IMMUNOLOGY AND AGRICULTURAL MICROBIOLOGY

M.Sc. MICROBIOLOGY

SEMESTER-II, PAPER-VI

LESSON WRITER

Prof. A. Amruthavalli
Professor
Department of Botany & Microbiology,
Acharya Nagarjuna University

EDITOR

Prof. V. Umamaheswara Rao
Professor
Department of Botany & Microbiology,
Acharya Nagarjuna University

DIRECTOR, I/c.

Prof. V. Venkateswarlu
M.A., M.P.S., M.S.W., M.Phil., Ph.D.
Professor
Centre for Distance Education
Acharya Nagarjuna University
Nagarjuna Nagar 522 510

Ph: 0863-2346222, 2346208
0863- 2346259 (Study Material)
Website www.anucde.info
E-mail: anucdedirector@gmail.com

Ravi

M.Sc. MICROBIOLOGY: IMMUNOLOGY AND AGRICULTURAL MICROBIOLOGY

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FOREWORD

Since its establishment in 1976, Acharya Nagarjuna University has been forging ahead in the path of progress and dynamism, offering a variety of courses and research contributions. I am extremely happy that by gaining 'A+' grade from the NAAC in the year 2024, Acharya Nagarjuna University is offering educational opportunities at the UG, PG levels apart from research degrees to students from over 221 affiliated colleges spread over the two districts of Guntur and Prakasam.

The University has also started the Centre for Distance Education in 2003-04 with the aim of taking higher education to the door step of all the sectors of the society. The centre will be a great help to those who cannot join in colleges, those who cannot afford the exorbitant fees as regular students, and even to housewives desirous of pursuing higher studies. Acharya Nagarjuna University has started offering B.Sc., B.A., B.B.A., and B.Com courses at the Degree level and M.A., M.Com., M.Sc., M.B.A., and L.L.M., courses at the PG level from the academic year 2003-2004 onwards.

To facilitate easier understanding by students studying through the distance mode, these self-instruction materials have been prepared by eminent and experienced teachers. The lessons have been drafted with great care and expertise in the stipulated time by these teachers. Constructive ideas and scholarly suggestions are welcome from students and teachers involved respectively. Such ideas will be incorporated for the greater efficacy of this distance mode of education. For clarification of doubts and feedback, weekly classes and contact classes will be arranged at the UG and PG levels respectively.

It is my aim that students getting higher education through the Centre for Distance Education should improve their qualification, have better employment opportunities and in turn be part of country's progress. It is my fond desire that in the years to come, the Centre for Distance Education will go from strength to strength in the form of new courses and by catering to larger number of people. My congratulations to all the Directors, Academic Coordinators, Editors and Lesson-writers of the Centre who have helped in these endeavors.

Prof. K. Gangadhara Rao
M.Tech., Ph.D.,
Vice-Chancellor I/c
Acharya Nagarjuna University.

M.Sc. MICROBIOLOGY

SEMESTER-II, PAPER-VI

206MB24-IMMUNOLOGY AND AGRICULTURAL MICROBIOLOGY

SYLLABUS

Immunology

1) Demonstration of Heamagglutination Reaction
2) Widal Test
3) VDRL Test
4) Morphology of PMN Cells
5) Immunodiffusion Techniques
    a) Ouchterlony Method
    b) Radial Method
6) Blood Grouping Test

Agricultural Microbiology

1) Isolation of Bacteria from Rhizospheric and Non-Rhizospheric Soils
2) Enumeration of Fungi from Rhizospheric and Non-Rhizospheric Soil
3) Isolation of Microflora from Rhizoplane
4) Isolation of Microflora from Phyllosphere
5) Clearing and Staining Technique for Observation of AM Fungi
6) Isolation of Rhizobium from Root Nodules

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IMMUNOLOGY

EXPERIMENT-1

DEMONSTRATION OF HEAMAGGLUTINATION REACTION

Aim:

To demonstrate the phenomenon of hemagglutination and understand its significance in various biological processes.

Principle:

Hemagglutination is a reaction that involves the clumping together (agglutination) of red blood cells (erythrocytes) due to specific interactions between surface antigens on the erythrocytes and corresponding antibodies or lectins. This reaction is commonly observed in various biological contexts, including blood typing, detection of antibodies or antigens, and viral infections. The interaction between antigens and antibodies or lectins leads to the formation of lattice-like structures, resulting in visible clumping or agglutination of the red blood cells.

