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ISOLATION OF BIOSURFACTANTS FROM SOIL SAMPLE, IT IDENTIFICATION TESTS AND ANTI-BACTERIAL ACTIVITIES

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Abstract:

Biosurfactants are surface-active compounds produced by microorganisms with diverse applications in pharmaceuticals, agriculture, and environmental remediation industries. This study aimed to isolate and characterize biosurfactants from soil samples and evaluate their antibacterial activities. Soil samples were collected from contaminated and uncontaminated sites and subjected to serial dilution and microbial enrichment techniques to isolate potential biosurfactant-producing microorganisms. Screening for biosurfactant production was conducted using standard methods, including the oil displacement test, emulsification index (E24), and drop collapse assay. The biosurfactants were extracted using solvent extraction methods and characterized by biochemical tests and Fourier-transform infrared spectroscopy (FTIR) analysis to identify functional groups. The antibacterial activities of the isolated biosurfactants were assessed against pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* using the agar well diffusion method. The results demonstrated that the isolated biosurfactants exhibited significant emulsification properties and broad-spectrum antibacterial activity, highlighting their potential for therapeutic and industrial applications. This study provides insights into the ecological potential of soil-derived biosurfactants as natural, eco-friendly alternatives for antimicrobial agents and industrial surfactants.

Keywords: Bacteria, Biosurfactants, Antibiotics, Anti-bacterial activity, Spread plate method.

INTRODUCTION:

BACTERIA:

Bacteria are single-celled microorganisms that belong to the prokaryotic domain. They are among the oldest forms of life on Earth and play a crucial role in various ecological processes, including nutrient cycling, digestion, and disease. Bacteria can be found in nearly every environment, ranging from extreme conditions like hot springs and deep-sea vents to more common environments like soil and water. [1]

Bacterial cells are generally characterized by their lack of a nucleus, a feature distinguishing them from eukaryotes. They possess a simple structure with a cell wall, plasma membrane, cytoplasm, and ribosomes. Some bacteria also have additional structures like flagella for movement or pili for attachment to surfaces. Bacteria reproduce asexually through binary fission, where one cell divides into two genetically identical cells. [2]

Bacteria can be classified based on various factors, such as shape (e.g., cocci, bacilli, spirilla), Gram stain characteristics (Gram-positive or Gram-negative), and metabolic pathways (e.g., aerobic or anaerobic). Their ability to adapt to diverse environments makes them highly versatile, and they can be either harmless or pathogenic to humans, animals, and plants. [3]

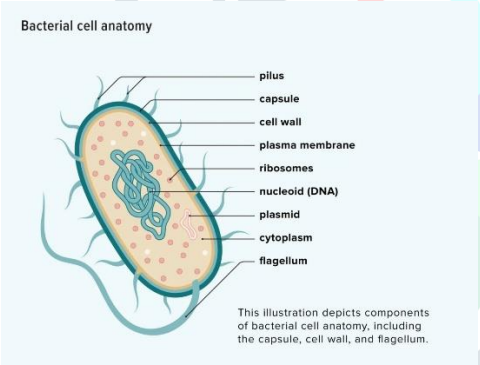

Fig: 1 Bacteria

TAXONOMY AND DIVERSITY:

Bacteria are classified into two primary groups based on their cell wall structure:

- **Gram-positive**
- **Gram-negative**

This classification is determined by the Gram stain procedure, which differentiates bacteria based on the composition of their cell walls.

GRAM-POSITIVE:

Gram-positive bacteria have a thick peptidoglycan layer in their cell walls, which retains the crystal violet dye, giving them a purple appearance under the microscope.

Examples: Staphylococcus aureus and Streptococcus pneumoniae.

GRAM-NEGATIVE:

Gram-negative bacteria have a thinner peptidoglycan layer and an outer membrane, which allows them to retain the red counterstain (safranin) in the Gram stain procedure.

Examples: Escherichia coli and Pseudomonas aeruginosa.[4]

PHYSIOLOGICAL CHARACTERISTICS:

Bacteria exhibit immense metabolic diversity, allowing them to thrive in a variety of environments. Some of the major metabolic categories include:

Autotrophs: These bacteria can synthesize their own food using inorganic compounds. **Heterotrophs:** Most bacteria are heterotrophic, obtaining carbon from organic compounds. **Aerobes and Anaerobes:** Some bacteria require oxygen (aerobes) for respiration, while others thrive in the absence of oxygen (anaerobes). There are also facultative anaerobes that can switch between aerobic and anaerobic respiration based on the presence of oxygen. [5]

ROLE IN THE ENVIRONMENT:

Bacteria play critical roles in environmental processes such as:

Nitrogen Fixation: Certain bacteria, such as those in the genus Rhizobium, form symbiotic relationships with plants and help convert atmospheric nitrogen into a form usable by plants, which is essential for plant growth.

Decomposition: Bacteria decompose organic matter, recycling nutrients in ecosystems and making them available for other organisms.

Bioremediation: Some bacteria can degrade pollutants and toxins, offering potential solutions for cleaning up oil spills, wastewater, and other environmental contaminants.

Symbiosis: Many bacteria live in mutualistic relationships with hosts, such as the human gut microbiota, where they aid digestion and protect against harmful pathogens. [6]

ANTIBIOTIC RESISTANCE:

The widespread use of antibiotics has led to the emergence of antibiotic-resistant bacteria, which pose a significant challenge to public health. Resistance mechanisms include the production of enzymes that degrade antibiotics, alterations to the bacterial cell membrane to prevent antibiotic entry, and the efflux of antibiotics out of the cell. The rise of antibiotic-resistant infections underscores the need for new therapeutic strategies and prudent use of existing antibiotics. [7]

BACTERIAL REPRODUCTION AND GENETIC EXCHANGE:

Bacteria primarily reproduce through binary fission, a form of asexual reproduction where a single bacterial cell divides into two identical daughter cells. However, bacteria can also exchange genetic material through several mechanisms, which contributes to their adaptability and genetic diversity:

Conjugation: Direct transfer of genetic material (usually plasmids) between two bacteria via a pilus.

