

**DISTRIBUTION OF ANTIMICROBIAL RESISTANCE MONITORING OF WATER  
ENVIRONMENT - A CASE STUDY**

**PROJECT WORK**

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

**By**

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**SATHYABAMA**

**INSTITUTE OF SCIENCE AND TECHNOLOGY**

**(DEEMED TO BE UNIVERSITY)**

**CATEGORY- 1 UNIVERSITY BY UGC**

**Accredited with Grade “A++” by NAAC12B Status Approved by AICTE**

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**APRIL - 2024**



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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **KEERTHI MOHAN (41770012) / M. FIRDOUS (41770009)** who carried out the project entitled "**DISTRIBUTION OF ANTIMICROBIAL RESISTANCE MONITORING OF WATER ENVIRONMENT - A CASE STUDY**" under my supervision from

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### DECLARATION

We, **KEERTHI MOHAN (41770012) / M. FIRDOUS (41770009)** hereby declare that the Project Report entitled "**DISTRIBUTION OF ANTIMICROBIAL RESISTANCE MONITORING OF WATER ENVIRONMENT- A CASE STUDY**" done by me under the guidance of **Dr. R. Thirugnanasambandam, Centre for Ocean Research, Sathyabama Research Park, Sathyabama Institute of Science and Technology** is submitted in partial fulfillment of the requirements for the award of Bachelor of Science degree in Biotechnology.



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## **ABSTRACT**

This Abstract presents a case study on the Distribution of antimicrobial resistance (AMR) in water environments. The study aimed to assess the prevalence and patterns of AMR, employing robust monitoring methods. Various water sources were examined, and a comprehensive analysis of resistance profiles was conducted. Results indicate significant variability in AMR across locations, emphasizing the need for vigilant monitoring. We have collected samples from different water sources like sewage(from urban areas), mangrove sediments, mangrove pure water, aquaculture discharge water, sewage (from the Sathyabama sewage treatment plant), and raw water(from the Sathyabama sewage treatment plant). We stored the sample in a cold storage room at -4°C. after the collection of samples, we did the serial dilution process. Then plating of samples with the use of nutrient agar, the plating method which we used was a spread plate. We have incubated the plates for 24 hours at 37°C. after the incubation, we got different types of bacterial colonies which were different in shape, color, etc. The different colonies were streaked in nutrient agar by the method of quadrant streaking. After streaking we inoculated the bacterial colonies in nutrient broth, the broth was then kept in an incubator shaker for 24 hours. at last, we slanted the inoculated colonies into test tubes. By this, we have completed the bacterial colony isolation method. After the process of isolation, we did the antibiotic-susceptibility test for this test we used 10 different antibiotics Vancomycin, Fluconazole, Penicillin-G, Methicillin, Clarithromycin, Streptomycin, Tetracycline, Ciprofloxacin, Ampicillin, Azithromycin. And then we checked If the bacterial strain produces biosurfactant or not. We have also isolated the bacteria by using the Gram staining method.

**Keywords:** Antimicrobial resistance, water environment, Sewage water, Culture dependent method, Antibiotics.

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## **LIST OF ABBREVIATIONS**

|     |   |                                   |
|-----|---|-----------------------------------|
| AMR | - | Antimicrobial Resistance          |
| ARB | - | Antimicrobial Resistant Bacteria  |
| ARG | - | Antimicrobial Resistant Gene      |
| AST | - | Antimicrobial Susceptibility Test |
| LBS | - | Pounds Per Square Inch            |

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# **CHAPTER 1**

## **INTRODUCTION**

### **1.1 BACTERIAL COMPOSITION**

In the intricate web of our planet's ecosystem, water serves as a fundamental connector, nurturing life in its myriad forms. Within this aqueous matrix, bacterial communities play pivotal roles, shaping the health and dynamics of the aquatic environment. The study of bacteria in environmental water sources is not nearly an academic pursuit; it is a crucial endeavor with far-reaching implications for human health, ecological balance, and sustainable resource management.

Understanding the composition, diversity, and behavior of bacterial populations in water sources is essential for various reasons. Firstly, these microorganisms act as indicators of water quality, reflecting the impact of anthropogenic activities, natural processes, and climatic changes on aquatic ecosystems. Monitoring bacterial communities provides invaluable insights into the presence of contaminants, pathogens, and pollutants, aiding in the formulation of effective water management strategies and public health interventions.

Furthermore, elucidating the interactions among the bacterial species and their environment enhances our comprehension of ecosystem functioning and resilience. From nutrient cycling to bioremediation, bacteria contribute to equilibrium and the mitigation of the adverse effects of human activities on aquatic ecosystems. Moreover, the study of bacterial communities in environmental water sources is inherently interdisciplinary, drawing upon insights from microbiology, ecology, hydrology, bioinformatics, and environmental science. Integrating diverse methodologies, such as culture-dependent techniques, molecular analyses, and bioinformatics tools, enables researchers to unravel the complexity of microbial ecosystems and decipher their implications for ecosystem health and human well-being.

In this introductory overview, we embark on a journey to explore the fascinating world of bacteria inhabiting environmental water sources. By delving into their diversity, dynamics, and ecological roles, we aim to underscore the significance of studying these profound influences on the sustainability of aquatic ecosystems and

the welfare of our planet. Through collaborative research endeavors and holistic approaches, we endeavor to deepen our understanding of bacterial communities in water sources and harness this knowledge for the betterment of both nature and society.

Waterborne diseases pose significant health risks to humans worldwide, largely due to harmful microbes present in water sources. These microorganisms can contaminate water supplies through various sources such as sewage, animal waste, and runoff from agricultural and industrial activities. Their presence in drinking water can lead to illnesses ranging from gastrointestinal infections to more severe conditions like cholera and hepatitis. Therefore, understanding and mitigating the risks associated with harmful microbes in water is essential for ensuring public health and safety

One notable incident highlighting this danger occurred in 1993 in Milwaukee, USA. *Cryptosporidium*, a microscopic parasite, contaminated the city's drinking water, leading to the largest documented waterborne disease outbreak in United States history. Over 400,000 people fell ill with symptoms including severe diarrhea, vomiting, and stomach cramps. Tragically, at least 69 individuals lost their lives, predominantly among the elderly or those with weakened immune systems. This incident underscores the grave consequences of waterborne pathogens and emphasizes the urgent need for robust water treatment and sanitation measures to safeguard against such outbreaks

Antibiotic-resistant genes (ARGs) in environmental water pose a growing concern due to their potential impact on public health. As a result of widespread antibiotic use, these genes can enter water sources through various pathways, such as agricultural runoff and discharge from pharmaceutical manufacturing. The persistence of ARGs in water environments raises alarms about the development of antibiotic resistance in bacteria, posing a threat to both aquatic ecosystems and the transfer of resistance to human pathogens. Understanding and monitoring the presence of antibiotic-resistant genes in environmental water is crucial for addressing this global health issue.

In an era marked by escalating concerns over antimicrobial resistance (AMR) in water environments. The study aimed to assess the prevalence and patterns

of AMR, employing robust monitoring methods. Various water sources were examined, and a comprehensive analysis of resistance profiles was conducted. Results indicate significant variability in AMR across locations, emphasizing the need for vigilant monitoring. This case study contributes valuable insights into the dynamics of antimicrobial resistance in the water environment, informing strategies for effective mitigation and public health management.

Antibiotic resistance is defined as the genetic ability of bacteria to encode the resistance genes that counterfeits the inhibitory effect of potential antibiotics for survival. It can be developed either intrinsically by natural recombination and integration into the bacterial genome, or it can be acquired through horizontal gene mutation events such as conjugation, transformation, and transduction. The prominent events in the generation of bacterial resistance include the inactivation of the porin channel, modification of antibiotic targets, and neutralizing of antibiotic efficacy through enzymatic action. Thus, the understanding of the genetic makeover and the morpho-anatomical changes in bacteria are of prime importance in counteracting the resistance mechanism.

## 1.2 ANTIBIOTIC-DISC DIFFUSION

The antibiotic disc diffusion method, also known as the Kirby-Bauer method, is a cornerstone technique in microbiology laboratories used to evaluate the susceptibility of bacteria to antibiotics. This method relies on the diffusion of antibiotics from paper discs onto an agar medium inoculated with a standardized bacterial culture. As the antibiotics diffuse outward from the discs, they create concentration gradients in the agar, leading to zones of inhibition where bacterial growth is inhibited. By measuring the diameter of these zones, healthcare professionals can determine the susceptibility or resistance of bacterial isolates to specific antibiotics. The simplicity, cost-effectiveness, and widespread applicability of the disc diffusion method make it an indispensable tool for guiding antibiotic therapy decisions and monitoring antibiotic resistance patterns in clinical and research settings.

