflammatory cytokines and growth factors, i.e., interleukin-10, and transforming growth factor ; and interaction with the brain-gut axis by regulation of endocrine and neurologic functions.

- Madhu Prakash Srivastava et al., [2018], their review study was based on the detection of Siderophore production from different cultural variables by CAS agar plate assay in reference the rate of colour change of CAS medium (in millimeters) as a function of time (in days) when microorganisms were grown in CAS agar plates and also evaluate the effect of the Inoculum type, iron concentration and pH.
- Nuredin Mohamedkassm Siraj et al.,[2017],their objective of this study was to isolate, screen and identify potential probiotic bacterial strains from soil samples collected from cattle farm areas in Dibrugarh district, Assam, India
- Pairat Sornplang et al.,[2016], their research work is based on selecting probiotic strains from unconventional sources with specific properties and, technologies that can be used to improve the probiotic production process.
- Sameer Singh Faujdar et al., [2016], their review work is based on , focus on the role of probiotics in health in general and oral health in particular.
- Sanders.M.E et al., [2018], their review work is based on the history of probiotics, their application in the health and food areas and new trends in probiotic products and processes.
- Sanders.M.E et al., [2018], their review work provides current perspective on the probiotic concept, discusses evidence-based probiotic interventions, considered fermented foods with regard to probiotics and addresses some regulatory challenges facing the probiotic field.
- Sayyed Kamaledin Allameh et al., [2017], their work was based on the effects of probiotic bacteria on rearing parameters, immune system response and inhibitor activities against pathogenic bacteria in fish.

CHAPTER-3

5. AIM AND OBJECTIVES

5.1 AIM :-

- To isolate probiotic species from marine sources and to estimate it's efficiency.

OBJECTIVES :-

- Sample collection
- Isolation of bacteria
- Screening of pure culture
- Growth analysis

5.2 PROBLEM STATEMENT

- Testing of the isolated species and the trial of the product developed, For the purpose of preventing and controlling diseases in aquatic animals and to promote the growth of microorganisms for numerous health benefits when consumed by improving the Human Immune system.

5.3 SCOPE OF THE PROJECT INVESTIGATION

The research of probiotics for aquatic animals is increasing with the demand for environment friendly aquaculture. The probiotics were defined as live microbial feed supplements that improve health of man and terrestrial livestock. The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut, with continuous intrusion of microbes coming from water and food. Some commercial products are referred to as probiotics, though they were designed to treat the rearing medium, not to supplement the diet.

This extension of the probiotic concept is pertinent when the administered microbes survive in the gastrointestinal tract. Otherwise, more general terms are suggested, like biocontrol when the treatment is antagonistic to pathogens, or bioremediation when water quality is improved. However, the first probiotics tested in fish were commercial preparations devised for land animals. Though some effects were observed with such preparations, the survival of these bacteria was uncertain in aquatic environment. Most attempts to propose probiotics have been undertaken by isolating and selecting strains from aquatic environment.

To meet global demand for fish protein, the aquaculture business produces a substantial amount of aquaculture species. To expand production in the foreseeable future, the sector is moving toward intensification. To advance aquaculture to the next level, numerous technologies and initiatives were implemented. However, disease restrictions are a barrier to the aquaculture industry's development.

As a result, large amounts of antibiotics and pesticides were used in aquaculture farming to reduce or decrease the risk of disease breakout. The aquaculture products and the environment were thereafter heavily exposed to these antibiotics and toxins. To overcome this problem, aquaculture species health management alternatives were pushed for use by fish producers.

CHAPTER- 4

6. APPARATUS REQUIRED AND METHODOLOGY

6.1 APPARATUS USED

➤ **Bacterial Isolates –**

The bacterial isolates used in this research study were isolated from two different sources: rhizosphere soil sample.

➤ **Chemicals –**

The chemicals used are Pikovskaya's Media, MRS agar (SRL), MRS broth (SRL), Nutrient agar(Hi-media), TOS-MUP agar, Muller Hinton agar (Hi-media), Phenol red Lactose Broth, LB broth, CAS agar, Antibiotic discs (Streptomycin, Chloramphenicol, Ampicillin, Tetracycline), Pathogens (Staphylococcus aureus and Escherichia coli), Glycerol, Calcium tri phosphate, distilled water.

➤ **Equipments –**

The equipments used are petri plates, test tubes, glass beakers, Micropipette, Weighing balance, autoclave, pH meter, micro-oven, inoculation loop, non-absorbent Cotton, shaker, Hot air oven, measuring cylinder, Cork borer, sterile cotton swabs.

6.2 METHODOLOGY

6.2.1 Sample Collection

The Sample used in the process was marine soil sample which is collected from the rhizosphere of the *Spreading dayflower* (Scientific Name – ***Commelina Benghalensis***).



Fig:4.1 Common name – Spreading dayflower

6.2.2 Isolation of Microbes from soil sample

➤ **SERIAL DILUTION -**

The sample (1 gram) is mixed with 10ml sterile pure water to make solution sample. This solution sample is diluted up to 10^{-5} serial dilution. Serial dilution is a process of diluting a sample several times.



Fig:4.2 serial dilution method

Then 9ml of sterile distilled water is added to the five test tubes. By using micropipette ($1000\mu\text{l}$), 1ml of solution sample is transferred into 10^{-1} tube. Mix the test tube properly. Continue dilution with aseptic technique for test 10^{-2} until 10^{-5} .

➤ **POUR AND STREAK PLATE METHOD -**

1ml of an appropriately diluted culture is added to the Petriplate using micropipette and Pikovskaya agar is added & mixed with the sample diluted. The plate is then

incubated until the colonies appear. The bacterial colonies is then streaked to achieve pure culture.