Materials Required:

- Red blood cells (erythrocytes) - preferably human or animal origin
- Antisera or lectins specific to the red blood cell antigens (e.g., anti-A, anti-B for ABO blood typing)
- Microscope slides
- Test tubes
- Microscope
- Buffer solution (e.g., phosphate-buffered saline)
- Pipettes
- Stirring rod

Procedure:

1) Prepare a suspension of red blood cells in a buffer solution. The concentration of red blood cells should be standardized and appropriate for the experiment.
2) Divide the red blood cell suspension into several test tubes, each containing the desired volume of the suspension.

3) To each test tube, add different antisera or lectins specific to the antigens present on the red blood cells. For example, add anti-A serum to one test tube and anti-B serum to another for ABO blood typing.
4) Mix the contents of each test tube thoroughly using a stirring rod.
5) Incubate the test tubes at an appropriate temperature for the reaction to occur. The specific temperature and incubation time may vary depending on the antigens and antibodies/lectins used.
6) After the incubation period, observe the contents of each test tube for the presence of agglutination. Agglutination will be visible as clumping or precipitation of red blood cells.
7) Optionally, prepare control tubes with red blood cells and buffer solution only to observe any nonspecific agglutination.
8) If available, examine the agglutination under a microscope to visualize the clumping of red blood cells in more detail.

Observation:

1) Presence or absence of agglutination in each test tube.
2) Degree of agglutination observed (e.g., strong, weak, or no agglutination).
3) Any differences observed between tubes containing different antisera or lectins.
4) Any nonspecific agglutination observed in the control tubes.
5) Microscopic appearance of agglutination, if observed under a microscope.

EXPERIMENT-2

WIDAL TEST

Aim:

To detect antibodies against Salmonella typhi and other serotypes causing typhoid fever.

Principle:

The Widal test is based on the principle of agglutination, where specific antibodies in the patient's serum react with antigens derived from Salmonella typhi and other serotypes. The agglutination reaction is visible as clumping of the bacteria-antibody complexes. The presence of antibodies against specific antigens indicates a recent or past infection with Salmonella typhi.

Materials Required:

- Test tubes
- Buffered saline solution
- Antigen suspensions of Salmonella typhi O and H antigens
- Patient serum samples
- Control sera
- Water bath or incubator
- Centrifuge (optional)
- Disposable pipettes

Procedure:

1) Label test tubes for each antigen and control.
2) Prepare antigen suspensions by adding appropriate volumes of buffered saline to lyophilized antigen vials.
3) Dilute patient serum samples and control sera with buffered saline solution.
4) Add diluted patient serum samples and control sera to labeled test tubes.
5) Add a drop of Salmonella typhi O antigen suspension to each test tube labeled with O antigen, and a drop of H antigen suspension to each test tube labeled with H antigen.
6) Mix the contents of each test tube thoroughly.

7) Incubate the tubes at 37°C for 1 hour.
8) After incubation, observe the tubes for agglutination, indicated by visible clumping.
9) Record the results.

Observation:

1) Presence or absence of agglutination in each test tube.
2) Titer of antibodies, if present (the highest dilution showing agglutination).
3) Compare results with controls to ensure accuracy.

EXPERIMENT-3

VDRL TEST

Aim:

To detect the presence of antibodies against Treponema pallidum, the causative agent of syphilis, in a patient's serum using the Venereal Disease Research Laboratory (VDRL) test.

Principle:

The VDRL test is a non-specific flocculation test used for the detection of reagin, which is a non-treponemal antibody produced in response to syphilis infection. The test involves mixing the patient's serum with a cardiolipin-cholesterol-lecithin antigen in the presence of a complex of antibodies, such as beef cardiolipin antigen and cholesterol. If antibodies to Treponema pallidum are present in the patient's serum, they will react with the antigen to form visible flocculation or clumping.