This method involves inoculating a standardized bacterial culture onto a solid agar medium, typically nutrient agar, and placing paper discs impregnated with specific antibiotics onto the surface. As the antibiotics diffuse outwards from the discs into the

agar, they create concentration gradients. If the bacteria are susceptible to the antibiotic, they will be inhibited from growing in the vicinity of the disc, resulting in a clear zone of inhibition around it. By measuring the diameter of these zones, healthcare professionals can interpret the results according to standardized guidelines provided by organizations such as the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). This method provides valuable information to clinicians, enabling them to make informed decisions regarding antibiotic treatment and aiding in the surveillance of antibiotic resistance patterns in bacterial populations. Despite its simplicity, the disc diffusion method remains a robust and widely used technique in both clinical and research settings for combating bacterial infections and preserving the efficacy of antibiotics

### 1.3 BIOSURFACTANT

Biosurfactants are surface-active molecules produced by microorganisms. They have diverse applications in various industries, including bioremediation, pharmaceuticals, cosmetics, and food processing. Analyzing biosurfactants involves several techniques aimed at characterizing their properties and assessing their potential applications. "Biosurfactant bacteria" refers to bacteria that can produce biosurfactants. Biosurfactants are surface-active molecules synthesized by microorganisms, including *bacteria*, *fungi*, and *yeast*. These molecules exhibit surfactant properties, meaning they can reduce the surface tension between two immiscible phases, such as oil and water.

Biosurfactant-producing bacteria have the genetic machinery to synthesize and secrete these molecules into their surrounding environment. Biosurfactants play diverse roles for bacteria in their natural habitats, including facilitating nutrient uptake, biofilm formation, motility, and survival in harsh environments. The production of biosurfactants by bacteria has garnered significant attention due to their potential industrial applications. These applications include enhanced oil recovery, bioremediation of oil-contaminated environments, detergency, cosmetics, pharmaceuticals, and food processing. As a result, biosurfactants-producing bacteria are of interest for both environmental and industrial biotechnology research

## CHAPTER 2

### LITERATURE SURVEY

#### 2.1 WATER BORN BACTERIA

Wells used for drinking water frequently have a large biomass and a high bacterial diversity. Current technologies are not always suitable to reduce the bacterial population, and the trouble of pathogen proliferation in drinking water sources is universal. The environmental conditions that shape the microbial communities in drinking water sources have to be illustrated so that pathogen proliferation can be prevented. In this work, the bacterial community in nine water wells of a groundwater aquifer in Northern Mexico was characterized and identified as environmental characteristics that might control them. Although a large variation was observed between the water samples, temperature, and iron attention were the characteristics that affected the bacterial community structure and composition in groundwater wells. Small increases in the attention of iron in water modified the bacterial communities and promoted the growth of the iron-oxidizing bacteria *Acidovorax*. The cornucopia of the rubrics *Flavobacterium* and *Duganella* was identified appreciatively with temperature and the Actinobacteria Gp4 and Gp1, and the genus *Acidovorax* with iron attention in the well-conditioned water. Large probabilities of *Flavobacterium* and *Pseudomonas* bacteria were set up, and this is of special concern as bacteria

#### 2.2 AMR (ANTIMICROBIAL-RESISTANCE)

AMR is a major trouble to planetary health, which requires an ecosystem approach and demands One Health results. Our study findings give sapience into the waterborne transmission of AMR by mapping and assaying the spatiotemporal distribution of ARB and ARGs in different submarine surroundings in Bangladesh. Discharge of undressed wastewater from pastoral homes, flesh granges, and civic requests to the open terrain is a significant contributor to pollution of face water bodies with antibiotic-resistant bacteria and genes. unborn interventions are demanded to reduce the burden of AMR in the terrain. similar interventions could include bettered biosecurity practices, particularly waste disposal systems, along with advanced water, sanitation, and hygiene structures in pastoral and civic areas, as well as advanced

practices for ferocious flesh husbandry. Civilians-grounded mapping of AMR using quantitative microbiological data from connected environmental chambers can give precious perceptivity into the dynamics of AMR in an ecological setting and this could be an important step in addressing the environmental confines of AMR through acclimatized mitigation measures.

## 2.3 ANTIBIOTIC- DISC

The term “antibiotic disc” typically refers to small, sterile paper disc that are impregnated with specific antibiotics. These discs are used in the antibiotic disc diffusion method, also known as the Kirby-Bauer method, which is a standard laboratory technique for testing the susceptibility of bacteria to antibiotics.

Antibiotic discs are commercially available and come in various formulations, each containing a specific antibiotic or combination of antibiotics at defined concentrations. These discs are placed onto the surface of an agar medium inoculated with a bacterial culture. As the antibiotic diffuses from the disc into the agar, it creates a concentration gradient. If the bacteria are susceptible to the antibiotics, they will be inhibited from growing around the disc, resulting in a clear zone of inhibition. The size of the zone of inhibition around the antibiotic disc is measured and compared to standardized interpretive criteria to determine the susceptibility or resistance of the bacterial isolate to the antibiotic being tested.

Antibiotic discs are essential in clinical microbiology laboratories for guiding antibiotic therapy decisions and monitoring antibiotic resistance patterns in bacterial pathogens. They allow healthcare professionals to quickly assess which antibiotics are most effective against specific bacterial infections. [Dickert. H, Braveny: 1981].

### **2.3.1 Types of antibiotic disc used in disc diffusion**

*Single antibiotic disc* - These discs contain a single antibiotic at a defined concentration. They are used to test the susceptibility of bacterial isolates to individual antibiotics. Common examples include disc-containing antibiotics such as penicillin, erythromycin, ciprofloxacin, and vancomycin. The size of the zone of inhibition around the single antibiotic disc was measured and compared to standardized interpretive criteria to determine the susceptibility or resistance of the bacterial isolate to the

antibiotic being tested. Single antibiotic discs are essential tools in clinical microbiology laboratories for guiding antibiotic therapy decisions and monitoring antibiotic resistance patterns in bacterial pathogens. They allow healthcare professionals to quickly assess which antibiotics are most effective against specific bacterial infections.

*Multi-drug combination discs* - These discs contain multiple antibiotics in combination at fixed concentrations. They are used to test bacterial susceptibility to multiple antibiotics simultaneously. Combination discs are useful for detecting resistance mechanisms, such as beta-lactamase production. Examples include discs containing ampicillin/sulbactam or amoxicillin/clavulanic acid. In contrast to single discs, which contain only one antibiotic, combination discs offer the advantage of simultaneously testing the susceptibility of bacterial isolates to multiple antibiotics. This can be particularly useful when dealing with bacteria that may exhibit resistance to multiple antibiotics or when assessing the effectiveness of combination therapy.

Amoxicillin/Clavulanic Acid: this combination is effective against beta-lactamase-producing bacteria, as clavulanic acid inhibits beta-lactamase enzymes, which can degrade penicillins and cephalosporins. Ampicillin/sulbactam: similar to amoxicillin/clavulanic acid, this combination is effective against beta-lactamase-producing bacteria.

*Specialized discs* - Some laboratories may use specialized discs containing less commonly tested antibiotics or antibiotics specific to certain bacterial species. Examples include discs containing trimethoprim-sulfamethoxazole, nitrofurantoin, or colistin. It contains less commonly tested antibiotics. Specialized disc may include, ***less commonly tested antibiotics***: some antibiotics may not be routinely included in standard antibiotic susceptibility testing panels but may be important in certain clinical or research contexts. Examples include antibiotics such as linezolid, colistin, nitrofurantoin, or trimethoprim/sulfamethoxazole. ***Antibiotics specific to certain bacterial species***: some bacterial species may have unique antibiotic susceptibility patterns or may be inherently resistant to certain antibiotics. Specialized discs may be used to test the susceptibility of these bacteria to specific antibiotics. For example, certain antibiotics may be used specifically to test for the susceptibility of Gram-positive bacteria like staphylococcus aureus or streptococcus pneumoniae. ***Niche***

**applications:** specialized discs may also be used for niche applications, such as research studies investigating the susceptibility of bacteria to experimental or investigational antibiotics, or for testing the susceptibility of bacteria isolated from specific environments or sources.