Transformation: Uptake of free DNA from the environment by a bacterial cell.

Transduction: Transfer of genetic material between bacteria via bacteriophages (viruses that infect bacteria). [8]

ANTIBIOTICS:

INTRODUCTION:

Antibiotics are a class of drugs used to treat bacterial infections by either killing or inhibiting the growth of bacteria. They have revolutionized medicine, significantly reducing mortality rates from infections and enabling complex surgeries and organ transplants to become safer. However, overuse and misuse of antibiotics have led to the emergence of antibiotic-resistant bacteria, which pose a major public health threat worldwide.[9]

TYPES OF ANTIBIOTICS:

Antibiotics can be classified into several categories based on their mechanism of action, spectrum of activity, or chemical structure. The main classes include:

Beta-lactams: This class includes penicillins, cephalosporins, carbapenems, and monobactams. They work by inhibiting bacterial cell wall synthesis.

Macrolides: These include drugs like erythromycin, azithromycin, and clarithromycin. They inhibit protein synthesis by binding to the bacterial ribosome.

Tetracyclines: These include tetracycline, doxycycline, and minocycline, which also inhibit protein synthesis.

Aminoglycosides: Examples include gentamicin and tobramycin, which interfere with protein synthesis by binding to bacterial ribosomes.

Fluoroquinolones: These include ciprofloxacin, levofloxacin, and moxifloxacin, which target bacterial DNA replication and repair.

Sulfonamides: These drugs inhibit folic acid synthesis, which is crucial for bacterial DNA synthesis.

Glycopeptides: Vancomycin is the most well-known drug in this category, which inhibits bacterial cell wall synthesis.

Lincosamides: Clindamycin is a common drug in this class, which also inhibits protein synthesis. [10]

MECHANISM OF ACTION:

Antibiotics can act in several ways to either kill or inhibit bacteria:

Inhibition of Cell Wall Synthesis: Antibiotics like penicillins and cephalosporins prevent the formation of bacterial cell walls, leading to cell lysis.

Inhibition of Protein Synthesis: Antibiotics such as macrolides, tetracyclines, and aminoglycosides bind to bacterial ribosomes, preventing the synthesis of proteins essential for bacterial survival.

Inhibition of Nucleic Acid Synthesis: Fluoroquinolones interfere with bacterial DNA replication, while rifamycins inhibit RNA synthesis.

Disruption of Cell Membrane Integrity: Some antibiotics, like polymyxins, disrupt the bacterial cell membrane, leading to leakage of cellular contents and cell death.

Inhibition of Metabolic Pathways: Sulfonamides inhibit the synthesis of folic acid, which bacteria need for growth and replication.

ANTIBIOTIC RESISTANCE:

Antibiotic resistance occurs when bacteria evolve mechanisms to resist the effects of drugs that once killed them or inhibited their growth. This resistance is a growing global concern, driven by factors such as:

Overuse and misuse of antibiotics: The inappropriate use of antibiotics in human medicine, agriculture, and animal farming accelerates the development of resistance.

Genetic mutations and horizontal gene transfer: Bacteria can acquire resistance genes through mutations or by transferring genetic material from other resistant bacteria.

Incomplete courses of treatment: When patients do not complete their antibiotic courses, some bacteria may survive and develop resistance. [11]

BIOSURFACTANTS:

INTRODUCTION:

Biologically synthesized surface active agents are known as biosurfactants. They are synthesized by small sized organisms known as microorganisms. Biosurfactants are amphiphilic compound that have hydrophilic and hydrophobic domains. The hydrophilic domains are usually consisting of carbohydrates, amino acids and phosphate groups. Hydrophobic domains are usually made up of long chain fatty acids.

Classification of Biosurfactants:

The Biosurfactants are classified on the basis of its molecular weight and on the basis of its chemical composition.

1. Basis of its molecular weight.
2. Basis of its chemical composition.

1. **Basis of its molecular weight:**
   - i. **Low molecular weight Biosurfactant**
   - ii. **High molecular weight Biosurfactant**

i. **Low molecular weight Biosurfactant:**

These compounds lower the surface and interfacial tension at the air/water interfaces.The low molecular-weight biosurfactants are generally glycolipids or lipopeptides. The glycolipids are till date best studied as rhamnolipids, trehalolipids and sophorolipids which are disaccharides that are acylated with long- chain fatty acids or hydroxyl fatty acids. [12]

ii. **High- molecular weight biosurfactants:** These are most commonly referred to as bioemulsan. They are more effective in stabilizing oil in water emulsions. They are highly efficient emulsifiers that work at low concentrations. It also bears extensive substrate specificity. Ron and Rosenberg reported that a large number of bacterial species from different genera produce exo-cellular polymeric surfactant composed of polysaccha-rides, proteins, lipopolysaccharides, lipo-proteins or complex mixtures of these biopolymers. When classification is based on their polar groupings most of the biosurfactants are either anionic or neutral and the hydrophobic moiety is based on long chain fatty acids or fatty acids derivatives whereas the hydrophilic portion can be carbohydrates, amino acid, phosphate orcyclic peptide.[13]

2. Classification based on Chemical Structure

i. **Glycolipids:** Most known biosurfactants are glycolipids, they are carbohydrates whose constituent mono-, di-, tri and tetrasaccharides include glucose, mannose, galactose, rhamnose, galactose sulphate and glucoronic acid.