Materials Required:

- VDRL antigen suspension
- VDRL test cards
- Syphilis control serum
- Patient's serum
- Mixing sticks
- Test tube rack
- VDRL test procedure guidelines
- Pipettes
- Distilled water
- Timer or clock

Procedure:

1) Label the VDRL test cards for each sample to be tested, including controls.
2) Dispense 0.05 ml of VDRL antigen suspension onto each circle on the VDRL test card.

3) Dispense 0.05 ml of patient's serum onto one circle and 0.05 ml of control serum onto another circle.
4) Using separate mixing sticks for each, mix the antigen and serum thoroughly, spreading the mixture evenly over the entire circle.
5) Incubate the cards at room temperature for 8-24 hours.
6) After the appropriate incubation period, examine the cards macroscopically for any visible flocculation or clumping.
7) Record the results as positive, negative, or inconclusive based on the presence or absence of flocculation.
8) Compare the reaction of the patient's serum with the reaction of the control serum. A positive reaction in the control serum is necessary to validate the test.
9) Perform appropriate confirmatory tests for positive results, such as Treponema pallidum particle agglutination (TPPA) or fluorescent treponemal antibody absorption (FTA-ABS) tests.

Observation:

Positive Result: The presence of clumping or flocculation in the patient's serum indicates the presence of antibodies against Treponema pallidum.

Negative Result: Absence of clumping or flocculation suggests the absence of antibodies against Treponema pallidum.

Inconclusive Result: Ambiguous or weak reactions that cannot be clearly interpreted as positive or negative. These may require retesting or further confirmation with other serological tests.

EXPERIMENT-4

MORPHOLOGY OF PMN CELLS

Aim:

To study the morphology of polymorphonuclear (PMN) cells from a blood smear sample.

Principle:

Polymorphonuclear cells, commonly referred to as neutrophils, eosinophils, and basophils, are white blood cells involved in the body's immune response. The study of their morphology can provide valuable insights into various pathological conditions. By examining the shape, size, granularity, and distribution of granules within these cells, one can gain information about the presence of infections, inflammation, or other disorders.

Materials Required:

- Blood smear slide
- Microscope
- Immersion oil (if using an oil immersion lens)
- Wright-Giemsa stain
- Fixative (e.g., methanol)
- Distilled water
- Staining dish
- Staining rack
- Cover slips
- Microscope slides
- Kimwipes or blotting paper
- Timer

Procedure:

1) Place a drop of blood on one end of a clean microscope slide.
2) Using another slide, spread the drop of blood across the slide's surface to create a thin smear.

3) Allow the smear to air dry completely. After drying, stain the blood smear with leishman stain.

4) After one-minute wash the smear with distilled water and allow the slide to dry.

5) Observe the slide under the microscope.

Observation:

Stained slide was observed under the microscope for the identification of PMN cells like basophils and neutrophils.

EXPERIMENT-5

IMMUNODIFFUSION TECHNIQUES

a) OUCHTERLONY METHOD

Aim:

To study the reaction pattern of an antigen with a set of antibodies by ouchterlony double diffusion method.

Principle:

In the test, an antigen solution or a sample extract of interest is placed in wells bore on gel plates while sera or purified antibodies are placed in other remaining wells (Mostly, an antibody well is placed centrally). On incubation, both the antigens in the solution and the antibodies each diffuse out of their respective wells. In case of the antibodies recognizing the antigens, they interact together to form visible immune complexes which precipitate in the gel to give a thin white line (precipitin line) indicating a reaction.

In case multiple wells are filled with different antigen mixtures and antibodies, the precipitate developed between two specific wells indicate the corresponding pair of antigen-antibodies.

Materials Required:

- Glass plate or Petri plate
- Agarose
- Gel borer
- Buffer
- Antiserum
- Antigen solutions

Procedure:

1) Dissolve 100 mg of agarose in 10 ml of the buffer by boiling to completely dissolve the agarose.
2) Cool solution to 55 °C and pour agarose solution to a depth of 1 – 2 mm on a clean glass plate (petri dish or rectangular plate) placed on a horizontal surface.
3) Allow the gel to set for 30 minutes.

4) Wells are punched into the gel using a gel borer corresponding to the marks on the template if used.

5) Fill wells with solutions of antigen and antiserum (of same or different dilutions) until the meniscus just disappears. Antiserum is usually placed in the central well and different antigens are added to the wells surrounding the center well.