*Quality control discs* - Quality control discs contain antibiotics at known concentrations and are used to monitor the performance of the disc diffusion assay. They ensure that the tests are accurate and reliable. Quality control discs may include reference strains with known susceptibility patterns. Quality control discs are employed to monitor the consistency of AST results over time and between different batches of media, antibiotics, and other reagents. By testing the quality control discs alongside patient samples during each AST run, laboratories can identify any variations or discrepancies in the results and take corrective actions as necessary. Any discrepancies between the observed and expected results from quality control discs may indicate errors in the AST procedure, such as incorrect inoculum density, inappropriate incubation conditions, or problems with the agar medium or antibiotic discs. By identifying and rectifying these errors promptly, laboratories can maintain the reliability and validity of their AST results.

*Customized discs* - In some cases, laboratories may prepare customized antibiotic discs containing specific antibiotics or concentrations not available in commercially prepared discs. These customized discs can be tailored to the specific needs of the laboratory or to investigate particular antibiotic resistance patterns. Laboratories may prepare discs containing antibiotics that are not readily available in commercially prepared discs. This could include antibiotics used for testing specific bacterial species or antibiotics of interest in research studies. In research studies, laboratories may prepare discs containing experimental compounds or novel antimicrobial agents for testing against bacterial isolates. These customized discs allow researchers to evaluate the effectiveness of new compounds in inhibiting bacterial growth and may contribute to the development of new antibiotics.

## 2.4 CHECKING PRODUCTION OF BIOSURFACTANT BY BACTERIAL STRAIN

Biosurfactant bacteria are bacteria that can produce biosurfactants. Biosurfactant-producing bacteria have the genetic machinery to synthesize and

secrete these molecules into their surrounding environment. Examples of biosurfactant-producing bacteria are *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Rhodococcus* spp., *Lactobacillus* spp., *Staphylococcus* spp. These examples represent just a few of the many bacterial species capable of biosurfactant production. The diversity of biosurfactant-producing bacteria highlights the potential for harnessing these molecules for various industrial and environmental applications.

#### ***2.4.1 Drop collapse assay***

In this qualitative assay, a small droplet of oil is placed on a hydrophobic surface. If the bacterial culture produces biosurfactants, it will cause the droplet to collapse due to decreased surface tension. Non-biosurfactant-producing strains will not cause the droplet collapse. The behavior of the droplets is observed over time. If the surfactant molecules in the solution reduce the surface tension of the droplet sufficiently, the droplet will spread and eventually collapse, forming a flat film on the hydrophobic surface. The degree of spreading and collapse of the droplet is qualitatively assessed. A more significant collapse indicates a higher surface activity of the surfactant molecules present in the solution.

#### ***2.4.2 Oil spreading assay***

This qualitative assay involves spreading a thin layer of oil on an aqueous agar surface. If the bacterial strain produces biosurfactant, it will spread the oil across the surface due to decreased surface tension. The oil spreading assay provides a simple and visual method for screening the surface-active properties of surfactants, including biosurfactants. It is often used in microbiology and biotechnology research to identify and characterize surfactant-producing microorganisms and assess the potential applications of biosurfactants in various industries, such as bioremediation, agriculture, and cosmetics. It's important to note that the oil spreading assay is qualitative and does not provide quantitative data on surface tension reduction. For quantitative measurements of surface tension reduction, more advanced techniques such as the Du Nouy ring method or the Wilhelmy plate method are to be used.

### **2.4.3 Emulsification assay**

In this qualitative assay, the bacterial culture is mixed with an oil phase and agitated. If the strain produces biosurfactants, it will emulsify the oil phase into small droplets, forming a stable emulsion. Emulsification assays provide valuable information about the emulsifying properties of surfactants, including biosurfactants, and their potential applications in various industries. These assays help researchers identify surfactant-producing microorganisms, characterize the types of surfactants produced, and optimize conditions for biosurfactant production. Additionally, emulsification assays play a crucial role in screening and selecting surfactants for specific industrial applications, such as enhancing oil recovery, emulsifying contaminants for bioremediation, or stabilizing emulsion in food and cosmetic products.

### **2.4.4 Hemolytic assay**

A hemolytic assay is a laboratory test used to assess the ability of substances to cause hemolysis, the rupture or destruction of red blood cells (erythrocytes). Hemolysis can be caused by various factors, including bacterial toxins, chemicals, drugs, and biological molecules such as peptides or proteins. There are several types of hemolytic assays, but the two most common are the blood agar plate method and the spectrophotometric method. Hemolytic assays provide valuable information about the potential toxicity, cytotoxicity, and pharmacological effects of substances on red blood cells. They are commonly used in pharmaceutical development, drug screening, environmental monitoring, and clinical diagnostics to assess the safety and efficacy of compounds and identify potential hazards associated with exposure to hemolytic agents. Additionally, hemolytic assays play a crucial role in the evaluation of hemolytic diseases, autoimmune disorders, and hemolytic transfusion reactions in clinical settings. *Blood Agar Plate Method* - In this assay, a sample containing the substance to be tested is streaked or spotted onto a blood agar plate, which is a nutrient-rich agar medium supplemented with red blood cells. If the substance has hemolytic activity, it will cause a visible zone of hemolysis around the area of application due to the destruction of red blood cells. The size and appearance of the zone of hemolysis can provide qualitative and semi-quantitative information about the hemolytic activity of the substance. *Spectrophotometric Method* - This assay involves measuring the release

of haemoglobin from lysed red blood cells using a spectrophotometer. Red blood cells are incubated with the substance to be tested, and the extent of hemolysis is determined by measuring the absorbance of released haemoglobin at a specific wavelength. This method provides quantitative data on hemolytic activity and can be more sensitive than the blood agar plate method.

Hemolytic assays are commonly used in various fields, including microbiology, pharmacology, toxicology, and biotechnology, to evaluate the potential toxicity or therapeutic effects of substances on red blood cells. They are also used in clinical diagnostics to detect certain types of bacterial pathogens that produce hemolysins, such as *Streptococcus pyogenes*, which causes strep throat and other infections.

## **CHAPTER 3**

### **AIM AND OBJECTIVES**

#### **3.1 AIM**

To study the antimicrobial resistance and monitoring the water environments

#### **3.2 OBJECTIVES**

- \* To Determine the regions of (or) water bodies with high levels of antimicrobial resistance (AMR) through comprehensive monitoring
- \* To improve awareness and understanding of antimicrobial resistance
- \* To identify the microbial colonies and to check the biosurfactant in the water bodies

# CHAPTER 4

## MATERIALS AND METHODOLOGY

### 4.1 MATERIALS REQUIRED

#### **4.1.1 Glasswares**

Petri plates, conical flasks, pipettes and tips, absorbent and non-absorbent cotton, spirit lamps, beakers, test tubes, micro tips, lighter, marker, Eppendorf, measuring cylinder, L-rod, spatula, forceps.

#### **4.1.2 Chemicals**

Nutrient agar, Agar powder, Nutrient broth, ethanol, spirit, distilled water, 70% ethanol, crystal violet, grams iodide solution, acetone, safranin, xylene, antibiotic discs

### 4.2 SAMPLE COLLECTION

We have collected six different types of environmental water samples Sewage water (urban areas), Sewage water (Sathyabama sewage water treatment plant), Raw water (Sathyabama sewage water treatment plant), Mangrove sediment water (muttukad), Mangrove pure water(muttukad), aquaculture discharge water (sathyabama aquaculture facility). These samples were collected and stored in a cold storage facility in the sathyabama Ocean Research Centre at -4°C.



**Fig: 4.1: Samples collection**

### 4.3 ISOLATION OF BACTERIA

For the preparation of nutrient agar here we are taking 14 grams of nutrient agar powder in a conical flask and adding 500ml of distilled water. and then we added 10 grams of agar powder for the easy solidification of the media. then we plugged it with cotton before autoclaving the media. We autoclaved the media for 20 minutes at 121° C at 15 LBS pressure. Once the agar solution has cooled to approximately 50-55° C, it is ready to be poured into the petri dishes. As we have completed the preparation of the media, we are ready to perform the screening of bacteria from the samples. For this process, we have conducted the spread plate method, two types of streaking and inoculation. The spread plate method was the first method that we used for the screening process. In this method, we have to spread the samples which we have already serially diluted. Here we have serially diluted each sample up to  $10^{-5}$ . From each dilution, we took 10 $\mu$ L of the sample with the help of a micropipette and poured it into the solidified agar medium in the Petri plates. Then we spread the sample by using an L-rod. After spreading we incubated the culture in an incubator for 24 hours

for the growth of the bacterial colonies at 37° C. after incubation we finally got different types of bacterial colonies which were different in colour and shape .