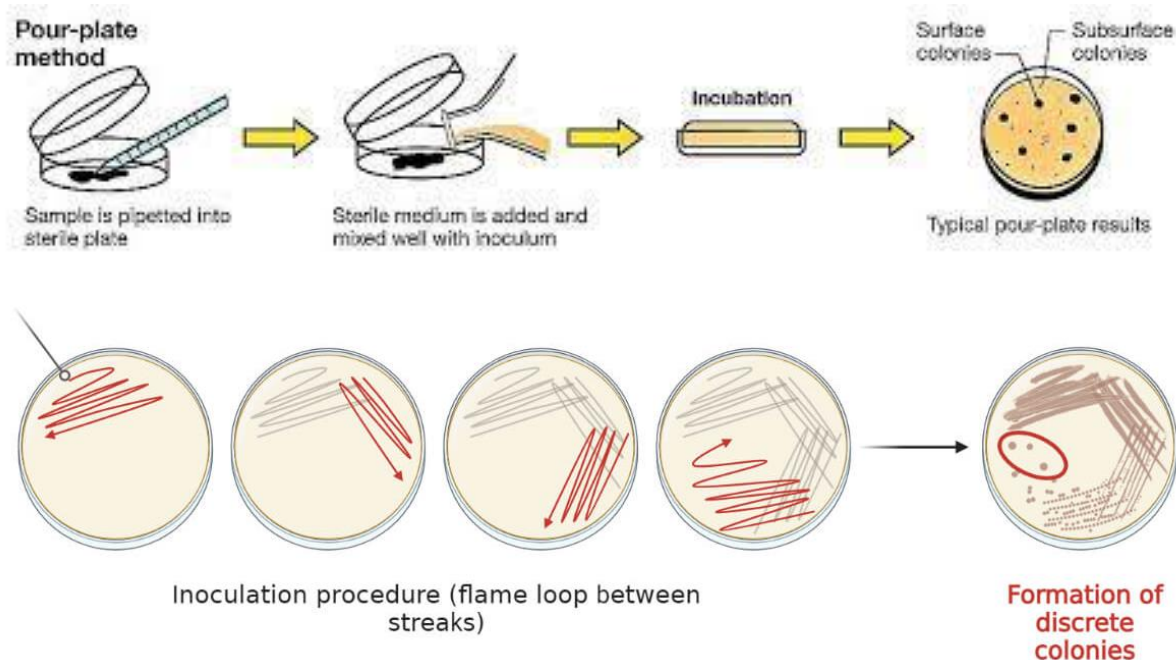


Fig:4.3 Pour and Streak plate method

6.2.3 CHARACTERIZATION OF BACTERIAL STRAINS

SCREENING TESTS –

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

6.2.3.1 Carbohydrate Fermentation Test

In accordance with the various enzymes present, many organisms like bacteria utilize carbohydrates in different ways to produce energy. While certain species can ferment carbohydrates like glucose anaerobically, others must employ the aerobic method. This test aims to identify an organism's capacity to produce acid from the fermentation of a specific carbohydrate.

Preparation of the media –

1. Insert a Durham tube to detect gas production.
2. Autoclave the prepared test media (at 121°C for 15 mins) to sterilize. The prepared broth media will be a light red color and the final pH should be 7.4 ± 0.2 .
3. Inoculate each test tube with the test microorganism.
4. Incubate tubes at 35-37°C for 18-24 hours

6.2.3.1.2 Indole acetic acid test

This test demonstrates the ability of certain bacteria to decompose the ammonia acid tryptophan to Indole, which accumulates in the medium. Indole production test is important in the identification of bacteria.

Principle -

Indole test is used to determine the ability of an organism to split amino acid tryptophan to form the compound Indole. Tryptophan is hydrolyzed by tryptophanase to form three end products – one of which is Indole. Indole production is detected by Kovac's reagent or Ehrlich's reagent which contains 4 (p)-dimethylamino benzaldehyde, this reacts with Indole to produce a red coloured compound.

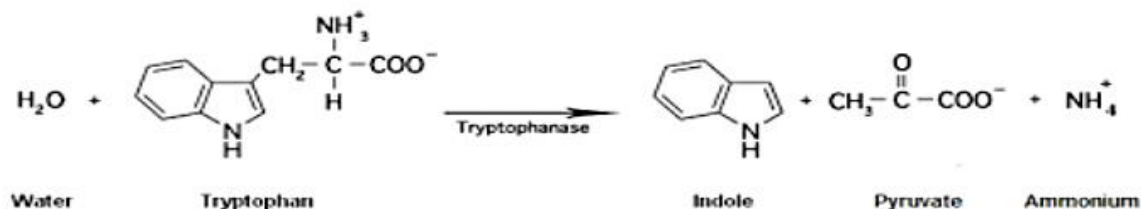


Fig:4 Indole test reaction

Procedure for Indole test -

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.

6.2.3.2 Antimicrobial Activity Test

Agar Well Diffusion Method

- **Preparation of Inoculum** - Stock cultures of the isolated bacterias 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** - Antibacterial activity of sample was determined by well diffusion method on Muller Hinton agar (MHA) medium. The Muller Hinton agar medium was weighed as 3.8 gms and dissolved in 100ml of distilled water and add 1gm of agar. Then the medium is kept for sterilization. After sterilization the media was poured into sterile petriplates and were allowed to solidify for 1hr. After the medium was solidified, the inoculums were spread on the solid plates with sterile swab moistened with the bacterial suspension. Wells were made using cork borer. Pathogens (*Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, and *Escherichia coli*) Samples of respective concentrations (50µl and 100 µl) were loaded in respective wells. These plates were incubated for 24 hrs at 37°C. Then the microbial growth was determined by measuring the diameter of zone of inhibition.

6.2.3.3 Antibiotic Susceptibility Test

Antibiotic sensitivity was determined by disc diffusion method on Muller Hinton agar (MHA) medium. The Muller Hinton Agar medium was weighed as 3.8gms and dissolved in 100ml of distilled water and add 1gm of agar. Then the medium is kept

for sterilization. After sterilization the media was poured in to sterile petriplates and were allowed to solidify for 1hr. After the medium was solidified, the inoculums were spread on the solid plates with sterile swab moistened with the bacterial suspension and antibiotic discs (Streptomycin, Nystatin, and Tetracycline) were placed. These plates were incubated for 24 hrs at 37°C. Then the microbial growth was determined by measuring the diameter of zone of inhibition.

6.2.3.4 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 basic principle underlying the test is that when a strong ligand (eg. Siderophore) is added to a highly coloured dye Fe^{3+} complex, the iron-ligand complex is formed and the release of free dye is accompanied by a colour change.
- **Procedure** -
 1. CAS agar plates were prepared by mixing 10 ml CAS reagent in 90 ml sterilized LB agar medium.
 2. Bacterial strains were spot inoculated on each plate.
 3. After inoculation, plates were incubated at 28 °C for 72 hrs and observed for the formation of orange zone around the bacterial colonies.

6.2.4 IN VIVO TEST

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

Probiotic treatment in fishes

The black moor , koi , gold fish and iridescent shark were collected. The fishes were

visually looked out for their active conditions and were stocked in plastic container for three days for adaptation to laboratory conditions.



Fig:4.4 Gold fish, Iridescent shark, Black moor

Six fishes were tested for each fresh water tank with aerator. Two tanks are taken, where one is considered as control and the other one as sample. The experiment was conducted for 30 days in 40 litre glass tank. The sample culture is being tested in the fishes by checking its growth analysis and water quality. The 5ml of culture was added to the sample tank for every 2 days. Water exchange was made for 20% of the culture water every morning. Feeding was given at a rate of 10% of the body weight, once daily.