ii. **Lipopeptides and Lipoproteins:** A large number of cyclic lipopeptides including decapeptide antibiotics (gramicidin) and Lipopeptide antibiotics (polymxin) produced by bacteria, Bacillus brevis and Bacillus polymyxa that had remarkable surface active properties. It is consist of a lipid attached to a polypeptide chain. [14]

iii. **Fatty Acids, phospholipids and neutral lipids:** several bacteria and yeast produces large quantities of fatty acids and phospholipids type of surfactant during growth on n-alkanes substrate. Fatty acids produced from alkanes are as a result of microbial oxidations that have been considered as surfactant.

iv. **Phospholipids:** Phospholipids are major components of microbial membranes, when certain hydrocarbon degrading bacteria or yeast are grown on alkane substrates the level of the phospholipids increases greatly. When bacteria Acinetobacter sp HOI-N grown on Hexadecane substrate it produces the phospholipids that is mainly phosphatidylethanolamine.[15]

v. **Polymeric microbial surfactants:** most of these biosurfactants are polymeric heterosaccharide containing proteins. The best studied polymeric biosurfactants are emulsan, liposan, mannoprotein and polysaccharide protein complexes. [14]

PROPERTIES OF BIOSURFACTANTS:

a. It reduces the surface tension of water.

b. They have excellent capacity of forming critical micelle concentration (CMC).

c. The lethality of biosurfactants is very low.

d. They have good compatibility and digestibility.

ADVANTAGE OF BIOSURFACTANTS:

Biosurfactants have many advantages when compared to chemically synthesized

Counterparts such as:

1. They are easily degraded by microbes.

2. It has low toxicity.

3. It has good compatibility and digestibility with other living organisms.

4. They can be produced from cheap raw materials that are easily available in large quantities.

5. It exhibits emulsification capacity.

6. They are ecologically accepted due to their property of maintaining sustainability. [16]

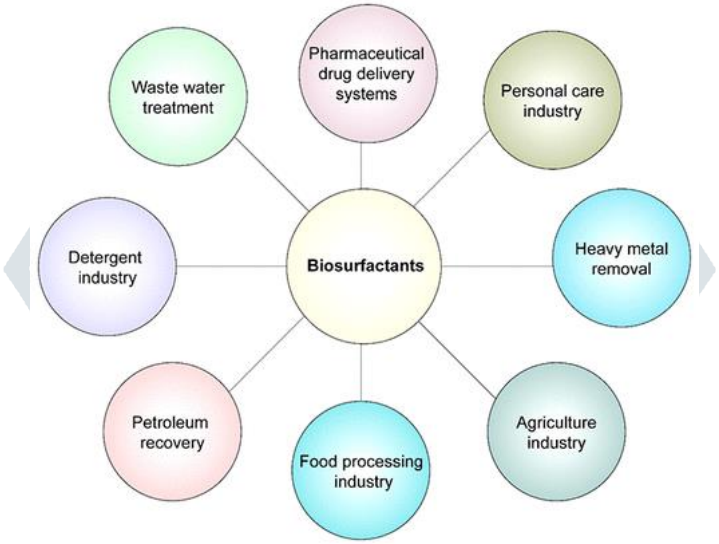

Fig: 2 Biosurfactant

MATERIALS AND METHODS:

Isolating bacteria from dairy farm soil involves a series of systematic procedures aimed at obtaining pure cultures for further study. This process is crucial for understanding microbial communities in agricultural settings and exploring potential applications in biotechnology and agriculture. Below is a detailed explanation of the isolation process, supported by insights from relevant studies.

1. SAMPLE COLLECTION:

- **Site Selection:** Choose sampling sites within the dairy farm that are representative of the area under study. This may include areas with varying soil types, moisture levels, and proximity to dairy waste sources.
- **Sampling Procedure:** Collect soil samples aseptically using sterile tools to prevent contamination. Samples are typically taken from the top 10 cm of soil, where microbial activity is most abundant.

Fig: 3 Sample Collection

2. SAMPLE HANDLING AND STORAGE:

- **Sterile Equipment:** Use **autoclaved** or pre-sterilized tools such as soil scoops, spatulas, or core samplers.
- **Aseptic Techniques:** Wear gloves and use sterile sampling bags (e.g., Whirl-Pak) to avoid cross-contamination.

3. PRE- TREATMENT BEFORE CULTURING:

- **Air Drying:** For spore-forming bacteria, let the soil air-dry before culturing.
- **Sieving:** Remove large debris and stones using a **2 mm sieve** to obtain uniform soil texture.
- **Dilution:** Prepare **serial dilutions** of soil suspensions in sterile saline or PBS for plating on agar media.

Fig: 4 Air Drying

4. PURPOSE OF SERIAL DILUTIONS:

- **To obtain countable bacterial colonies**: Many soil samples have a high bacterial load. Directly plating them can lead to overcrowded growth, making it difficult to distinguish individual colonies.
- **To reduce bacterial concentration systematically**: It allows microbiologists to culture bacteria at different dilutions, making colony enumeration feasible.
- **To improve accuracy**: It helps in estimating bacterial density in a sample by enabling **colony-forming unit (CFU) calculations**. [18]

5. STEP-BY-STEP SERIAL DILUTION PROCEDURE:

Step 1: Prepare the Soil Suspension

1. Take **1 gram** of soil from the dairy farm sample.

2. Add it to **9 mL** of sterile saline or buffer (this is the **stock solution**).
3. Vortex or shake the solution well to distribute bacteria evenly.