**Fig: 4.2: Media preparation**



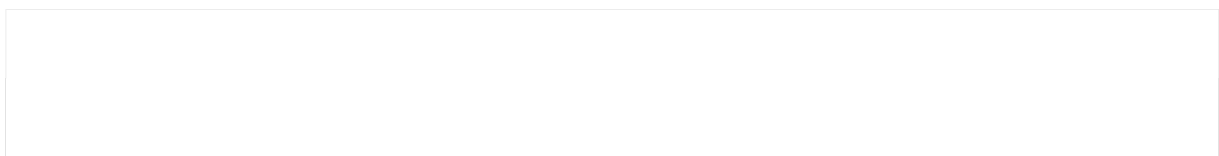
**Fig: 4.3: Microbial colonization**

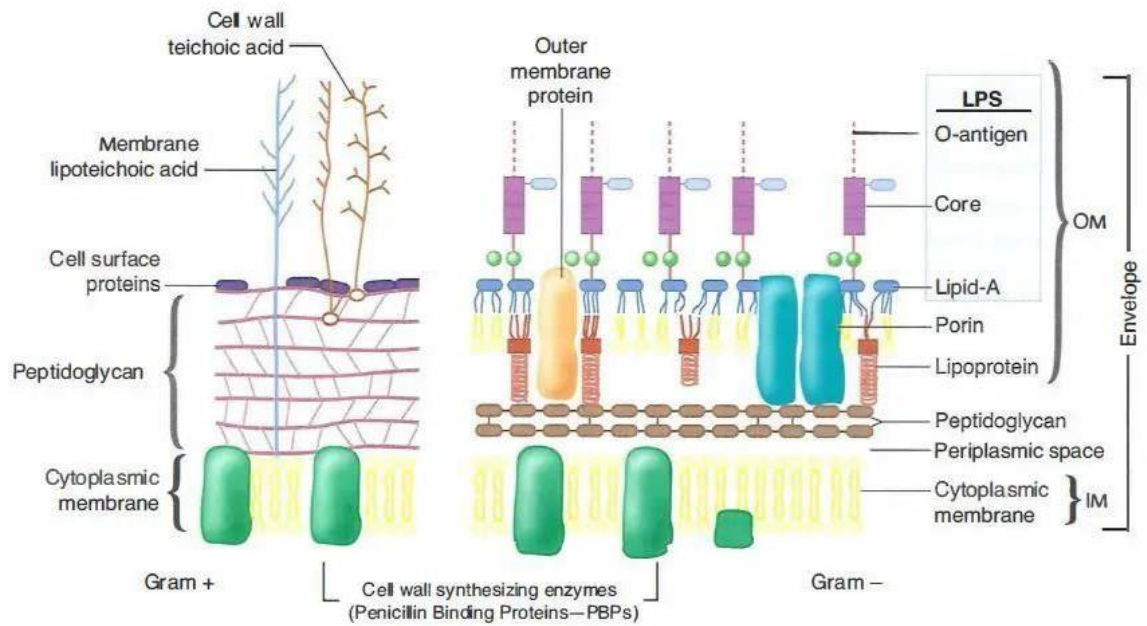
After the spread plate method, we did the sub-culturing process that is we did the streaking process. Here we have streaked the colonies two times, first, we did it with the quadrant streaking method then we streaked the colonies with the slanting method. After the streaking process, we incubated the culture in an incubator for 24 hours at

37° C. We got different colored bacterial colonies after the incubation. We have inoculated the different colored colonies into nutrient broth.

#### 4.4 GRAM STAINING

Danish physician Hans Christian Gram developed the gram staining method in 1884. The gram staining procedure uses four chemicals; crystal violet, iodine, alcohol, and safranin, to stain bacteria. Gram staining is still the cornerstone of bacterial identification and taxonomic division. This differential staining technique separates most bacteria into two groups based on cell wall composition. Gram-positive bacteria stain purple color and Gram-negative bacteria stain red/pink color. Gram staining is a valuable tool for microbiologists to quickly classify bacteria into two main groups based on their cell wall characteristics. This classification has important implications for bacterial identification, treatment selection, and understanding of bacterial pathogenesis. Gram-positive bacteria have a thick layer of peptidoglycan in their cell wall, which retains the crystal violet-iodine complex during the Gram staining process. This gives them a purple or blue color. Gram-negative bacteria have a thinner layer of peptidoglycan in their cell wall, which is located between an inner cytoplasmic membrane and an outer membrane. The outer membrane contains lipopolysaccharides (LPS) and other complex molecules. During Gram staining, the outer membrane of Gram-negative bacteria is disrupted by the decolorizing agent, causing them to lose the crystal violet-iodine complex and take up the counterstain, appearing pink or red.





**Fig: 4.4: Gram staining principle**

#### **4.4.1 Preparation of the smear**

First, we have to take a clean grease-free slide and then transfer a loop of the sample to the microscope slide. Spread the sample to an even-thin film over a circle of 15mm diameter. After this air dry the sample, and once the sample gets air dried, heat fix the smear by passing it through a Bunsen burner three times. Heat application helps the cell adhesion to the glass slide and prevents its loss during rinsing.

#### **4.4.2 Methanol fixation**

Place or hold the slide over a paper towel and flood the slide with absolute methanol for two minutes. Once two minutes have passed, tilt the slide drain off the excess methanol and let the slide air dry. Do not wipe or blot the slide, As this can remove cells.

#### **4.4.3 Procedure**

This procedure is done by spreading a thin layer of bacterial colonies in a glass slide. Then add some crystal violet dye and let it stand for one minute. Then add grams of iodine solution (usually a potassium iodide- iodine complex) and let it stand for One minute. After this process, we need to rinse the slide with a contrasting stain, this will help to visualize the bacteria that do not retain the primary stain (crystal violet). Here we are using safranin as the contrasting stain. Flood the smear with safranin and let it stand for 30 to 1 minute. Rinse and wash the slide gently. After that observe the stained bacteria under a light microscope using oil immersion at 1000x magnification.

### **4.5 ANTIBIOTIC- SUSCEPTIBILITY TEST**

The antibiotic susceptibility test, also known as antimicrobial susceptibility testing (AST) or antibiogram, is a laboratory technique used to determine the susceptibility of bacterial isolates to specific antibiotics. This test helps healthcare providers choose the most effective antibiotic treatment for bacterial infections.

#### **4.5.1 Preparation of inoculum from bacterial culture**

Here we have used colonies grown within 18 to 24 hours. We have inoculated the bacterial colonies in a nutrient agar medium. The dilution of the suspension was 1:20. Then the colonies were swabbed from the plate and inoculated into the nutrient broth. This was kept in an incubator shaker for 24 hours at 300 rpm.

#### **4.5.2 Antibiotic discs used**

- Vancomycin -30mcg
- Fluconazole- 10mcg
- Clarithromycin- 15mcg
- Azithromycin- 15mcg
- Streptomycin- 10mcg

- Tetracycline- 30mcg
- Ciprofloxacin- 5mcg
- Penicillin-G - 10mcg
- Ampicillin- 10mcg
- Methicillin- 5mcg

#### **4.5.3 Procedure**

- Preparation of agar plates: Here we have taken nutrient agar medium we have prepared 1000ml of nutrient agar media which consists of 28 grams of nutrient agar and 20 grams of agar powder to make the media easily solidify. And then we autoclaved it under 121° C for 20 minutes at 15 LBS pressure. After autoclaving we let the media cool down and then we poured it into the Petri plates and let it solidify.
- Inoculation of bacterial culture: The standardized suspension of the test bacteria is spread uniformly over the surface of the agar using a sterile L-rod, creating a bacterial lawn.
- Placement of antibiotic discs: paper discs impregnated with specific concentrations of antibiotics are placed onto the surface of the agar using sterile forceps
- Incubation: The plates are then incubated at 37° C for 24 hours to allow bacterial growth and antibiotic diffusion.
- Interpretation: After incubation, the plates are examined for zones of inhibition around each antibiotic disc. The diameter of the zone is measured using a ruler or calipers.

#### **4.6 ANALYSIS OF BIOSURFACTANT PRODUCTION BY BACTERIAL STRAIN**

Here we have used three different methods to check the production of biosurfactants by the bacterial strain. It's important to perform multiple assays to confirm the production of biosurfactants by a bacterial strain, as different assays provide complementary information. Additionally, negative results in one assay may not necessarily rule out biosurfactant production, so a combination of qualitative and quantitative approaches is recommended for comprehensive analysis.