6.2.4.1 Analysis of Physio-Chemical Parameters

6.2.4.1.1 TEMPERATURE -

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

6.2.4.1.2 pH -

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

6.2.4.1.3 Determination of Ammonia Level

The fish release ammonia into the water from their gills, urine and excrement. Ammonia damages gill membranes and prevents respiration and reduces the immune system. Ammonia kit analyses define the amount of ammonia level present

in the water sample.

Method –

1. Fill a clean test tube with 5ml of water to be tested.
2. Add 8 drops from Ammonia Test Solution 1, by holding the dropper bottle upside down in a completely vertical position to assure uniformity of drops.
3. Add 8 drops from Ammonia Test Solution2, by holding the dropper bottle upside down in a completely vertical position to assure uniformity of drops.
4. Cap the test tube and shake vigorously for 5 seconds.
5. Wait for the color to develop. 6. Read the test results by comparing the color of the solution to the appropriate ammonia color card. The closet match indicates the ppm (mg/l) of ammonia in the water sample.

6.2.4.1.4 Determination of Oxygen Level

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

Method –

1. Fill a clean test tube with 5ml of water to be tested.
2. Add 4 drops from Dissolved oxygen Test Solution 1, by holding the dropper bottle upside down in a completely vertical position to assure uniformity of drops.
3. Add 4drops from Dissolved oxygen Test Solution2, by holding the dropper bottle upside down in a completely vertical position to assure uniformity of drops.
4. Cap the test tube and shake vigorously for 5 seconds.
5. Wait for the color to develop.
6. Read the test results by comparing the color of the solution to the appropriate dissolved oxygen color card. The closet match indicates the (mg/l) of dissolved oxygen in the water sample.

6.2.4.2 Fish Growth Analysis

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

6.2.4.2.1 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.

6.2.4.2.2 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.

6.2.4.3 Microbial analysis

The water samples collected from the sample and control fish tank was checked for bacterial growth. The bacterial growth is calculated using the TPC (Total plate count) method. The samples are serially diluted and pour plate method is done to count the number of colonies grown. Total plate count gives us the colony forming unit per ml.

6.2.5 Identification of Bacterial Strain

Identification of Microbial culture using 16srRNA sequencing method –

Sample Name : 1

Sample Type : Bacteria

Method

- DNA Isolation
- PCR Amplification
- Data Analysis

Steps followed

Genomic DNA was isolated from the sample provided by the scientist. The ~1.5 kbp, 16S-rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was sequenced Bi-directionally. The sequence data was aligned and analyzed to identify the Bacteria and its closest neighbors.

Protocol

DNA Extraction –

- The sample was picked up and placed in a mortar and homogenized with 1 ml of extraction buffer and the homogenate was transferred to a 2 ml-microfuge tube.
- An equal volume of Phenol: Chloroform: Isoamylalcohol (25:24:1) was added to the tubes and mixed well by gently Shaking the tubes.
- The tubes were centrifuged at room temperature for 15 min at 14,000 rpm.
- The upper aqueous phase was collected in a new tube and an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed.
- The upper aqueous phase obtained after centrifuging at room temperature for 10 min at 14,000 rpm was transferred to a new tube.
- The DNA was precipitated from the solution by adding 0.1 volume of 3 M Sodium acetate pH 7.0 and 0.7 volume of Isopropanol.
- After 15 min of incubation at room temperature the tubes were centrifuged at 4°C for 15 min at 14,000 rpm.
- The DNA pellet was washed twice with 70% ethanol and then very briefly with 100% ethanol and air dried.
- The DNA was dissolved in TE (Tris-Cl 10 mM pH 8.0, EDTA 1 mM).
- To remove RNA 5 µl of DNase free RNase A (10 mg/ml) was added to the DNA.

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 MgCl₂
- 4) PCR Enzyme Buffer

Sequencing –

1. The purified PCR amplicon was sequenced using either of the Universal forward and reverse primers.
2. Sequencing was done using BDT v3.1 Cycle sequencing kit on ABI 3500 Genetic Analyzer.

6.2.6 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.

6.2.6.1 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.

6.2.6.2 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.

CHAPTER-5

7. RESULT AND DISCUSSIONS

➤ ISOLATION OF MICROBES FROM SOIL SAMPLE

Several distinct colonies were formed in the petri plates, out of which the dilution 10^{-2} and 10^{-5} shows the clear colony which provided inhibition to the surrounding bacterial colonies.

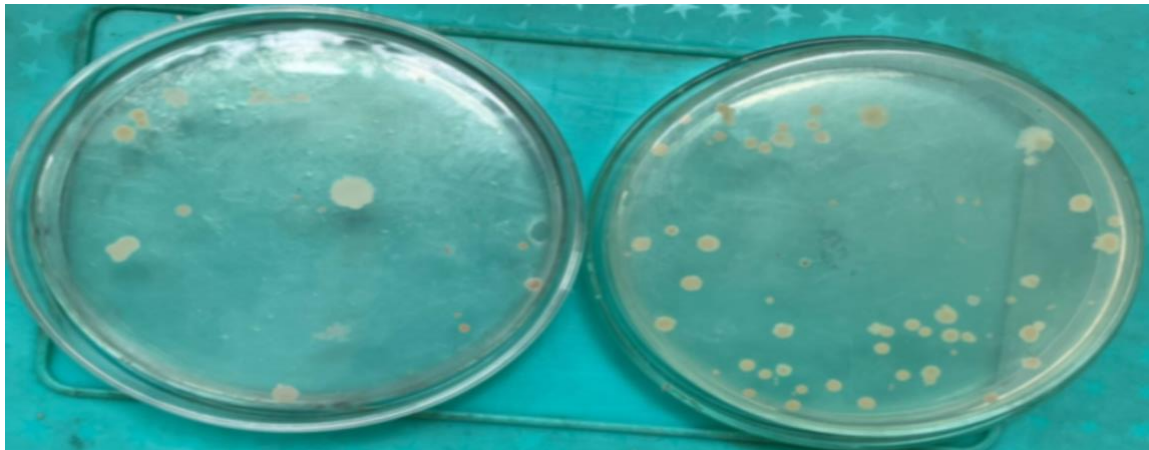


Fig:5.1 Isolation of bacterial colonies

The colonies from these dilutions were continuously streaked onto a nutrient agar medium plate to form pure colonies. The loop of microorganisms are taken and streaked on the slants and stored for future use.