Step 2: Perform Serial Dilutions

1. Label **test tubes** from **10^{-1} to 10^{-6}** (depending on the expected bacterial load).
2. Transfer **1 mL** from the stock solution into **9 mL of sterile saline** in the first test tube (10^{-1} dilution).
3. Mix thoroughly and transfer **1 mL** from the first tube into the second tube (10^{-2} dilution). Repeat the process until the desired dilution (e.g., 10^{-6}).

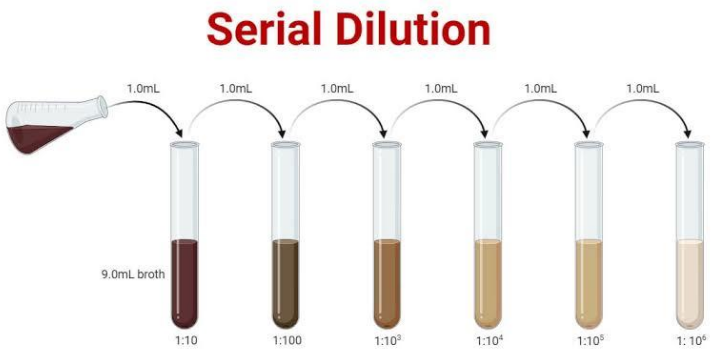

Fig: 5 Serial Dilution

Dilution Factor	Soil Sample (mL)	Diluent (mL)	Final Concentration
10^0 (Stock)	1.0	0.0	Original Sample
10^{-1}	1.0	9.0	1:10 dilution
10^{-2}	1.0	9.0	1:100 dilution
10^{-3}	1.0	9.0	1:1000 dilution
10^{-4}	1.0	9.0	1:10,000 dilution
10^{-5}	1.0	9.0	1:100,000 dilution
10^{-6}	1.0	9.0	1:1,000,000 dilution

Table1: Serial dilutions

Step 3: Preparation of Nutrient Agar Medium

Step-by-Step Procedure:

❖ **Weighing the Ingredients:**

1. Weigh **0.5g of peptone**, **0.3g of yeast**, **0.5g of NaCl**, and **5g of agar** using a digital balance.

2. Measure **100mL of distilled water** in a measuring cylinder.

❖ **Dissolving the Components:**

1. Transfer the measured ingredients into a **conical flask or beaker**.
2. Add **100 mL** of distilled water first and stir until all ingredients dissolve.
3. Add the remaining **100 mL** of distilled water and continue stirring.

❖ **Adjusting the pH:**

1. Check the pH using a **pH meter**.
2. Adjust the pH to **7.0 ± 0.2** using **1N NaOH (if acidic) or 1N HCl (if basic)**.

❖ **Boiling the Medium:**

1. Heat the solution on a **hot plate** or **Bunsen burner** until the agar completely dissolves.
2. Stir continuously to prevent burning.

❖ **Sterilization by Autoclaving:**

1. Transfer the medium into **sterile conical flasks** or **bottles**.
2. Cover the flask with **cotton plugs** or **aluminum foil**.
3. Autoclave at **121°C, 15 psi pressure for 15–20 minutes** to **kill contaminants**.

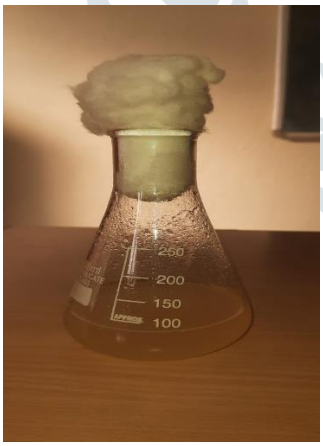

Fig: 6 Nutrient Agar Medium

❖ **Pouring the Agar Plates:**

1. Allow the agar to **cool to about 45–50°C** (to avoid killing microbes when inoculated).
2. Pour **20–25 mL** into **sterile Petri plates** inside a **laminar airflow cabinet**.
3. Let the plates **solidify at room temperature** for 15–20 minutes.Store the plates **inverted** at **4°C** until use.

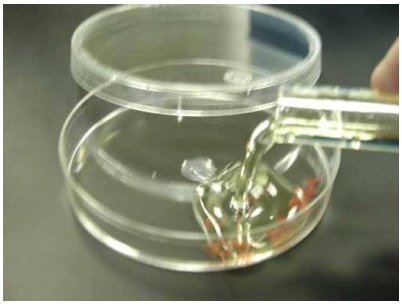

Fig: 7 Pouring the Agar plates

Step 4: Plating the Diluted Samples:

1. Take **0.1 mL** from each dilution and spread it onto separate **nutrient agar plates** using a **sterile glass spreader**.
2. Incubate the plates at **37°C for 24–48 hours** (or at room temperature for soil bacteria).
3. Observe colony growth after incubation. [19]

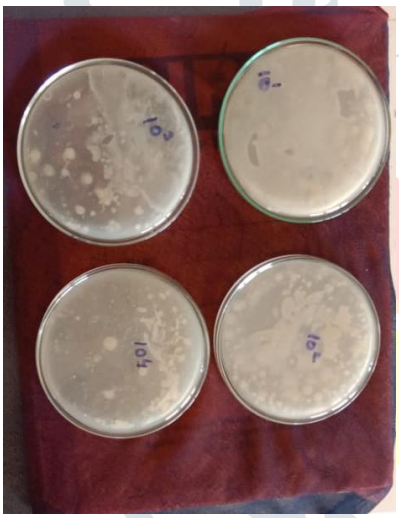

Fig: 8 Plating the diluted sample

Step 5: Subculturing Technique in Isolation of Bacteria

Subculturing is the process of transferring microorganisms from one growth medium to another to maintain viability, isolate pure colonies, or continue microbial studies. It is an essential technique in microbiology for bacterial isolation, identification, and preservation.