#### ***4.6.1 Drop collapse assay procedure***

The drop collapse assay is a simple and effective method used to assess the surface activity of surfactant molecules, including biosurfactants. It provides a quick and qualitative assessment of the surface activity of surfactant molecules and is widely used in microbiology and biotechnology research to screen for biosurfactant-producing microorganisms and evaluate the effectiveness of biosurfactants for various applications.

- **Preparation of surfactant solution:** we have taken xylene which contains surfactant.
- **Preparation of hydrophobic surface:** we have to use a clean, flat surface that repels water. This could be a glass slide treated with a hydrophobic coating or a material like polystyrene or polypropylene. Ensure that the surface is clean and free of any contaminants. We used plastic Petri plates.
- **Dispensing Droplets:** using a micropipette, dispense small droplets here we took 1.5µl of the surfactant solution onto the hydrophobic surface. Be sure to place the droplets close together but not touching. And add 0.5µl of the sample. The sample should be centrifuged and the supernatant should be used.
- **Observation:** observe the behavior of the droplets immediately after dispensing them onto the surface. If the surfactant molecules in the solution reduce the surface tension sufficiently, the droplets will spread out and eventually collapse, forming a thin film on the surface.

#### ***4.6.2 Oil displacement assay procedure***

The oil displacement assay is a method used to evaluate the emulsification or surfactant activity of compounds, including biosurfactants. This assay assesses the ability of a substance to displace or spread oil from a water surface, indicating its surface-active properties. Here's a step-by-step guide on how to perform an oil displacement assay:

- **Preparation of experimental set up:** fill the plastic petri plates with water to create a water surface. Then place a small volume of oil i.e. xylene on to the

water surface, forming a thin layer of oil. we have added some methylene blue in the surfactant solution to easily differentiate the displacement.

- **Addition of test substance:** apply a drop of the supernatant of the sample which we have already centrifuged. add it to the center of the oil layer using a micropipette. ensure that the drop is carefully placed on top of the oil layer.
- **Observation:** Observe the behavior of the oil and test substance immediately after the drop is added. If the substance possesses surfactant activity, it will spread rapidly across the oil-water interface, displacing the oil and forming a clear zone where the oil was previously located.

#### ***4.6.3 Hemolytic assay procedure:***

A hemolytic assay is a laboratory test used to measure the ability of a substance to cause the rupture of red blood cells known as hemolysis. It is often used in research and clinical settings to assess the toxicity of compounds, study immune responses, or diagnose certain medical conditions.

- **Preparation of media:** Here we have used 50ml of nutrient agar as the media, after autoclaving the nutrient agar media we mixed 2.5ml of Human blood in it.
- **Streaking:** We have streaked the bacterial strain in the media by using the zig-zag streaking method. We have streaked the bacterial strain with the use of an inoculation loop.
- **Incubation:** Then we have incubated the culture in an incubator at 37° C for 24 hours.

## CHAPTER 5

### RESULTS AND DISCUSSION

#### 5.1 COLLECTION OF SAMPLE

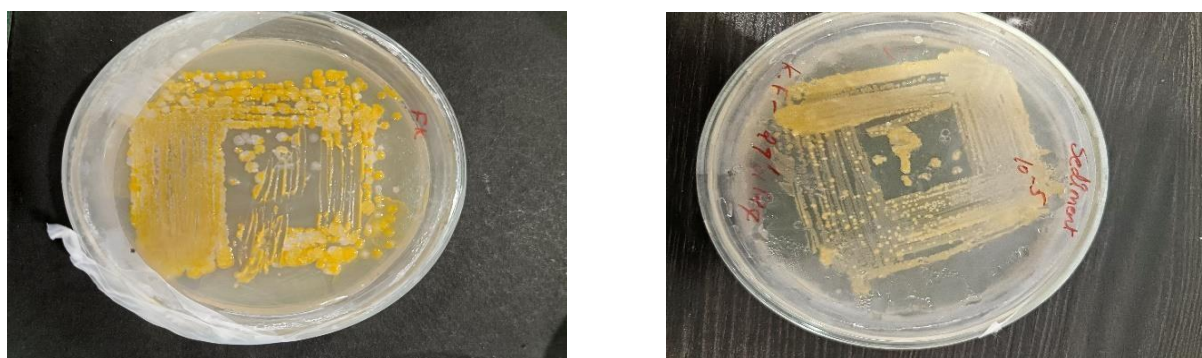
We have collected six different types of water samples from different water sources we have collected sewage water from two different places one we collected from the urban areas and another sewage water sample was collected from the sathyabama sewage water treatment plant, we have collected mangrove pure water, and mangrove sediment water from muttukad, Then we have collected raw water from sathyabama sewage water treatment plant, and aquaculture discharge water sample from sathyabama aquaculture facility.

| Places                                  | No: of samples | Types                            |
|-----------------------------------------|----------------|----------------------------------|
| Muttukad                                | 2              | Mangrove sediment And pure water |
| Sathyabama sewage water treatment plant | 2              | Sewage water and raw water       |
| Sathyabama Aquaculture Field facility   | 1              | Aquaculture discharge water      |
| Urban Areas                             | 1              | Sewage water                     |

**Table: 5.1: Sample collection**

## 5.2 ISOLATION OF BACTERIA

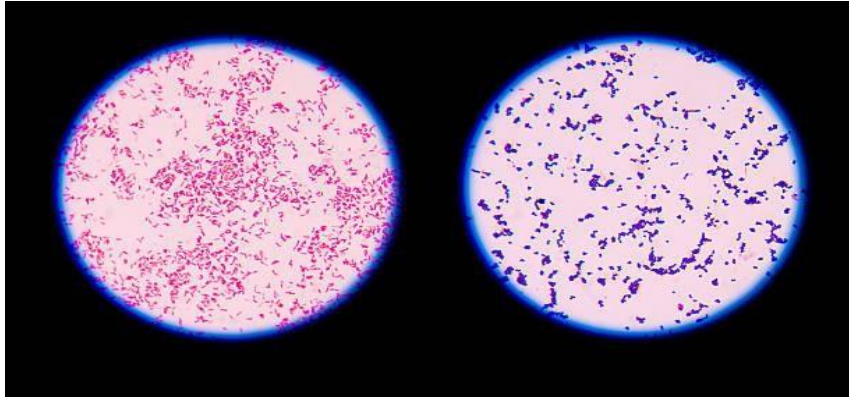
Isolation of bacteria is the process of separating or obtaining pure cultures of bacteria from a complex mixture or sample containing multiple microorganisms. We have isolated the bacteria by different screening methods like streaking, slant streaking, and inoculation. We have different types of bacterial strains. It was different according to their color and shape. We got yellow, pink, and gray colored bacterial colonies. After streaking we have done the slant streaking method for isolating pure colonies. Then for the growth of bacteria, we have inoculated the bacterial strains.



**Fig: 5.1: Colored colonies**

## 5.3 Gram staining

Here we have stained the bacterial colonies that we have isolated from the different environmental water sources. We have used  $10^{-5}$  power for all the samples. and we have got gram-positive bacteria and gram-negative bacteria. We got gram-positive bacteria from the Mangrove sediment water sample from this we got rod-shaped bacteria in a cluster cell arrangement. Then for the mangrove pure water sample, we got a rod-shaped single-cell arrangement. For both the sewage water sample we got rod-shaped cluster cell arrangement bacteria. Then for the aquaculture discharge water sample, we got cocci-shaped cluster cell arranged bacteria. The gram-positive and gram-negative bacteria are differentiated by the cell wall structure and composition.