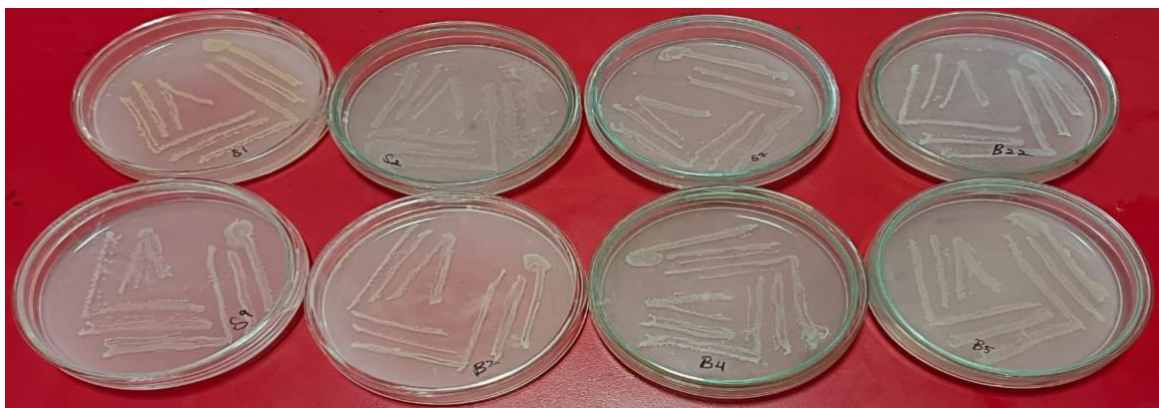


Fig:5.2 Pure colonies of isolates

➤ CHARACTERIZATION OF BACTERIAL COLONIES

1. 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.

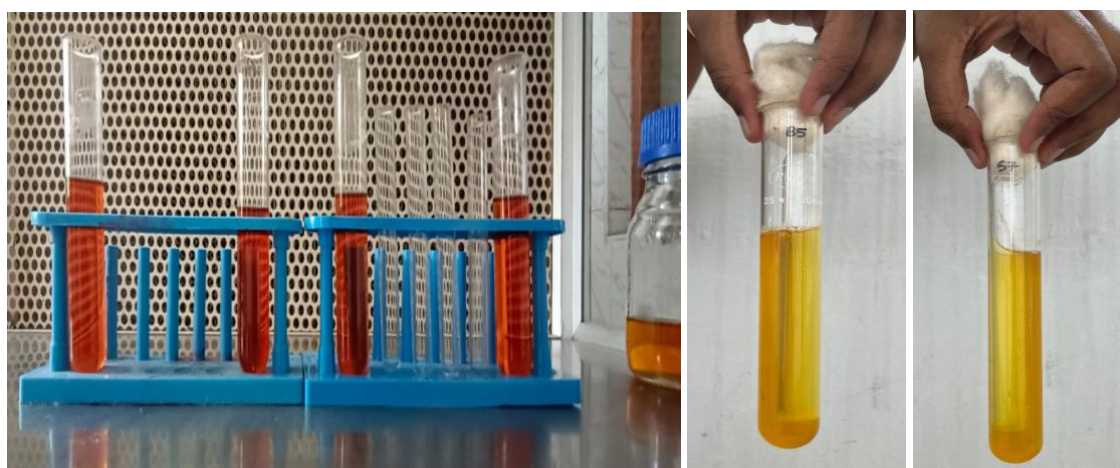


Fig:5.3 Sample-1 and sample-2 carbohydrate fermentation test

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 (sugar) present in the media.

1.1 Indole acetic acid production test –

There is no colour change even after the addition of Kovac's reagent in the sample test tube-1. Therefore, it is negative result.



Figure:5.3.1 Indole acetic acid (IAA) production test

2. Antimicrobial activity test -

Antibacterial assay of sample-1 and sample-2 was investigated by agar well diffusion susceptibility method. Two bacterial species, that is, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), were used for antibacterial bioassay. The bacterial species were immersed in the wells at 50 μ l and 100 μ l concentrations and incubated for 24 hrs. The results indicate that there is zone of inhibition for sample-1 the zone of inhibition is 13mm and for sample-2 the zone of inhibition is 18 mm for 100 μ l ,11mm and 15mm for 50 μ l concentration against *Escherichia coli*.

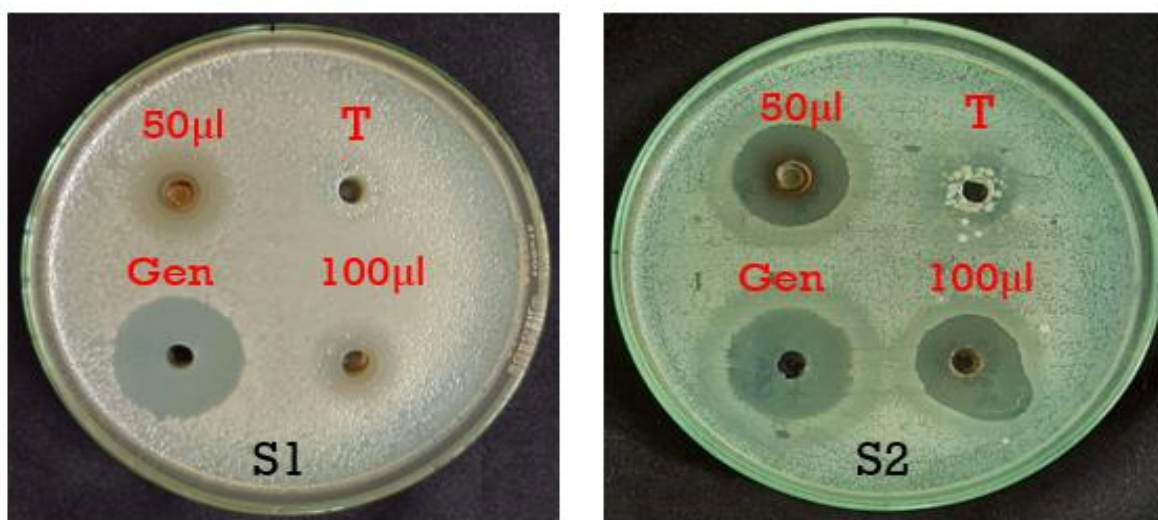


Fig:5.4 Sample-1 and sample-2 Antimicrobial activity test of *Escherichia coli*

Table:5.1 zone of Inhibition

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

3. Antibiotic Susceptibility test -

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

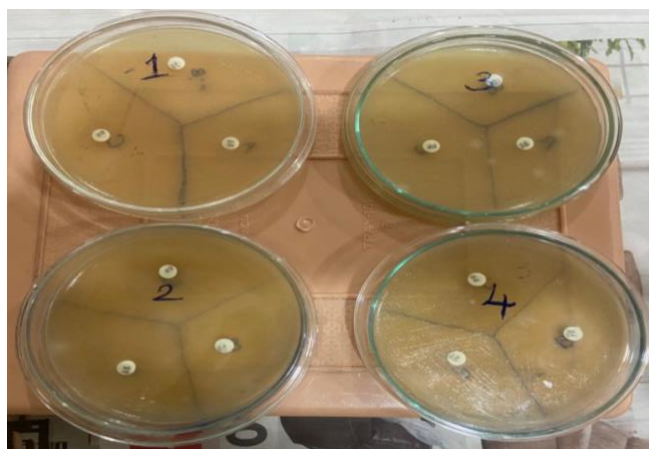


Fig:5.5 Antibiotic susceptibility test

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.