6) Incubate the glass plate in a moist chamber overnight at 37 °C.

Results:

The presence of an opaque precipitant line between the antiserum and antigen wells indicates antigen-antibody interaction.

Absence of precipitant line suggests the absence of reaction.

When more than one well is used there are many possible outcomes based on the reactivity of the antigen and antibody selected.

The results may be either of the following:

A full identity (i.e. a continuous line): Line of precipitation at their junction forming an arc represents serologic identity or the presence of a common epitope in antigens.

Non-identity (i.e. the two lines cross completely): A pattern of crossed lines demonstrates two separate reactions and indicates that the compared antigens are unrelated and share no common epitopes.

Partial identity (i.e. a continuous line with a spur at one end): The two antigens share a common epitope, but some antibody molecules are not captured by the antigen and traverse through the initial precipitin line to combine with additional epitopes found in the more complex antigen.

The pattern of the lines that form can determine whether the antigens are the same.

b) RADIAL METHOD

Aim:

To study the radial immunodiffusion technique by single radial immunodiffusion

Principle:

Radial immuno-diffusion is a type of precipitation reaction. It is thus based on the principles of the precipitin curve which states that antigen-antibody interact forming visible cross-linked precipitate when the proper ratio of antigen to antibody is present. In the test, antibody is incorporated into agar and poured into a glass plate to form a uniform layer. Circular wells are cut into the agar and antigen is introduced into the wells. Specific antigens to the impregnated antibodies diffuse through the agar in all directions from the well and react with the antibody present forming visible precipitate or a precipitin ring. Ring shaped bands of precipitates from concentrically around the well indicating reaction. The diameter of the precipitate ring formed, corresponds to the amount of antigen in the solution.

Materials Required:

- Glass plate or Petri plate
- Agarose
- Gel borer
- Buffer
- Antiserum
- Antigen solutions

Procedure:

1) An agar containing an appropriate antiserum (antibody) is poured in plates.
2) Carefully circular wells are cut and removed from the plates.
3) A single or series of standards containing known concentration of antigen are placed in separate wells, while control and “unknown” samples are placed in other remaining wells.
4) As the antigen diffuses radially, a ring of precipitate will form in the area of optimal antigen – antibody concentration.
5) The ring diameters are measured and noted.
6) A standard curve is prepared using the ring diameters of the standards versus their concentrations. This curve is then used to determine the concentration of the control and unknown samples.

Results:

1) The presence of a precipitin ring around the antigen wells indicate specific antigen-antibody interaction.
2) Absence of precipitin ring suggest absence of reaction.
3) The greater the amount of antigen in the well, the farther the ring will form from the well.

EXPERIMENT-6

BLOOD GROUPING TEST

Aim:

The main purpose of conducting this experiment is to understand the basic concept of the ABO blood group system and to know our blood group and type.

Materials Required:

- Toothpicks
- Blood sample
- Alcohol Swabs
- Lancet
- Clean glass slide
- Sterile cotton balls
- Biohazard disposal container
- Monoclonal Antibodies (Anti-A, B, and D)

All this equipment will be readily available in a blood test tool kit.

Procedure:

- Take a clean glass slide and draw three circles on it.
- Unpack the Monoclonal Antibodies (MAB) kit. In the first circle add Anti-A, to the second circle add Anti-B and to the third circle add Anti-D with the help of a dropper.
- Keep the slide aside safely without disturbing.
- Now wipe the ring finger with the alcohol swabs and rub gently near the fingertip, where the blood sample will be collected.
- Prick the ring fingertip with the lancet and wipe off the first drop of the blood.
- As blood starts oozing out, allow it to fall on the three circles of the glass slide by gently pressing the fingertip.
- Apply pressure on the site where it was pricked and to stop blood flow. Use the cotton ball if required.
- Mix the blood sample gently with the help of a toothpick and wait for a minute to observe the result.

Conclusion:

Here is the chart which predicts the different types of blood groups along with its Rh factor.

Blood Type	A	B	O	AB
Rh-positive	A+	B+	O+	AB+
Rh-negative	A-	B-	O-	AB-

Precautions:

Discard the alcohol swabs, lancet, cotton balls and toothpick after their use. Drop all the materials, including the glass slide into the biohazard disposal container after observing the result.