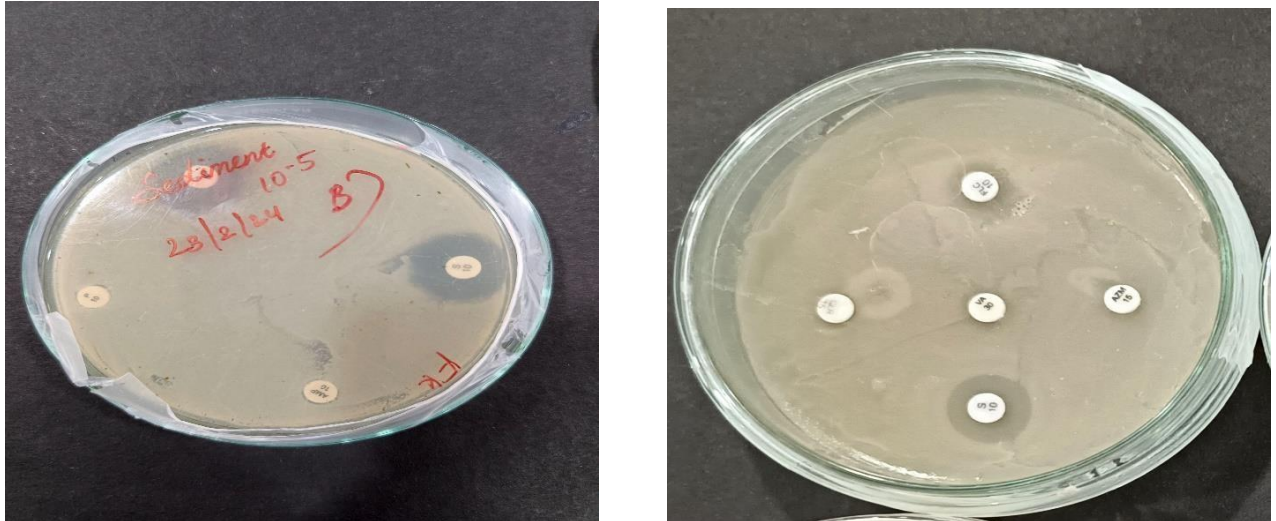
**Fig:5.2: visualization gram staining**

| <b>Strains<br/>(10<sup>-5</sup>)</b> | <b>Shape</b>            | <b>Grams<br/>Class</b>   | <b>Cell<br/>Arrangement</b> |
|--------------------------------------|-------------------------|--------------------------|-----------------------------|
| <b>Mangrove<br/>pure</b>             | <b>Rod<br/>Shaped</b>   | <b>Gram<br/>Negative</b> | <b>Cluster</b>              |
| <b>Sediment</b>                      | <b>Rod<br/>Shaped</b>   | <b>Gram<br/>Positive</b> | <b>Cluster</b>              |
| <b>Aquaculture</b>                   | <b>Cocci<br/>Shaped</b> | <b>Gram<br/>Negative</b> | <b>Cluster</b>              |
| <b>Sewage</b>                        | <b>Rod<br/>Shaped</b>   | <b>Gram<br/>Negative</b> | <b>Cluster</b>              |
| <b>Raw</b>                           | <b>Rod<br/>Shaped</b>   | <b>Gram<br/>Negative</b> | <b>Cluster</b>              |

**Table:5.2 : Gram staining**

#### 5.4 ANTI- BACTERIAL SUSCEPTIBILITY TEST

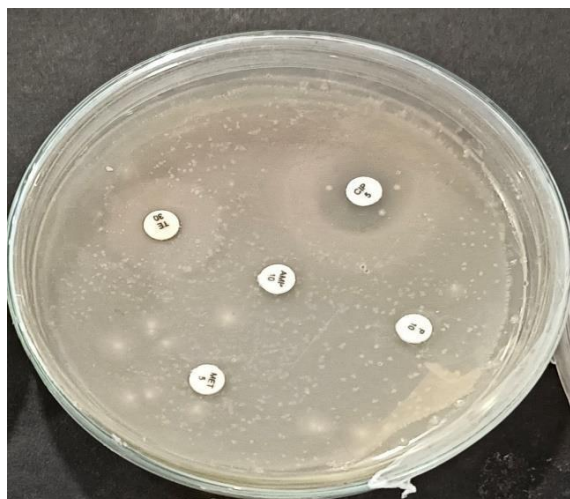
The Antibacterial susceptibility test was done by using the disc-diffusion method. Here we have diffused 10 different types of antibiotic disc in each bacterial strain from the environmental water sources. For different strains, it showed a different result. It showed the result ie susceptible or not was shown by forming a zone of inhibition around the antibiotic disc. The measurement of the zone indicates the effectiveness of the antibiotic against the bacteria being tested. A large zone generally suggests better antibiotic efficacy, while a smaller zone might indicate resistance. In this, we have susceptible and resistant characters. If the zone of inhibition diameter falls within the susceptible range, it indicates the bacteria are susceptible to the antibiotic. This means the antibiotic should be effective in treating infections caused by these bacteria. The results of the antibacterial susceptibility test can provide valuable information for guiding treatment decisions and can also be used for surveillance and monitoring of antibiotic resistance patterns in bacterial populations. The results are reported as susceptible, intermediate, or resistant, guiding clinicians in selecting the most appropriate antibiotic therapy for bacterial infections while helping to prevent the spread of antibiotic resistance. Overall, the antibiotic disc results provide valuable information about the susceptibility of the test bacteria to specific antibiotics and help inform treatment decisions to combat bacterial infections effectively while minimizing the development of antibiotic resistance.



**Fig: 5.3: Antibiotic disc diffused in Mangrove sediment water sample**

| Antibiotics    | Disc content<br>Mcg/disc | Sensitivity | Inhibition zone<br>diameter (mm) |
|----------------|--------------------------|-------------|----------------------------------|
| Vancomycin     | 30                       | Resistant   | Nill                             |
| Penicillin-G   | 10                       | Resistant   | Nill                             |
| Fluconazole    | 10                       | Susceptible | 18                               |
| Clarithromycin | 15                       | Resistant   | Nill                             |
| Azithromycin   | 15                       | Resistant   | Nill                             |
| Streptomycin   | 10                       | Susceptible | 26                               |
| Tetracycline   | 30                       | Resistant   | Nill                             |
| Ciprofloxacin  | 5                        | Susceptible | 24                               |
| Ampicillin     | 10                       | Resistant   | Nill                             |
| Methicilin     | 5                        | Resistant   | Nill                             |

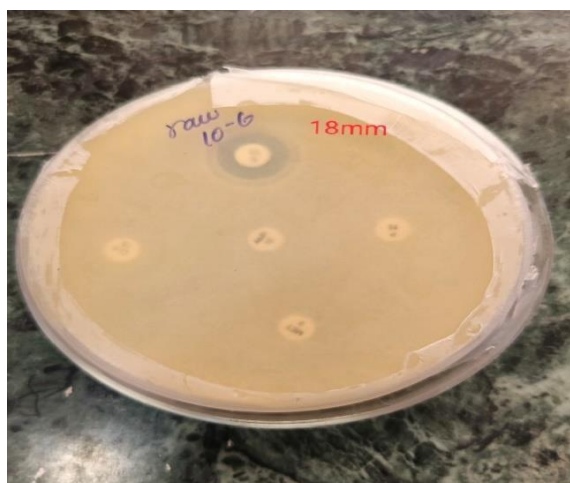
**Table: 5.3: Mangrove sediment water sample**



**Fig: 5.4: Disc diffusion in Aquaculture Discharge Water**

| Antibiotics    | Disc content<br>Mcg/disc | Sensitivity | Inhibition zone<br>diameter (mm) |
|----------------|--------------------------|-------------|----------------------------------|
| Vancomycin     | 30                       | Resistant   | Nill                             |
| Penicillin-G   | 10                       | Resistant   | Nill                             |
| Fluconazole    | 10                       | Resistant   | Nill                             |
| Clarithromycin | 15                       | Susceptible | 27                               |
| Azithromycin   | 15                       | Resistant   | Nill                             |
| Streptomycin   | 10                       | Susceptible | 20                               |
| Tetracycline   | 30                       | Resistant   | Nill                             |
| Ciprofloxacin  | 5                        | Susceptible | 24                               |
| Ampicillin     | 10                       | Resistant   | Nill                             |
| Methicilin     | 5                        | Resistant   | Nill                             |

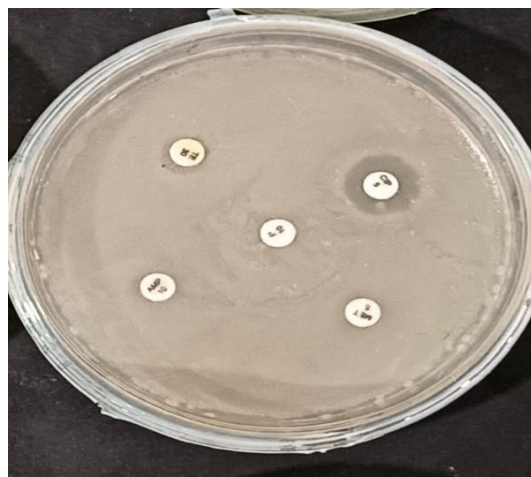
**Table: 5.4: Aquaculture Discharge Water Sample**