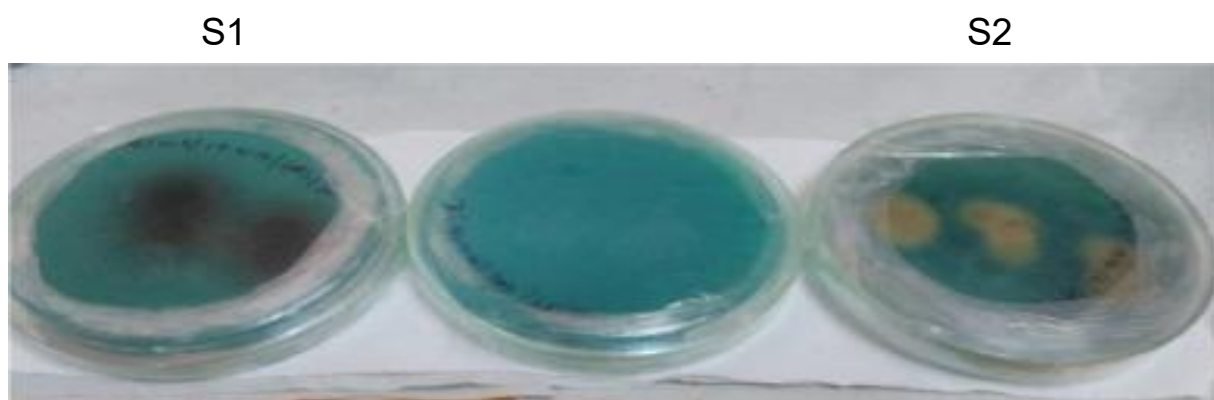


Fig:5.6 Sample-1 and sample-2 siderophore production test

SAMPLES		RESULTS	
Sample - 1		+	
Sample - 2		-	

Table:5.2 siderophore production test

Gram staining –

The bacterial strain is gram stained to know the shape and nature of the bacterial strain.

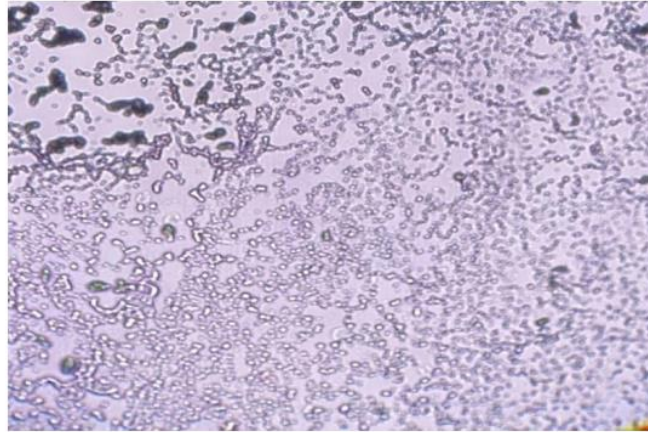


Fig:5 gram staining of isolated bacterial species

The bacteria when observed under the 100x light microscope is gram-positive, rod shaped bacterium.

➤ 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 is best suited Sample is being given to fishes along with feed. The fishes used in testing are Gold, Black moor and iridescent shark fishes.