As mentioned above, there are four major blood groups and eight different blood types, collectively called the ABO Blood Group System. The groups are based on the presence or absence of two specific antigens and antibodies-A and B:

1) Group A- Antigen A and Antibody B.
2) Group B- Antigen B and Antibody A.
3) Group AB- Antigen A and B both and no Antibodies
4) Group O- No Antigens and both A and B Antibodies.

Other than this, there is a third kind of antigen called the Rh factor. Based on the presence or absence of this antigen (Rh factor), the four blood groups are classified into eight different blood types:

1) A positive – Presence of Rh+
2) A negative- Presence of Rh-
3) B positive- Presence of Rh+
4) B negative- Presence of Rh-
5) AB positive- Presence of Rh+
6) AB negative- Presence of Rh-
7) O positive- Presence of Rh+
8) O negative- Presence of Rh-

AGRICULTURAL MICROBIOLOGY

EXPERIMENT-1

ISOLATION OF BACTERIA FROM RHIZOSPHERIC AND NON-RHIZOSPHERIC SOILS

Aim:

To isolate and compare bacterial populations present in rhizospheric soil and non-rhizospheric soil by serial dilution and plating techniques.

Principle:

Rhizospheric soil is the soil closely adhering to plant roots and is rich in nutrients released as root exudates, leading to a higher microbial population. **Non-rhizospheric soil** is collected away from plant roots and has comparatively lower microbial density. Bacteria are isolated by **serial dilution** followed by **spread plate or pour plate method** on a suitable growth medium.

Materials Required:

Samples:

- Fresh rhizospheric soil (soil adhering to plant roots)
- Fresh non-rhizospheric soil (soil collected away from roots)

Media and Reagents

- Nutrient Agar (NA) / Tryptic Soy Agar (TSA)
- Sterile distilled water or saline (0.85% NaCl)
- 70% ethanol

Glassware and Equipment

- Sterile test tubes
- Sterile Petri plates
- Micropipettes and sterile tips
- Conical flasks
- Glass spreader (L-rod)
- Bunsen burner / Laminar airflow chamber
- Incubator (28–30 °C)
- Weighing balance

Procedure:

A. Collection of Soil Samples

1. Rhizospheric Soil

- Carefully uproot a healthy plant.
- Shake gently to remove loosely attached soil.
- Collect the soil **closely adhering to the roots** into a sterile container.

2. Non-Rhizospheric Soil

- Collect soil from the same field but **10–15 cm away from plant roots** and from similar depth.
- Transfer to a sterile container.

B. Preparation of Soil Suspension

1) Weigh **1 g of soil** (rhizospheric or non-rhizospheric).
2) Transfer into a test tube containing **9 mL sterile distilled water**.
3) Shake vigorously to obtain a **10^{-1} dilution**.

C. Serial Dilution

1) Transfer **1 mL** from 10^{-1} dilution to another tube containing 9 mL sterile water to obtain **10^{-2}** dilution.
2) Continue serial dilution up to **10^{-5} or 10^{-6}**.
3) Prepare separate dilution series for rhizospheric and non-rhizospheric soils.

D. Plating

Spread Plate Method (preferred)

1. Pour sterile nutrient agar into Petri plates and allow to solidify.
2. Pipette **0.1 mL** from selected dilutions onto agar surface.
3. Spread evenly using sterile L-rod.

(Alternatively, pour plate method may be used with 1 mL inoculum.)

E. Incubation

- Incubate plates at **28–30 °C for 24–48 hours** in an inverted position.

Observation

- Observe plates for **bacterial colony formation**.
- **Note:**
    - Number of colonies
    - Colony morphology (size, shape, color, margin, elevation)
- Rhizospheric soil generally shows **higher colony count** than non-rhizospheric soil.

EXPERIMENT-2

ENUMERATION OF FUNGI FROM RHIZOSPHERIC AND NON-RHIZOSPHERIC SOIL

Aim:

To enumerate and compare the fungal population present in rhizospheric and non-rhizospheric soils using the serial dilution and plate count method.