**Fig: 5.5: Disc diffusion in Raw water strain**

| Antibiotics    | Disc content<br>Mcg/disc | Sensitivity | Inhibition zone<br>diameter (mm) |
|----------------|--------------------------|-------------|----------------------------------|
| Vancomycin     | 30                       | Resistant   | 20                               |
| Penicillin-G   | 10                       | Resistant   | 27                               |
| Fluconazole    | 10                       | Susceptible | 16                               |
| Clarithromycin | 15                       | Resistant   | Nill                             |
| Azithromycin   | 15                       | Resistant   | Nill                             |
| Streptomycin   | 10                       | Susceptible | 18                               |
| Tetracycline   | 30                       | Resistant   | Nill                             |
| Ciprofloxacin  | 5                        | Susceptible | Nill                             |
| Ampicillin     | 10                       | Resistant   | Nill                             |
| Methicilin     | 5                        | Resistant   | Nill                             |

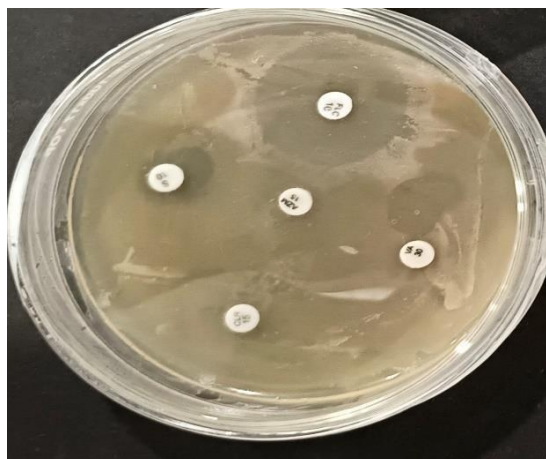
**Table: 5.5: Result of Disc Diffusion in Aquaculture Strain**



**Fig: 5.6: Disc diffusion in mangrove pure water Strain**

| Antibiotics    | Disc content<br>Mcg/disc | Sensitivity | Inhibition zone<br>diameter (mm) |
|----------------|--------------------------|-------------|----------------------------------|
| Vancomycin     | 30                       | Resistant   | Nill                             |
| Penicillin-G   | 10                       | Susceptible | 27                               |
| Fluconazole    | 10                       | Resistant   | Nill                             |
| Clarithromycin | 15                       | Resistant   | Nill                             |
| Azithromycin   | 15                       | Susceptible | 24                               |
| Streptomycin   | 10                       | Resistant   | Nill                             |
| Tetracycline   | 30                       | Resistant   | Nill                             |
| Ciprofloxacin  | 5                        | Resistant   | Nill                             |
| Ampicillin     | 10                       | Susceptible | 13                               |
| Methicilin     | 5                        | Resistant   | Nill                             |

**Table: 5.6: Result of Disc Diffusion in Mangrove pure Strain**



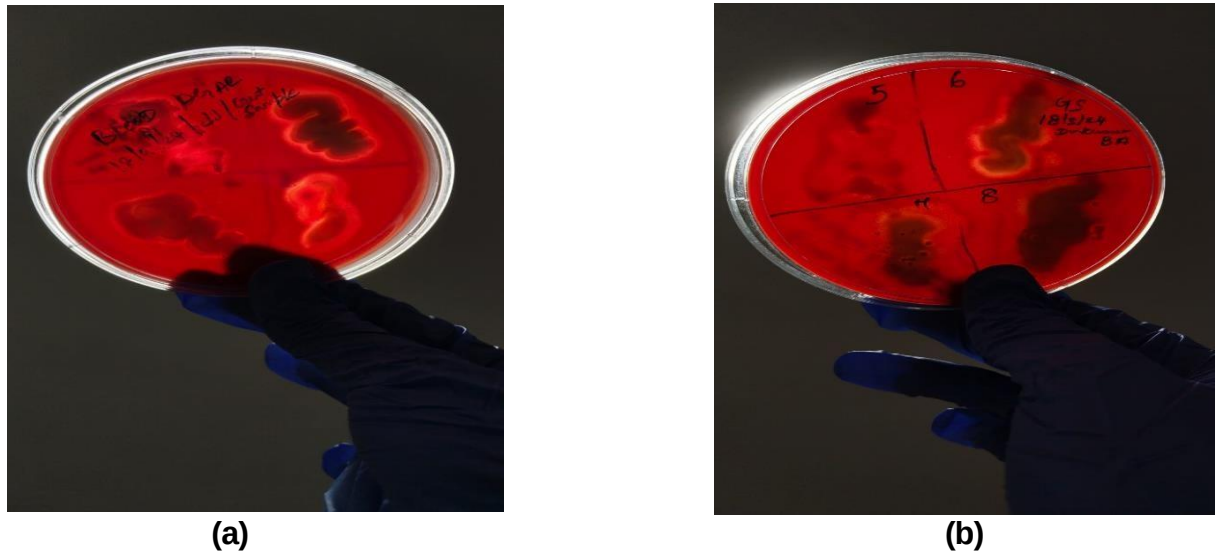
**Fig: 5.7: Disc diffusion in Sewage water Strain**

| Antibiotics    | Disc content<br>Mcg/disc | Sensitivity | Inhibition zone<br>diameter (mm) |
|----------------|--------------------------|-------------|----------------------------------|
| Vancomycin     | 30                       | Resistant   | Nill                             |
| Penicillin-G   | 10                       | Resistant   | Nill                             |
| Fluconazole    | 10                       | Susceptible | 35                               |
| Clarithromycin | 15                       | Resistant   | Nill                             |
| Azithromycin   | 15                       | Resistant   | Nill                             |
| Streptomycin   | 10                       | Susceptible | 18                               |
| Tetracycline   | 30                       | Susceptible | 23                               |
| Ciprofloxacin  | 5                        | Susceptible | 18                               |
| Ampicillin     | 10                       | Resistant   | Nill                             |
| Methicilin     | 5                        | Resistant   | Nill                             |