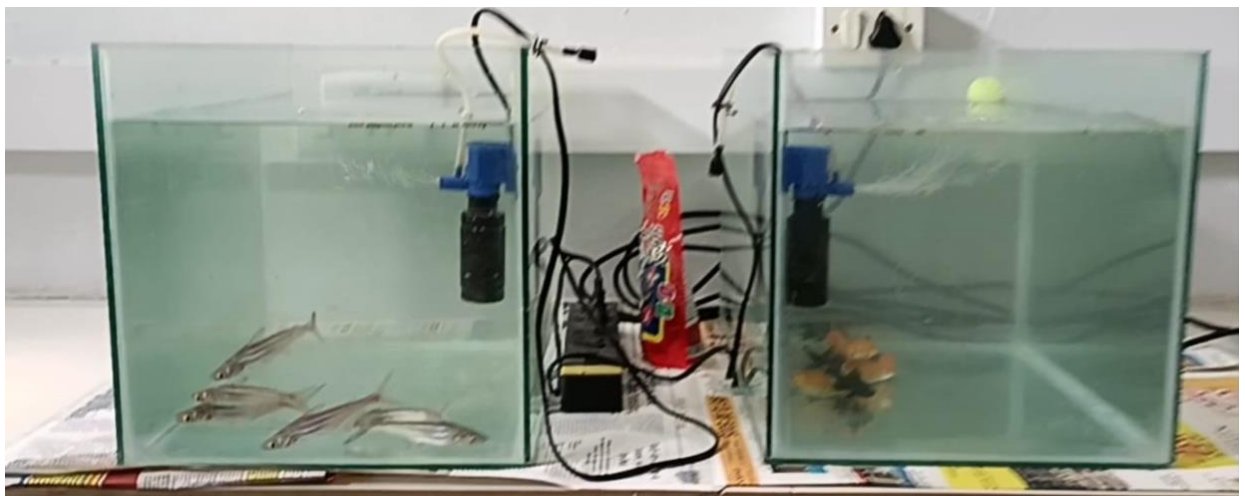


Fig:5.7 Control and Sample - Fish tanks

➤ ANALYSIS OF PHYSIO-CHEMICAL PARAMETERS

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 & Sample tank

<i>NO. OF DAYS</i>	<i>TEMPERATURE °C IN CONTROL</i>	<i>TEMPERATURE °C IN SAMPLE</i>
5	26	26
10	26.5	27
20	26.5	27

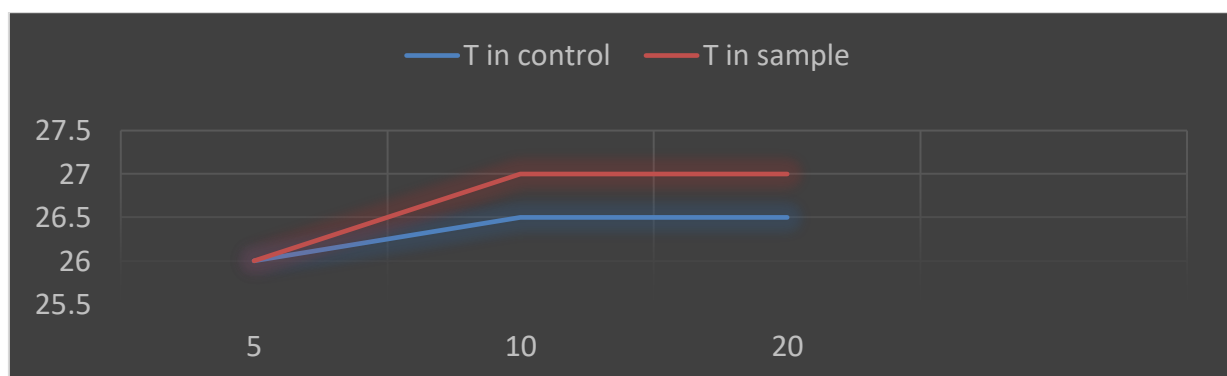


Fig:5.8 Temperature analysis in fish tank

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. The rise in ammonia raises the pH level. Optimum pH to be maintained in fresh water aquarium is 6.8 - 7.6.

Table: 5.4 pH in Control tank & sample tank

NO. OF DAYS	pH IN CONTROL	pH IN SAMPLE
5	6.8	6.8
10	6.8	7
20	7	7.2

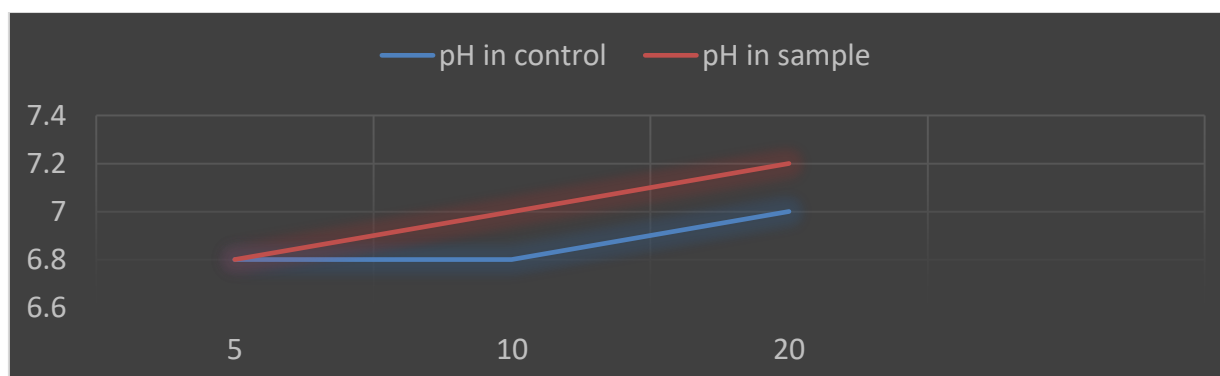


Fig: 5.9 pH analysis in fish tank

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: 5.5 Ammonia level in Control & Sample tank

NO. OF DAYS	AMMONIA LEVEL IN CONTROL	AMMONIA LEVEL IN SAMPLE
5	0.10	0.10
10	0.15	0.20
20	0.20	0.20

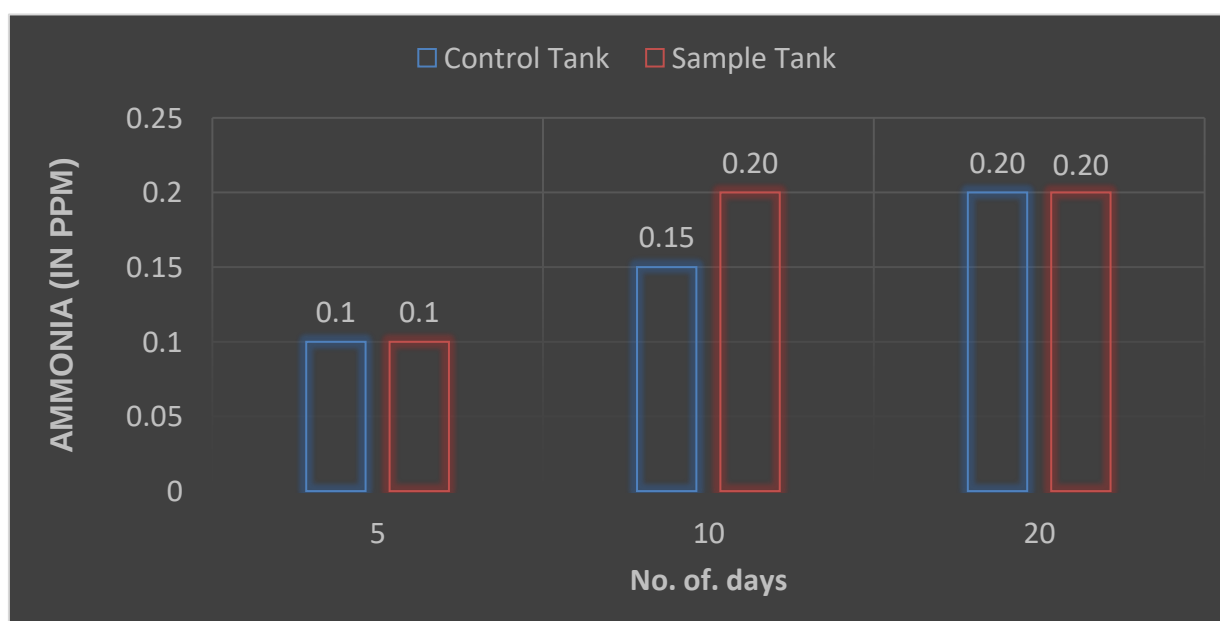


Fig: 5.10 Ammonia level analysis in fish tank

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: 5.6 Dissolved oxygen level in control & Sample tank