Steps in Subculturing Bacteria

1. Preparation of Materials

- Agar plate/slant or broth medium (as required)
- Inoculating loop/needle (sterile)
- Bunsen burner (to maintain sterility)

- Sterile pipette (if using liquid cultures).

2. Aseptic Transfer of Bacteria

i. Sterilization of Tools:

- Flame the inoculating loop or needle in a Bunsen burner until red-hot.
- Allow it to cool to avoid killing the bacteria.

ii. Picking the Bacterial Colony:

- Open the Petri dish with one hand while keeping the lid close to minimize contamination.
- Pick a single colony using the sterile inoculating loop.

iii. Transfer to New Medium:

- **For agar slants:** Streak the loop gently along the slant surface in a zigzag pattern.

Fig: 9 Agar slants

iv. Sterilization of the Loop:

- Flame the inoculating loop again before placing it down.

Fig: 10 Sterilization of the Loop

3. Incubation

- Incubate at an appropriate temperature (usually 35–37°C for pathogenic bacteria).
- Check for growth after 24–48 hours.

IDENTIFICATION TESTS:

Gram Staining Procedure

1. Prepare a bacterial smear on a clean glass slide and heat-fix it by passing it through a flame.
2. Apply crystal violet and let it sit for 30–60 seconds. Rinse with water.
3. Add iodine solution and let it sit for 30–60 seconds. Rinse with water.
4. Decolorize with alcohol or acetone for 5–10 seconds (critical step). Rinse immediately.
5. Apply safranin and let it sit for 30–60 seconds. Rinse with water.
6. Blot dry and examine under a microscope using oil immersion (100x objective).

Interpretation of Results

- **Gram-positive bacteria → Purple** (e.g., *Staphylococcus aureus, Streptococcus pneumoniae, Bacillus subtilis*).
- **Gram-negative bacteria → Pink/Red** (e.g., *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi*).

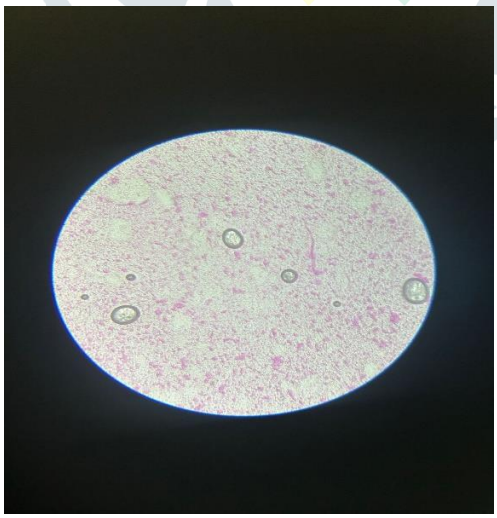

Fig: 11 Gram Negative Bacteria

Enzyme-Based Biochemical Tests

Identification of bacteria, especially through biochemical tests, involves evaluating various metabolic activities and enzymatic reactions that bacteria exhibit when exposed to specific substrates or conditions. These tests are crucial for identifying bacterial species and understanding their characteristics.

CATALASE TEST:

The Catalase Test is a biochemical test used to determine whether a bacterium produces the enzyme catalase. Catalase breaks down hydrogen peroxide (H_2O_2), a toxic by-product of aerobic metabolism, into water and oxygen. The test is commonly used to distinguish between Staphylococcus (catalase-positive) and Streptococcus (catalase-negative) species, among other bacteria.

Purpose:

- To detect the presence of catalase enzyme in a bacterial sample.

Procedure:

1. **Inoculate:** A small amount of bacterial culture (a colony from an agar plate or a liquid culture) is placed on a clean glass slide or in a test tube.
2. **Add Hydrogen Peroxide:** A few drops of 3% hydrogen peroxide (H_2O_2) are added directly to the bacterial sample.
3. **Observe for Bubbling:** The production of bubbles (oxygen) is observed immediately.

Results:

- **Positive Result:** Bubbling or effervescence (fizzing) occurs, indicating the presence of catalase, which breaks down hydrogen peroxide into water and oxygen.
  Example: Staphylococcus aureus, Streptococcus pneumoniae.
- **Negative Result:** No bubbling occurs, indicating the absence of catalase activity. **Example:** Streptococcus species, Enterococcus species.[20]

Fig: 12 Catalase Test

INDOLE TEST:

The Indole Test is a biochemical test used to determine whether a bacterium can produce indole from the amino acid tryptophan. This is done by testing for the activity of the enzyme tryptophanase, which breaks down tryptophan into indole, pyruvic acid, and ammonia.

Purpose:

- To identify bacteria that can produce indole, which is an important characteristic for differentiating bacterial species.

Procedure:

1. **Inoculate:** The bacterium is inoculated into a liquid medium containing tryptophan, such as tryptic soy broth (TSB) or tryptophan broth.
2. **Incubation:** The culture is incubated at 35-37°C for 24-48 hours.
3. **Add Reagent:** After incubation, **Kovac's reagent* (a combination of p-dimethylaminobenzaldehyde, hydrochloric acid, and amyl alcohol) is added to the broth.
4. **Observe:** The reaction is observed for the formation of a color change in the reagent layer.