Principle:

Fungi present in soil can be quantitatively estimated by **serial dilution followed by plating** on a selective medium. **Potato Dextrose Agar (PDA)** or **Rose Bengal Agar (RBA)** is used for fungal growth. **Antibiotics (streptomycin/chloramphenicol)** are added to suppress bacterial growth. Rhizospheric soil generally contains a **higher fungal population** due to nutrients released by plant roots.

Materials Required:

Soil Samples

- Rhizospheric soil
- Non-rhizospheric soil

Media and Reagents

- Potato Dextrose Agar (PDA) / Rose Bengal Agar
- Antibiotic solution (streptomycin or chloramphenicol)
- Sterile distilled water or 0.85% saline
- 70% ethanol

Glassware and Equipment

- Sterile Petri plates
- Test tubes (9 mL capacity)
- Micropipettes and sterile tips
- L-rod / glass spreader
- Weighing balance
- Incubator (25–28 °C)
- Laminar airflow chamber

Procedure:

A. Collection of Soil Samples

1. Rhizospheric Soil

- Uproot a healthy plant carefully.
- Remove loosely attached soil.
- Collect soil **tightly adhering to the roots**.

2. Non-Rhizospheric Soil

- Collect soil **10–15 cm away from plant roots** at the same depth.

B. Preparation of Soil Suspension

1. Weigh **1 g of soil sample**.
2. Transfer into **9 mL sterile distilled water**.
3. Shake well to obtain **10^{-1} dilution**.

C. Serial Dilution

1. Transfer **1 mL** from 10^{-1} dilution to 9 mL sterile water → **10^{-2}**.
2. Continue dilution up to **10^{-4} or 10^{-5}**.
3. Prepare separate dilution series for both soil samples.

D. Plating (Spread Plate Method)

1. Pour sterile PDA/RBA into Petri plates and allow to solidify.
2. Add antibiotic (if not already mixed) to prevent bacterial growth.
3. Pipette **0.1 mL** of appropriate dilution onto the agar surface.
4. Spread evenly using sterile L-rod.

E. Incubation

- Incubate plates at **25–28 °C for 3–5 days** in an upright position.

Observation

- Observe fungal colonies daily.
- Count plates with **30–300 colonies**.
- Note colony characteristics:
    - Color
    - Texture (cottony, velvety, powdery)
    - Growth pattern

Calculation:

CFU/g of soil=Number of colonies×Dilution factor/Volume plated (mL)

Example

- Colonies observed = 45
- Dilution = 10^{-4}
- Volume plated = 0.1 mL

  CFU/g = $45X10^{4}/0.1$

  $=4.5X10^{6}$ CFU/g

EXPERIMENT-3

ISOLATION OF MICROFLORA FROM RHIZOPLANE

Aim:

To isolate microflora (bacteria and fungi) present on the **rhizoplane** (root surface) of plants using washing and plating techniques.

Principle:

The **rhizoplane** is the root surface and the soil particles firmly adhering to it. It harbors a dense and specific microbial population influenced by root exudates. Microorganisms present on the rhizoplane can be isolated by **washing the roots to detach microbes**, followed by **serial dilution and plating** on suitable selective media.

Materials Required:

Plant Material

- Healthy plant with intact roots

Media

- Nutrient Agar (NA) for bacteria
- Potato Dextrose Agar (PDA) for fungi
- Antibiotics (streptomycin/chloramphenicol for PDA)

Reagents

- Sterile distilled water or 0.85% saline
- 70% ethanol

Glassware and Equipment

- Sterile test tubes (9 mL)
- Sterile Petri plates
- Micropipettes and sterile tips
- Laminar airflow chamber
- Shaker or vortex
- Incubator

Procedure:

A. Collection of Root Samples

1) Uproot a healthy plant carefully without damaging roots.
2) Remove loosely adhering soil by gentle shaking.
3) Cut root segments (2–3 cm) using sterile scissors.

B. Preparation of Rhizoplane Microbial Suspension

1) Transfer **1 g of root segments** into a test tube containing **9 mL sterile distilled water**.
2) Shake vigorously or vortex for **5–10 minutes** to dislodge microbes from the root surface.
3) This forms the **10^{-1} dilution**.

C. Serial Dilution

1) Transfer **1 mL** from 10^{-1} dilution into another tube with 9 mL sterile water to get **10^{-2} dilution**.
2) Continue serial dilution up to **10^{-5}**.