NO. OF DAYS	D.O LEVEL IN CONTROL	D.O LEVEL IN SAMPLE
5	6	6
10	6.5	6
20	6.5	6.5

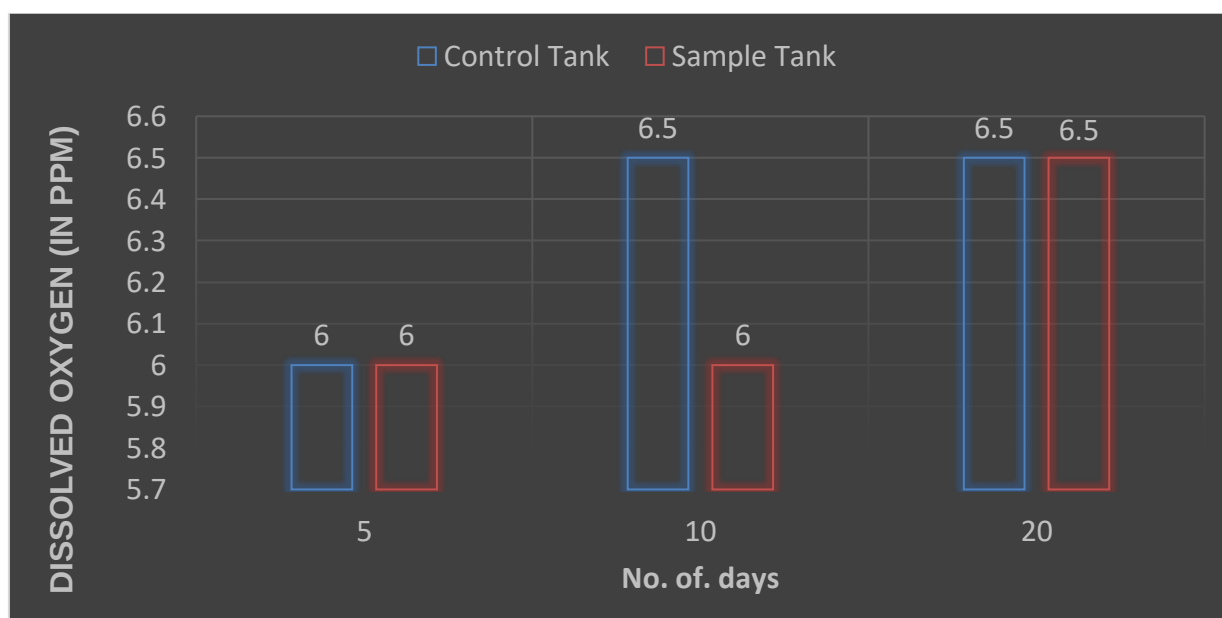


Fig: 5.11 Dissolved oxygen level analysis in fish tank

➤ IDENTIFICATION OF THE BACTERIAL STRAINS

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 various *Lactococcus lactis* strains.

Microbial Identification Report

Descriptions	Graphic Summary	Alignments	Taxonomy					
Sequences producing significant alignments								
Download ▼ New Select columns ▼ Show 100 ▼ ?								
☒ select all 100 sequences selected								
GenBank Graphics Distance tree of results New MSA Viewer								
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per Ident	Acc. Len	Accession
☒ Lactococcus lactis strain RTCMS10 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1472	1472	99%	0.0	97.58%	1313	KM654554.1
☒ Uncultured Lactococcus sp. gene for 16S ribosomal RNA, partial sequence, clone_BCC3-21	uncultured Lactococcus sp.	1472	1472	99%	0.0	97.58%	1372	AB828418.1
☒ Uncultured Lactococcus sp. gene for 16S ribosomal RNA, partial sequence, clone_BCC3-26	uncultured Lactococcus sp.	1469	1469	99%	0.0	97.47%	1373	AB828421.1
☒ Lactococcus lactis strain 4355 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1467	1467	99%	0.0	97.46%	1440	MT645510.1
☒ Lactococcus lactis subsp. lactis strain SDCM 5128 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1467	1467	99%	0.0	97.46%	1472	MT634254.1
☒ Lactococcus lactis subsp. lactis strain SDCM 5123 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1467	1467	99%	0.0	97.46%	1473	MT634253.1
☒ Lactococcus lactis subsp. lactis strain SDCM 5121 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. lactis	1467	1467	99%	0.0	97.46%	1475	MT626062.1
☒ Lactococcus lactis subsp. hordniae strain 1681 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1441	MT597554.1
☒ Lactococcus lactis strain TO17p 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1467	1467	99%	0.0	97.46%	958	MT579932.1
☒ Lactococcus lactis subsp. hordniae strain 4558 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1436	MT545070.1
☒ Lactococcus lactis subsp. hordniae strain 4555 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1437	MT545067.1
☒ Lactococcus lactis subsp. hordniae strain 4476 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1448	MT545001.1
☒ Lactococcus lactis subsp. hordniae strain 4401 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1448	MT544931.1
☒ Lactococcus lactis subsp. hordniae strain 4360 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1446	MT544898.1
☒ Lactococcus lactis subsp. hordniae strain 4359 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1453	MT544897.1
☒ Lactococcus lactis subsp. hordniae strain S-12 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1459	MT416439.1
☒ Lactococcus lactis subsp. hordniae strain S-9 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1455	MT416436.1
☒ Lactococcus lactis subsp. hordniae strain S-3 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1454	MT416432.1
☒ Lactococcus lactis subsp. hordniae strain S-2 16S ribosomal RNA gene, partial sequence	Lactococcus lactis subsp. hordniae	1467	1467	99%	0.0	97.46%	1457	MT378133.1
☒ Lactococcus lactis strain IMAU58307 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1467	1467	99%	0.0	97.46%	1461	MT473662.1
☒ Lactococcus lactis strain IMAU58266 16S ribosomal RNA gene, partial sequence	Lactococcus lactis	1467	1467	99%	0.0	97.46%	1458	MT473621.1

16sRNA forward sequence

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GATGAT
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TGGAAGC
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AATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGC
AGGTGGTTT
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16sRNA reverse sequence

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AAAACGTT
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AATGCTCG
TACAACTAACCCCCCCCCCGCCACTTAGTGAAGGATGGAGTGGGGTGGCG
GATGTGTTCTATA

➤ PRODUCT DEVELOPMENT

1. Powder –

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.



Fig:5.12 Probiotic powder and Liquid form

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.

2. Liquid –

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.

DISCUSSION

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. Several tests are performed to know the activity of the specific bacterial strains isolated. These tests provide the results for activity of the specific strains against several antibiotics and pathogens. The bacterial strains are tested for in vitro analysis in fishes. The bacterial sample is given as probiotic feed to the fishes to check the activity of the fishes. In aquaculture practices, the quality of water degenerates mainly due to accumulation of metabolic wastes. When the water quality is impaired, the bacterial culture becomes liable to diseases because of stress. In such a situation, it has been suggested that some commercial preparations can be added like probiotics, which are a group of beneficial microorganisms.