Results:

- **Positive Result:** A red or pink layer forms at the top of the broth (after adding Kovac's reagent), indicating the presence of indole. This means the bacterium produced tryptophanase and metabolized tryptophan into indole.
  Example: Escherichia coli (E. coli) is indole positive.
- **Negative Result:** No color change (i.e., the reagent remains yellow or unchanged), indicating the bacterium did not produce indole.
  Example: Enterobacter species are indole negative. [21]

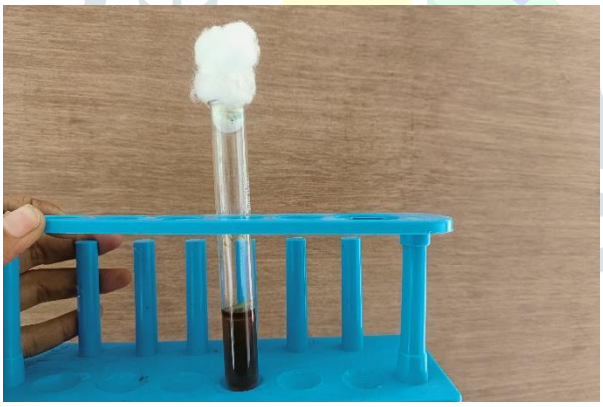

Fig: 13 Indole Test

METHYL RED:

The Methyl Red (MR) Test is a biochemical test used to determine if a bacterium can produce stable acids from glucose fermentation. This test is particularly useful for differentiating Enterobacteriaceae based on their ability to ferment glucose and produce acidic by-products.

Purpose:

- The MR test checks whether a bacterium ferments glucose to produce a significant amount of mixed acids, which lowers the pH of the medium.

Procedure:

1. **Inoculate:** The bacterium is inoculated into MR-VP broth (a medium that contains glucose, peptones, and phosphate buffer).
2. **Incubation:** The culture is incubated at 35-37°C for 48 hours to allow for fermentation.
3. **Add Reagent:** After incubation, a few drops of methyl red reagent are added to the broth.
4. **Observation:** The test result is observed for a color change.

Results:

- **Positive Result:** A red color forms in the broth after adding methyl red. This indicates that the bacterium has fermented glucose to produce a mixture of stable acids (such as lactic, acetic, and formic acid), lowering the pH of the medium below 4.4.
  Example: Escherichia coli (E. coli) is typically methyl red positive.
- **Negative Result:** No color change (i.e., the broth remains yellow or orange). This means that the bacterium did not produce stable acids but likely produced neutral products (e.g., acetoin).
  Example: Enterobacter species are often methyl red negative. [22]

Fig: 14 Methyl red Test

NITRATE REDUCTION TEST:

The Nitrate Reduction Test is a biochemical test used to determine whether a bacterium can reduce nitrate (NO_3^-) to nitrite (NO_2^-) or other nitrogenous compounds, such as nitrogen gas (N_2). This ability is due to the presence of enzymes like nitrate reductase and nitrite reductase, which facilitate the reduction process.

Purpose:

- To assess the bacterium's ability to reduce nitrate to nitrite or further reduced forms (e.g., ammonia or nitrogen gas).

Procedure:

1. **Inoculate:** The bacterium is inoculated in a nitrate broth (typically containing potassium nitrate).
2. **Incubation:** The culture is incubated at 35-37°C for 24-48 hours to allow bacterial growth and possible nitrate reduction.
3. **Add Reagents:** After incubation, nitrate reagents are added to the broth:

- **Reagent A:** sulfanilic acid.
- **Reagent B:** α-naphthylamine.

4. **Observation:**

- If the broth turns red after the addition of these reagents, it indicates that nitrite is present, confirming nitrate reduction to nitrite.
- If no color change occurs, it suggests that the nitrate may have been reduced beyond nitrite to nitrogen gas or ammonia.

5. **Confirm with Zinc:**

- If no red color forms, zinc dust is added. Zinc catalyzes the reduction of any remaining nitrate to nitrite.If the broth turns red after adding zinc, it means nitrate was not reduced by the bacteria, and nitrate remained in the broth.
- If there is no color change after adding zinc, this indicates that the nitrate was completely reduced (probably to nitrogen gas or ammonia), and the bacterium is a strong reducer of nitrate.

Results:

- **Positive Result**:

  Red color after adding nitrate reagents: The bacterium reduced nitrate to nitrite.

  No color change after adding nitrate reagents, but no color change after adding zinc: The bacterium completely reduced nitrate to non-detectable nitrogen forms (like nitrogen gas).

- **Negative Result:**

  Red color after adding nitrate reagents and zinc: Nitrate was not reduced by the bacterium, and the color change indicates that nitrate remained in the medium. [23]

Fig: 15 Nitrate reduction Test

URASE TEST:

The Urease Test is a biochemical test used to determine whether a bacterium produces the enzyme urease, which breaks down urea into ammonia (NH_3) and carbon dioxide (CO_2). The production of ammonia raises the pH of the medium, turning it pink or red, which indicates a positive result.

Purpose:

- To identify bacteria that can hydrolyze urea into ammonia, which increases the pH of the medium and results in a color change.

Procedure:

1. **Inoculate:** The bacterium is inoculated into a urea broth or urea agar containing a pH indicator, typically phenol red, which is yellow at acidic pH and pink at alkaline pH.
2. **Incubation:** The culture is incubated at 35-37°C for 24-48 hours.
3. **Observe for Color Change:** After incubation, the color of the medium is observed.

Results:

- **Positive Result:** A pink color develops in the medium, indicating that the bacterium produced urease and hydrolyzed urea into ammonia, raising the pH (pH > 8.4).
  Example: Proteus vulgaris, Helicobacter pylori, Klebsiella pneumoniae.
- **Negative Result:** The medium remains yellow or orange, indicating that the bacterium did not produce urease and thus could not hydrolyze urea.
  Exaple: Escherichia coli, Enterococcus faecalis. [24]

Fig: 16 Urease Test

BIO SURFACTANT PRODUCTION:

Biosurfactants are surface-active compounds produced by microorganisms, such as bacteria and yeast. They have various applications in industries like petroleum, agriculture, and pharmaceuticals.