D. Plating

For Bacteria (Spread Plate Method)

1) Pour sterile Nutrient Agar into Petri plates and allow to solidify.
2) Pipette **0.1 mL** of selected dilutions onto agar plates.
3) Spread evenly using a sterile L-rod.

For Fungi

1) Use PDA supplemented with antibiotic.
2) Plate **0.1 mL** of dilution as above.

E. Incubation

- **Bacterial plates:** 28–30 °C for 24–48 hours
- **Fungal plates:** 25–28 °C for 3–5 days

Observation

- Observe plates for microbial colonies.
- Record:
    - Number of colonies
    - Colony morphology (shape, size, color, texture)
- Mixed populations of bacteria and fungi are usually observed.

EXPERIMENT-4

ISOLATION OF MICROFLORA FROM PHYLLOSPHERE

Aim:

To isolate and study microflora (bacteria and fungi) present on the **phyllosphere** (surface of leaves) using washing and plating techniques.

Principle:

The **phyllosphere** refers to the aerial parts of plants, especially leaf surfaces, which harbor diverse microorganisms. These microbes can be isolated by **washing leaf surfaces to detach epiphytic microflora**, followed by **serial dilution and plating** on appropriate culture media. Selective media and antibiotics help differentiate bacteria and fungi.

Materials Required:

Plant Material

- Fresh, healthy leaves

Media

- Nutrient Agar (NA) – for bacteria
- Potato Dextrose Agar (PDA) – for fungi
- Antibiotics (streptomycin/chloramphenicol)

Reagents

- Sterile distilled water or 0.85% saline
- 70% ethanol

Glassware and Equipment

- Sterile Petri plates
- Sterile test tubes (9 mL)
- Conical flasks
- Micropipettes and sterile tips
- Laminar airflow chamber
- Shaker / vortex
- Incubator

Procedure:

A. Collection of Leaf Samples

1) Collect fresh, healthy leaves using sterile forceps.
2) Place leaves in sterile polythene bags or containers.
3) Process samples as soon as possible.

B. Preparation of Phyllosphere Microbial Suspension

1) Weigh **1 g of leaf material** or cut into small pieces.
2) Transfer into a conical flask containing **9 mL sterile distilled water**.
3) Shake vigorously for **5–10 minutes** to detach surface microflora.
4) This suspension represents the **10^{-1} dilution**.

C. Serial Dilution

1) Transfer **1 mL** from 10^{-1} dilution to 9 mL sterile water → **10^{-2} dilution**.
2) Continue serial dilution up to **10^{-5}**.

D. Plating

For Bacteria

1) Pour sterile Nutrient Agar into Petri plates and allow to solidify.
2) Pipette **0.1 mL** of appropriate dilution onto the agar surface.
3) Spread evenly using a sterile L-rod.

For Fungi

1) Use PDA supplemented with antibiotics to suppress bacterial growth.
2) Plate **0.1 mL** of dilution as above.

E. Incubation

- **Bacterial plates:** Incubate at **28–30 °C for 24**

  Observation

- Observe plates for bacterial and fungal colonies.
- Record:
  - Number of colonies
  - Colony morphology (shape, size, color, texture)
- Mixed microbial populations are commonly observed.

EXPERIMENT-5

CLEARING AND STAINING TECHNIQUE FOR OBSERVATION OF AM FUNGI

Aim:

To clear and stain plant roots for microscopic observation of **arbuscular mycorrhizal (AM) fungal structures** such as hyphae, vesicles, and arbuscules.

Principle:

Plant roots are opaque due to cellular contents and pigments. **Clearing with potassium hydroxide (KOH)** removes cytoplasmic materials, making roots transparent. **Acidification with HCl** improves dye penetration. **Staining with Trypan blue (or cotton blue)** selectively stains fungal structures, allowing clear visualization under a microscope.