By maintaining the water quality parameters at the optimum level, probiotics are reported to be helpful in enhancing the growth rate among the cultured organisms. The first application of probiotics in aquaculture seems relatively recent (Kozasa, 1986) but the interest in such environment-friendly treatment is increasing rapidly. 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. The oxygen stage inside the culture medium can be maintained inside the desired range through aeration.

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 bacteria.

CHAPTER-6

8. SUMMARY OF THE INVESTIGATION

The bacterial samples are collected from the plant rhizosphere soil. The bacterial samples are isolated and the pure colonies are stored in slants and broth medium for further analysis. Further study was designed for characterization of the bacterial strain. Phenotypic characterizations of these isolates were done based on gram staining and different biochemical & carbohydrate tests. Various biochemical tests carried out where indole acetic acid production test and carbohydrate (lactose) test. Selected strain was identified as *Lactococcus Lactis* species level based on their morphological, biochemical characteristics and 16s rRNA sequencing. Bacterial culture was added to the tank under lab conditions for one month. Water quality parameters including ammonia and dissolved oxygen were recorded; the bacterial cultures were inoculated in the culture tanks individually on the 1st, 5th, 10th, 15th, 20th and 25th day of culture. The experiment was terminated on 31st day of culture. Wet weight and total length of the fish were measured.

The results of these tests show how some strains react to certain anti-toxins and infections. The included bacterial strains were subjected to several assays, including those for siderophore synthesis, biochemical activity, antimicrobial action, and antibacterial affectability. The results separate the characteristics of the specific strain. There were bacterial detaches.also examined for seed germination. The isolates from the soil sample were identified as Enterobacter species based on the 16S rRNA gene sequence.The bacterial strains are tested in vitro for research purposes. To monitor the fishes' behaviour, the bacterial sample is fed to them as probiotic feed. The study of the pharmacokinetic disposition (distribution and elimination) of the biotherapeutic agents with time after their administration to man and animals is necessary for the evaluation of the possible health effects of probiotic cultures in vitro. The growth parameters like weight and length are checked.

CHAPTER-7

9. CONCLUSION

The isolated strains were purified through sub culturing and identified biochemically. The isolates were identified to be *Lactococcus lactis* strain RTCMS10, complete genome with GenBank Accession Number KM654554 ,subjected for the safety experiment, where there were no diseases or mortalities in all groups.

Class: Bacilli

Domain: Bacteria

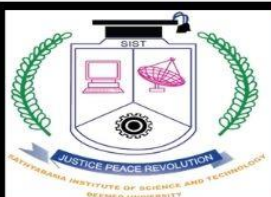
Species: *L. lactis*

Family: *Streptococcaceae*

The survival percent and body height and weight gain of the experimental fish with probiotics was recorded to be higher than that of the control tank during the first and also the second month of experiment.

In general, application of probiotic bacteria along with feed had given good results. Water application also significantly reduced the levels of ammonia. In general, water probiotic treatment showed better growth response than the feed application. The bacterial strain *Lactococcus lactis* species showed beneficial effect on the Golden fish, Iridescent shark and Black moor fish culture tank, by enhancing the growth and water quality of the fishes.

Lactococcus lactis has outstanding prospects as a probiotic because it is generally recognized as safe (GRAS) and has previously been shown to exert its probiotic potential in aquaculture through different mechanisms, such as competitively excluding pathogenic bacteria, increasing food nutritional value, and enhancing the host immune response against pathogenic microorganisms.



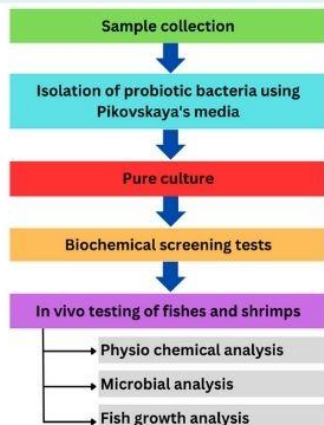
PROBIOTICS FOR FISH CULTURE

Dr.Masilamani Selvam.M and Pooja. V , Subhashini. R

INTRODUCTION

Aquaculture, comprising all forms of culture of aquatic animals and plants in fresh, brackish and marine environments, is the fastest-growing food-producing sector worldwide. Infectious disease remains a significant problem to aquaculture worldwide and many approaches are available to alleviate the effect of pathogens in farmed aquatic animals. The benefits of such probiotics include improving feed value, enzymatic contribution to digestion, inhibition of pathogenic microorganisms, anti-mutagenic, anti-carcinogenic activity, and also increasing immune response. These probiotics are harmless bacteria that help the host animal and contribute, directly or indirectly to protect the host animal against harmful pathogens. The present study aimed to evaluate the effects of the isolated field strains of probiotics by determining their inhibitory effect against pathogen.

METHODOLOGY



CONCLUSION

The isolated strains were purified through sub culturing and identified biochemically. The isolates were identified to be *Lactococcus lactis* strain RTCMS10, complete genome with GenBank Accession Number KM654554 ,subjected for the safety experiment, where there were no diseases or mortalities in all groups.

The survival percent and body weight gain of the experimental fish with probiotics was recorded to be higher than that of the control tank during the first and also the second month of experiment.

RESULTS

SOIL SAMPLE WAS COLLECTED FROM THE ROOTS OF THE PLANT SPREADING DAYFLOWER WAS SERIAL DILUTED (SCIENTIFIC NAME - *COMMELINA BENGHALENSIS*)

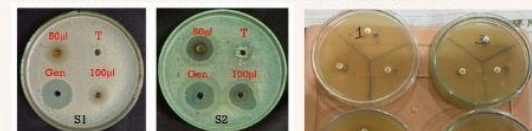


ISOLATION OF BACTERIAL PURE COLONIES FROM MARINE SOIL



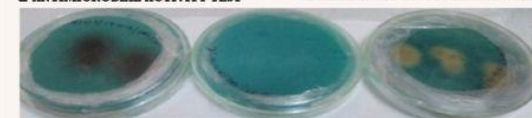
BIOCHEMICAL TESTING -

CARBOHYDRATE FERMENTATION TEST INDOLE ACETIC TEST



ANTIMICROBIAL ACTIVITY TEST

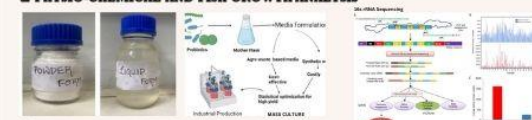
ANTIBIOTIC SUSCEPTIBILITY TEST



SIDEROPHORE PRODUCTION TEST



PHYSIO-CHEMICAL AND FISH GROWTH ANALYSIS



PROBIOTIC PRODUCT LIQUID AND POWDER

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CHAPTER-8

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