Ingredients:

1. **Microorganism:**
   Select a sample.
2. **Carbon Source:** Provide a carbon source.
   Examples: Glucose (10-20g /L), Sucrose (10-20g/L), Vegetable oil (e.g., soyabean oil, 10-20g/L).
3. **Nitrogen Source:** Add a nitrogen source.
   Examples: Ammonium sulphate (1-2g/L), Urea (1-2g/L), Yeast extract (1-2g/L).
4. **Minerals:** Include essential minerals.
   Examples: Magnesium sulphate (0.1-0.5g/L), Potassium phosphate (0.1-0.5g/L)

Iron (III) chloride (0.01-0.1g/L).

5. **Water:** Use distilled or deionized water as the solvent. [25]

Preparation:

- **Inoculum Preparation:** Grow the selected microorganism in a suitable medium, such as nutrient broth, to prepare an inoculum.
- **Medium Preparation:** Prepare a production medium containing the carbon source, nitrogen source, minerals, and water.
- **Inoculation:** Inoculate the production medium with the prepared inoculum.
- **Incubation:** Incubate the inoculated medium under suitable conditions, such as:
  - Temperature: 25-37°C
  - pH: 6-8
  - Agitation: 100-200 rpm
  - Incubation time: 24-72 hours.
- **Harvesting:** Harvest the biosurfactant by separating the cells from the medium through centrifugation or filtration.
- **Purification:** Purify the biosurfactant using techniques like solvent extraction, chromatography, or crystallization.[26]

Example of Biosurfactant Production Media:

- **Glucose-based medium:**
  - Glucose: 10 g/L
  - Ammonium sulfate: 1 g/L
  - Magnesium sulfate: 0.1 g/L
  - Potassium phosphate: 0.1 g/L
  - Water: 1 L[27]

Isolation of Biosurfactants:

Step 1: Selection and Cultivation of Microorganism

- Select a biosurfactant-producing microorganism, such as Pseudomonas aeruginosa, Bacillus subtilis, or Candida lipolytica.
- Cultivate the microorganism in a suitable medium, such as nutrient broth or a defined medium, under optimal conditions (temperature, pH, agitation, etc.). [27]

Step 2: Cell Harvesting

- Harvest the cells by centrifugation (5,000-10,000 rpm, 10-30 minutes) or filtration.
- Wash the cells with distilled water to remove any residual medium. [29]

Fig: 17 Centrifugation Sample

Step 3: Extraction of Biosurfactant

- Extract the biosurfactant from the cell-free broth or cell pellet using a solvent, such as:
  - Ethyl acetate
  - Methanol
  - Chloroform
  - Hexane
- Use a separation funnel or a rotary evaporator to separate the solvent from the aqueous phase. [30]

Step 4: Purification of Biosurfactant

- Purify the extracted biosurfactant using various techniques, such as:
  - Column chromatography (silica gel, Sephadex)
  - Thin-layer chromatography (TLC)
  - High-performance liquid chromatography (HPLC)
  - Crystallization
- Use a combination of these techniques to achieve high purity. [31]

Step 5: Characterization of Biosurfactant

- Characterize the purified biosurfactant using various techniques, such as:
  - Fourier transform infrared spectroscopy (FTIR)
  - Nuclear magnetic resonance spectroscopy (NMR)
  - Mass spectrometry (MS)
  - Surface tension measurement
- Determine the chemical structure, molecular weight, and surface-active properties of the biosurfactant. [32]

IDENTIFICATION TEST FOR BIOSURFACTANT:

1. **Drop collapsing test:**
   - Drop collapsing test is the qualitative process useful for the screening of biosurfactants.
   - The isolated strains were placed on the surface of hydrocarbon.

- The destabilizations of cell free broth drop indicate positive result. A drop of water acted as a control.

Fig: 18 Drop collapsing Test

2. Oil spreading test:

- On empty petri plate two different layers were formed.
- First layer would be of water and second layer would be of hydrocarbon.
- The 24 hrs old cell free extract broth of isolate was added surface on petri plate.
- The clear zone around the culture indicates positive result.
- The diameter of the clear zone was measured.
- A drop of water acted as a control.

Fig: 19 Oil spreading test

3. Emulsification index test:

- Emulsification index is the quantitative process.
- In test tubes 2mL of hydrocarbon was added along with 2mL of 48 hrs grown culture broth.
- It was further vortex for 2 min and allowed to stand by 24 hrs.
- After 24 hrs of incubation emulsification index was calculated according to standard methodologies.

Fig: 20 Emulsification index test

4. **Foaming test:**
    - Grow the microorganism in a suitable medium and collect the culture broth or supernatant.
    - Dilute the culture broth or supernatant with distilled water to a concentration of 1-5% (v/v).
    - Pipette 1-2 mL of the diluted culture broth or supernatant into a test tube or cylinder.
    - Shake the test tube or cylinder vigorously for 1-2 minutes or vortex for 10-30 seconds.
    - Observe the test tube or cylinder for foam formation and stability.
    - Record the height of the foam and its stability over time.[33]

Fig: 21 Foaming Test

ANTIBIOTIC RESISTANCE:

Antibiotic resistance in bacteria occurs when bacteria evolve mechanisms to survive exposure to antibiotics that would normally kill them or inhibit their growth. This makes bacterial infections harder to treat and increases the risk of severe illness or death.

Spread Plate Method (Displate Method) for Bacterial Growth Inhibition Testing Through Antibiotics:

The **spread plate method** is a widely used technique to study bacterial inhibition by antibiotics, including their effect on Biosurfactant-producing bacteria. This method allows for even distribution of bacterial cultures on an agar plate, followed by the application of antibiotics to observe their inhibitory effects.