**Table: 5.7: Result of Disc Diffusion in Sewage water Strain**

## 5.5 BIOSURFACTANT

### 5.5.1 Hemolytic assay

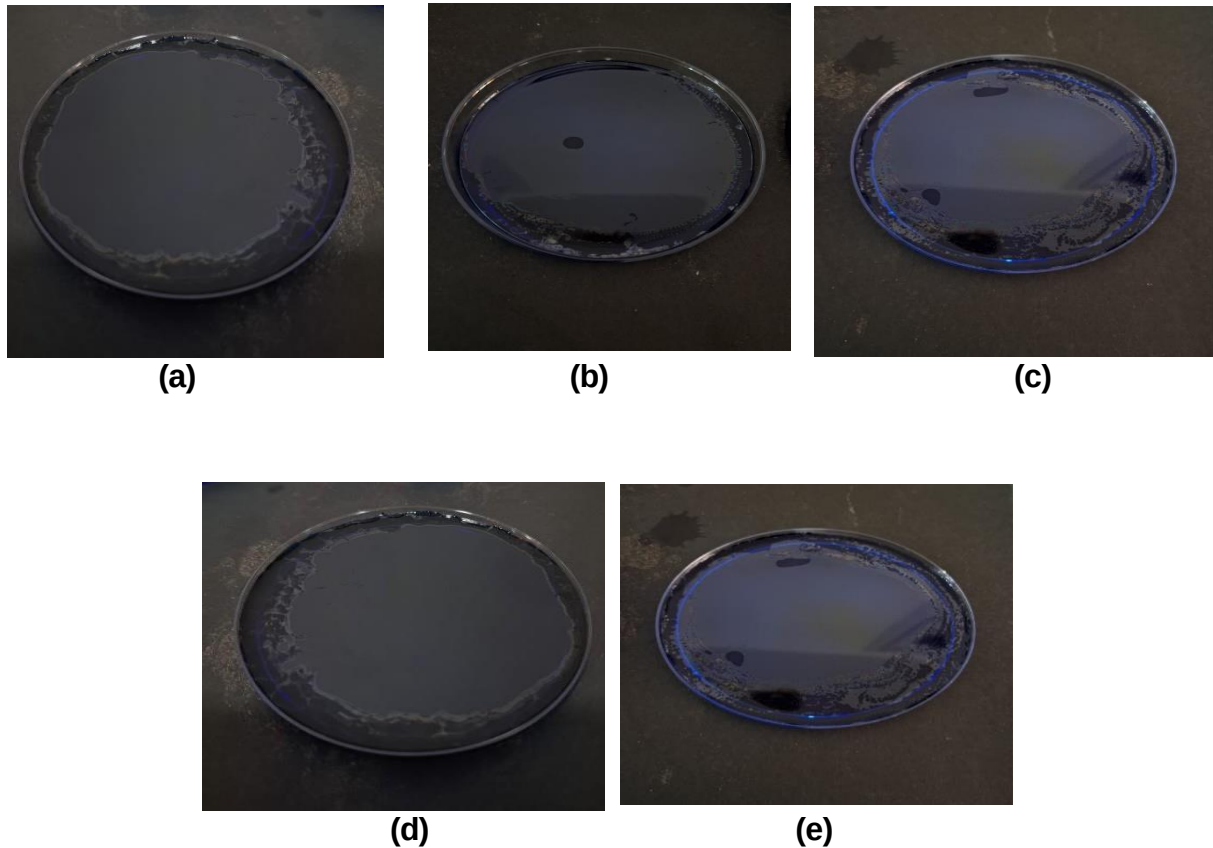


**Fig:5.8:hemolytic assay of (a) aquaculture discharge water**

**(b) Sewage water sample in petri plates**

Here we got the zone of inhibition around all the quadrants. The powers which we used for each sample were Aquaculture discharge water we used  $10^{-3}$ , sewage water  $10^{-5}$ , Mangrove sediment water  $10^{-5}$ , Mangrove pure water  $10^{-3}$ , Raw water  $10^{-3}$ , Sewage water (Sathyabama sewage water treatment plant)  $10^{-5}$ .

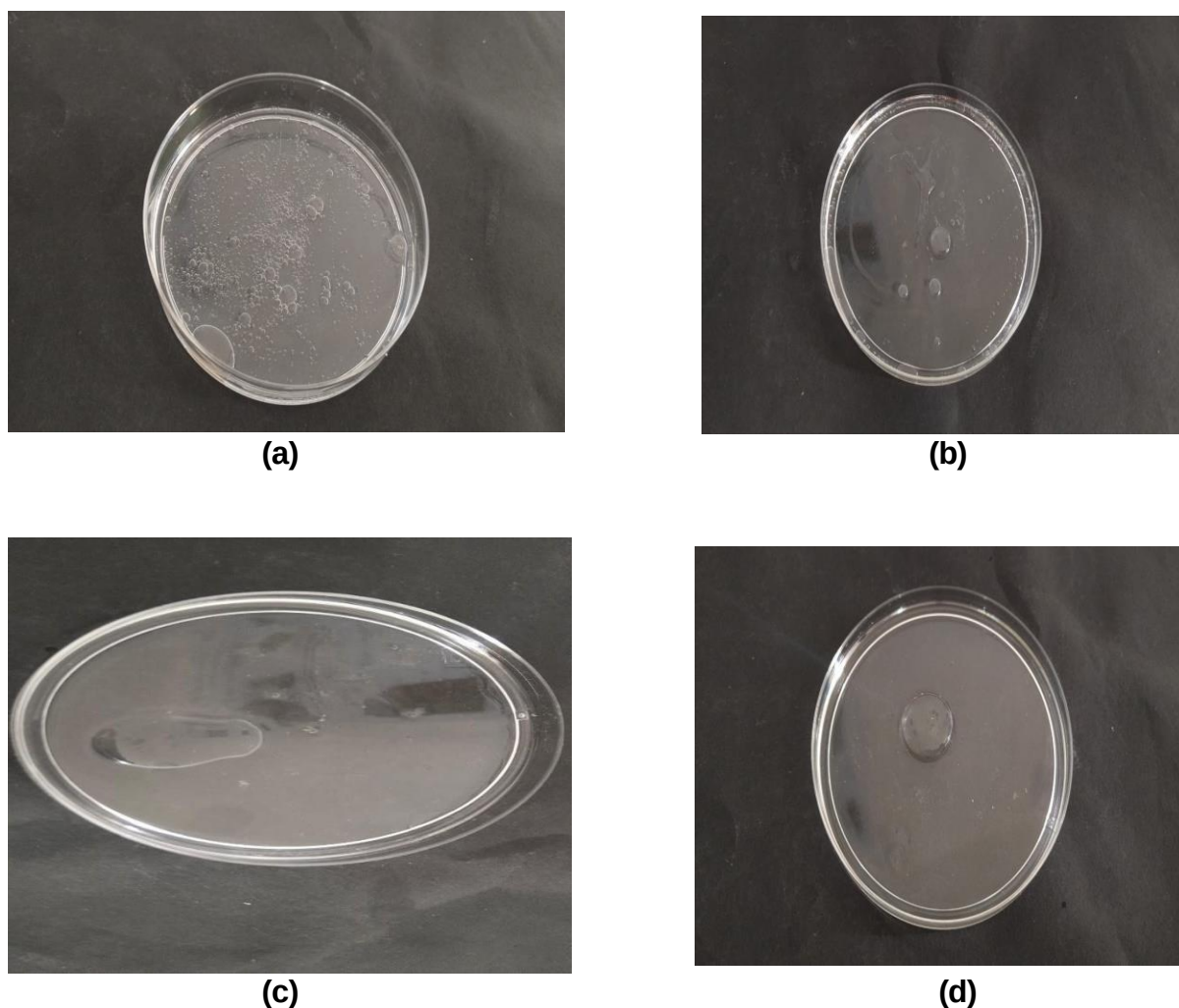
### 5.5.2 Oil- Displacement



**Fig: 5.9: Drop–collapse assay (a) Aquaculture discharge water (b) Sewage water ( from roadside) (c) Mangrove sediment water (d) Mangrove pure water (d) Raw water (e) Sewage water ( sathyabama sewage treatment plant).**

Here we have performed a Oil- displacement assay for the conformation of the production of biosurfactant from our different water source bacterial strains. Here we have used  $10^{-5}$  power of all the bacterial strains. Here the oil get displaced to the corner of the petri plate. Here we have used xylene which was mixed with methylene blue dye for the clear appearance of the displacement. For all the six samples the oil get displaced.

### 5.5.3 Drop-collapse assay



**Fig: 5.10: Visualization of oil-displacement assay result (a) Sewage water strain (b) Mangrove pure water strain (c) Mangrove sediment water strain (d) Raw water strain**

We have performed the drop-collapse test for analyzing the biosurfactant production. For this procedure, we have taken  $10^{-5}$  for all the bacterial strains. Here in this procedure we first dropped the oil in a clean and clear surface as a droplet but after adding the bacterial strain the oil got collapsed. Thus it was confirmed that all the bacterial strains produce biosurfactant.

| SAMPLE           | OIL-<br>DISPLACEMENT | DROP-<br>COLLAPSE | HEMOLYTIC<br>ASSAY |
|------------------|----------------------|-------------------|--------------------|
| CONTROL          | 0+0                  | -                 | -                  |
| AQUACULTURE      | 1.01±0.03            | +                 | +                  |
| SEWAGE           | 2.15±0.03            | +                 | +                  |
| RAW              | 1.05±0.01            | +                 | +                  |
| SEDIMENT         | 1.03±0.03            | +                 | +                  |
| MANGROVE<br>PURE | 1.03±0.03            | +                 | +                  |

**Table: 5.8: Biosurfactant**

## **CHAPTER 6**

### **SUMMARY AND CONCLUSION**

In conclusion, the distribution of antimicrobial resistance (AMR) in water environments is a complex and multifaceted issue with significant implications for public health and environmental sustainability. Through the case study presented it becomes evident that AMR is not confined to clinical settings but extends into natural ecosystems, including freshwater bodies, wastewater treatment plants, and coastal or marine waters. The monitoring of AMR in water environments is crucial for understanding the spread and dynamics of resistant bacteria and genes, identifying potential hotspots of resistance, and assessing associated risks to human health and ecosystems. Furthermore, the case study underscores the need for proactive strategies and interventions to mitigate the spread of AMR in water environments. This may include improving wastewater treatment processes, implementing antibiotic stewardship programs, promoting antimicrobial alternatives, and enhancing public awareness and education on the responsible use of antibiotics. Collaborative efforts at the local, national, and global levels are essential to combat AMR effectively and preserve the efficacy of antimicrobial agents for future generations. By this case study, we confirm the presence of antibiotic-resistant microorganisms are present in environmental water bodies and they can even produce biosurfactants. In summary, the distribution of antimicrobial resistance in water environments presents a complex challenge that requires a concerted and coordinated response from stakeholders across multiple sectors. By enhancing surveillance, understanding transmission dynamics, and implementing targeted interventions, we can mitigate the risks associated with AMR and safeguard human health, environmental integrity, and the sustainability of our water resources.

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