Expt. No. ..... Page No. 1

• Practicle -1 Isolation of nitrogen-fixing Bacteria from soil.

• Objective-
To isolate and study nitrogen-fixing bacteria from soil samples

• Material required:-

1. Soil sample - collected from a garden, agricultural field, or near the roots of leguminious plants.
2. Media for Isolation-
   - Ashby's Mannitol Agar (for free-living nitrogen fixers like (Azotobacteria (
   - Yeast Extract Mannitol Agar (YEMA) with congo Red (for symbiotic nitrogen finers like Rhizobium)
3. Sterile Materials -
   - Test Tubes and sterile saline or distilled water (for preparing soil suspension)
   - Petri dishes.
   - sterile pipettes.
4. Equipment -
   - Incubator (set at 28-30°C)
   - Sterile Inoculating loop or spreader.
5. Personal protective Equipment - gloves, lab coats, goggles.



Azotobacter colonies on jensen medium.

Colony morphology - slims Translucent, Irregular, pale white.

Page No. 2

- Procedure-

1. Soil suspension preparation-
   1. collect approximately 10 g of soil from the root zone of plants.
   2. suspend 1 g of soil in 9 ml of sterile saline or distilled water to prepare at 10^{-1} dilution.
   3. Perform serial dilutions up to 10^{-4} using sterile saline

2. Inoculation on selective media-

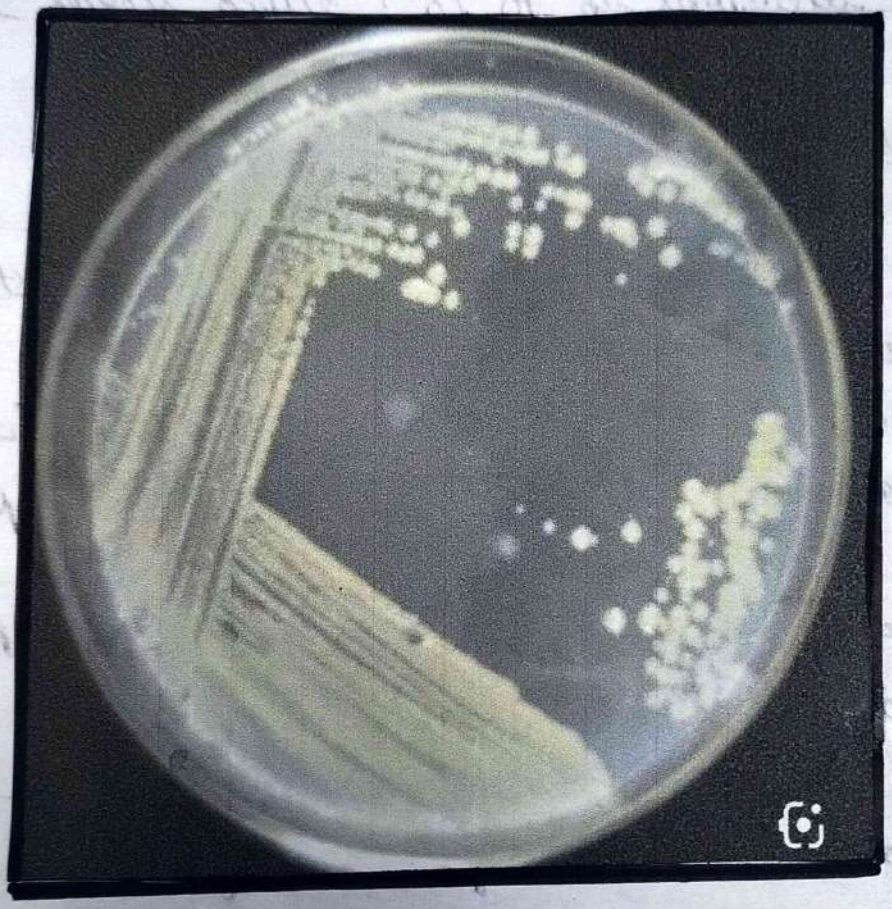
- for Azotobacter- ◦ Pour Ashby's mannitol Agar into sterile Petri dishes and allow it to solidify.
  ◦ pipette 0.1 ml of the 10^{-3} or 10^{-4} dilution onto the agar surface
  ◦ spread the inoculum evenly using a sterile spreader.

- for Rhizobium- ◦ Pour YEMA with congo red into Petri dishes.
  ◦ Repeating the plating process as above.

- Incubates the plates at 28-30°C for 3-5 days.