Materials Required:

1. Bacterial culture (sample)
2. Nutrient Agar (NA)
3. Sterile saline (to prepare bacterial suspension)
4. Antibiotic solutions
5. Sterile glass spreader (hockey stick) or L-spreader
6. Ethanol (for sterilization)
7. Petri dishes
8. Micropipette and sterile tips

Procedure:

1. Preparation of Bacterial Suspension:

- Pick a fresh bacterial colony and suspend it in **sterile saline** or **broth**.

2. Inoculation of the Agar Plate:

- Take **100 µL of bacterial suspension** and pipette it onto the **sterile agar plate**.
- Spread the suspension evenly using a **sterilized glass spreader** in a circular motion to ensure uniform distribution.
- Allow the plate to dry for **5-10 minutes** to let the bacteria adhere.

3. Application of Antibiotic Treatment:

- **For antibiotic discs:** Place commercially available antibiotic discs onto the agar surface using sterile forceps.
- **For liquid antibiotics:** Spot **10-20 µL** of antibiotic solution onto designated sections of the plate.

4. Incubation:

- Invert the plates and incubate at **37°C for 18-24 hours**.

5. Observation & Measurement:

- After incubation, examine the plates for **zones of inhibition** (clear areas around antibiotic applications indicating bacterial growth inhibition).
- Measure the **diameter of inhibition zones** using a ruler or calipers.

This method helps determine whether antibiotics can inhibit Biosurfactant-producing bacteria and whether the bacteria show resistance, which is crucial in antibiotic resistance research and biotechnological applications. [34]

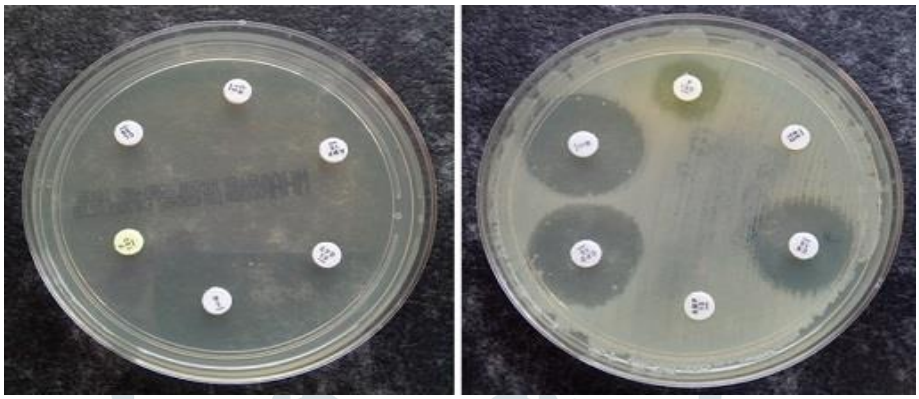

Fig: 22 Growth of Inhibition

RESULTS AND DISCUSSION:

BIOCHEMICAL TESTS:

S.No	Biochemical Test	Results
1.	Catalase Test	+
2.	Indole Test	+
3.	Methyl red Test	+
4.	Nitrate Reduction Test	+
5.	Urase Test	+

Table1: Biochemical Tests

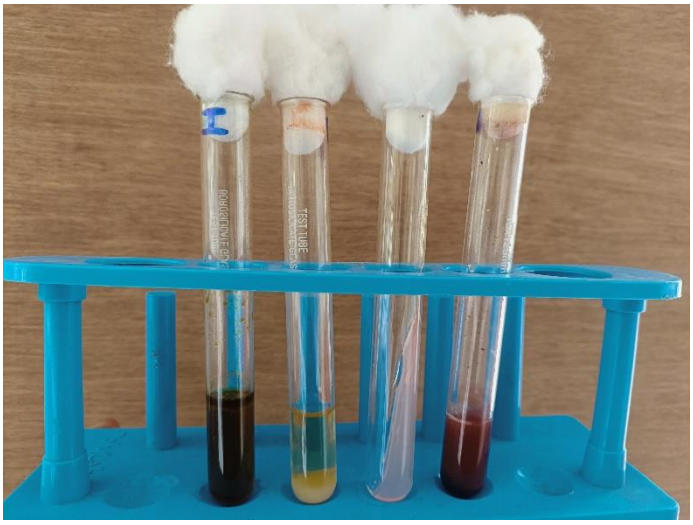

Fig 23: Results of Biochemical tests

IDENTIFICATION TESTS:

S.No	Methods	Samples			
		1	2	3	4
1.	Drop collapse test	++	+	-	+++
2.	Oil spreading method	+++	++	-	+
3.	Emulsifying index test	+++	+	++	++
4.	Foaming test	-	+	+++	++

Table 2: Identification Test for Biosurfactant

ZONE OF INHIBITION:

Zone of Inhibition (mm)	Interpretation
No zone (<6 mm)	Resistant
6-12 mm	Intermediate
>12 mm	Susceptible

Table 3: zone of inhibition

CONCLUSION:

In this study, bacteria were successfully isolated from dairy farm soil using serial dilution and the spread plate method. The bacterial colonies were subcultured and subjected to various biochemical tests, including the catalase, Indole, Methyl red, and Nitrate tests, confirming their identity and metabolic characteristics. Following bacterial identification, a production medium was prepared to evaluate biosurfactant production. Various tests were conducted to confirm biosurfactant activity, demonstrating the potential of the isolated strains in biosurfactant synthesis. Additionally, antibiotic resistance was assessed using the disc plate method, where zones of inhibition were observed, indicating varying levels of resistance among the bacterial isolates. The results highlight the diverse bacterial population in dairy farm soil and their potential applications in biosurfactant production. The study also emphasizes the importance of monitoring antibiotic resistance, which is crucial for understanding microbial adaptation in agricultural environments. Further research can explore optimizing biosurfactant production and evaluating its industrial applications.

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