Materials Required:

Plant Material

- Fine feeder roots (fresh or preserved)

Chemicals and Reagents

- Potassium hydroxide (KOH), 10% (w/v)
- Hydrochloric acid (HCl), 1%
- Trypan blue (0.05%) in lactoglycerol
  (or Cotton blue)
- Lactoglycerol (lactic acid : glycerol : water = 1:1:1)

Glassware and Equipment

- Test tubes or glass vials
- Water bath or hot plate
- Forceps
- Microscope slides and coverslips
- Compound microscope

Procedure:

A. Collection and Preparation of Roots

1) Collect fine roots from plants carefully.
2) Wash roots gently under running water to remove soil particles.
3) Cut roots into **1–2 cm segments**.

B. Clearing of Roots

1) Transfer root segments into test tubes containing **10% KOH**.
2) Heat in a water bath at **90 °C for 30–60 minutes**
   (or autoclave at 121 °C for 15 minutes for thick roots).
3) Discard KOH and wash roots thoroughly with distilled water until clear.

C. Acidification

1) Immerse cleared roots in **1% HCl** for **10–15 minutes**.
2) Decant HCl without washing.

D. Staining

1) Add **0.05% Trypan blue in lactoglycerol** to the roots.
2) Heat at **90 °C for 10–15 minutes** or leave overnight at room temperature.

E. Destaining

1) Replace stain with **lactoglycerol**.
2) Allow roots to destain for **12–24 hours** to remove excess stain.

F. Mounting and Observation

1) Place stained root segments on a clean glass slide.
2) Add a drop of lactoglycerol.
3) Cover with coverslip and gently press.
4) Observe under a compound microscope (10× and 40×).

Observation:

- **Fungal structures observed include:**

  Hyphae (thin, thread-like)

  Vesicles (round/oval storage structures)

  Arbuscules (branched tree-like structures)

EXPERIMENT-6

ISOLATION OF RHIZOBIUM FROM ROOT NODULES

Aim:

To isolate *Rhizobium* bacteria from leguminous plant root nodules using surface sterilization and selective culturing techniques.

Principle:

Rhizobium forms a **symbiotic association** with leguminous plants and fixes atmospheric nitrogen inside root nodules. To isolate *Rhizobium*, root nodules are **surface-sterilized** to remove external microorganisms, crushed aseptically, and streaked on **Yeast Extract Mannitol Agar (YEMA)**, which supports selective growth of *Rhizobium*.

Materials Required:

Plant Material

- Healthy legume plants with pink, intact root nodules
  (pea, groundnut, soybean, chickpea, etc.)

Media

- Yeast Extract Mannitol Agar (YEMA)
- YEMA + Congo red (optional for confirmation)

Reagents

- 70% ethanol
- 0.1% mercuric chloride ($HgCl_2$) **or** 2–3% sodium hypochlorite
- Sterile distilled water

Glassware and Equipment

- Sterile Petri plates
- Test tubes
- Forceps and scalpel
- Mortar and pestle / sterile glass rod
- Laminar airflow chamber
- Incubator (28–30 °C)

Procedure:

A. Collection of Root Nodules

1) Carefully uproot healthy legume plants.
2) Wash roots gently under running water to remove soil.
3) Select **young, pink, healthy nodules**.

B. Surface Sterilization of Nodules

1) Transfer nodules into a sterile test tube.
2) Treat with **70% ethanol for 30 seconds**.
3) Immerse in **0.1% $HgCl_2$ for 1–2 minutes**
   (or 2–3% sodium hypochlorite for 3–5 minutes).
4) Wash nodules **5–6 times** with sterile distilled water to remove sterilant.

C. Crushing of Nodules

1) Transfer sterilized nodules onto a sterile glass slide or mortar.
2) Crush gently using sterile glass rod or pestle.
3) Add a drop of sterile distilled water to make a suspension.

D. Inoculation on Medium

1) Streak the nodule suspension onto **YEMA plates** using sterile inoculating loop.
2) Optionally, streak on **YEMA + Congo red** plates for differentiation.

E. Incubation

- Incubate plates at **28–30 °C for 3–5 days** in an inverted position.

Observation:

- **Colonies appear as:**
  - Circular, translucent, gummy, white to cream colored
  - Do **not absorb Congo red** (remain pale)
- Growth is slow compared to many soil bacteria.

Confirmation Tests (Optional)

- **Gram staining:** Gram-negative rods
- **Congo red test:** Poor absorption
- **Acid production on YEMA + bromothymol blue:** Yellow color
- **No growth on glucose peptone agar**