- objectives-

1. Examine colonies- ◦ on Ashby's Mannitol Agar: Look for large, mucoid, glistening colonies; characteristics of Azotobacter.
   ◦ on YEMA with congo Red: Look for white or pale colonies, as Rhizobium does'nt absorb congo Red.



Rhizobium colonies
on YE MA medium

colony morphology - small, round, milky, concave.

2. Gram staining- ◦ Pick a single colony and prepare a smear.
◦ Perform gram staining to observe cell morphology under a microscope.

3. Confirmatory Tests- ◦ perform biochemical test (e.g motility, catalase) or nitrogenase activity tests (e.g acetylene reduction assay) to confirm nitrogen-fixing ability.

• Expected Result-

1. free- living nitrogen-fixers- ◦ Acetobacter colonies are large, opaque
◦ Microscopic observations reveals gram-negative, rod-shaped bacteria.

2. Symbiotic Nitrogen-fixers- ◦ Rhizobium colonies are white or pale on YEMA with congo red.
◦ Microscopy reveals gram-negative, rod-shaped bacteria.

Expt. No. .....

• **Practical-2** **Isolation of Azotobacter / Azospirillum from Root Nodules.**

• **objectives-**
To isolate and study Acetobacter or Azospirillum from plants roots nodules.

• **Materials required-**

1. **Plant samples-** leguminous plants (e.g. Cgloves, beans, peas) with intact root nodules.
2. **culture media-** ◦ nitrogen-free malate medium (NFMM) for Azospirillum.
   ◦ glucose-pepton-yeast extract Agar (GPY) for Acetobacter.
3. **Sterile material-** ◦ Test Tubes with sterile saline (0.85% Nacle) for washing and dilutions.
   ◦ Petri dishes.
   ◦ scalpel and forceps.
   ◦ sterile pipettes and inoculating loop.
4. **Reagents-** ◦ 70% ethanol (for surface sterilization)
   ◦ sodium hypochlorite solution (1%) for root disinfection.
   ◦ sterile distilled water.
5. **Equipment-** ◦ Incubator (set as 28-30°C)
   ◦ light microscope (for observation of bacterial morphology.)

Teacher's Signature : .....

Expt. No. ..... Page No. 5

6. personal protective equipment- (PPE)- gloves, lab coat, goggles.

• procedure-

1. collection and prepration of Root nodules-
• collect plant samples with root nodules intact.
• detatch root nodules carefully using a sterile scalpel.
• wash the nodules thoroughly with sterile distilled water To remove disinfectants.

2. crushing and dilution-
• Place sterilized nodules in a sterile test Tube containing 1ml of sterile saline.
• crush the nodules gently using a sterile glass rod To release the bacterial contents.
• prepraare serial dilutions (e.g 10^{-1} to 10^{-3}) using sterile saline.

3. Inoculation on selective media-

1. for Azospirillum- • Pour NFMM into sterile petri dishes and allow it to solidify.
• pipette 0.1 ml of the diluted nodule extract onto the NFMM plate.
• spread the inoculum evenly using a sterile spreader.

2. For Acetobacter- ◦ pour GYP agar into petri dishes and allow it to solidify.
◦ Repeat the platting process as above.
3. ◦ Incubate the plates at 28-30°C for 3-5 days.

3. Incubates the plates at 28-30° c for 3-5 days.

4. Observation and Identification -

• Examine colonies- ◦ on NFMM : Azospirillum colonies appear small, circular and slimy.
◦ on GYP : Acetobacter colonies appear round and translucent or pale.

• Microscopic observation - ◦ Pick a single colony and prepare a smear.
◦ stain the smear with gram staining and observe under a microscopic
▪ Azospirillum : Gram-negative, spiral or vibrio-shaped rods.
▪ Acetobacter : Gram-negative, rod-shaped bacteria.

• confirmatory Test - ◦ Test for nitrogenase activity (eg acetylene reduction assay).
◦ For Acetobacter, perform the oxidation of ethanol to acetic acid.

Teacher's Signature : .....

Expt. No. ..... Page No. 7

Expected Result-

Spirillum - Growth on NFMM, spiral or vibrio-shaped Gram-negative rods under the microscope.

A.calcoaceticus - Growth on CAPY, rod-shaped Gram-negative bacteria visible under the microscope.

Expt. No. ..... Page No. 8

• Practical-8 Microbial Analysis of Soil.

• **Objective-**
To perform microbial analysis of soil to determine the total microbial load and identify specific microbial groups.

• **Materials required-**

1. **Soil sample-** collected from an agriculture field, garden or forest.
2. **culture media-** ◦ nutrient agar (NA) for total visible bacteria.
   ◦ Potato dextrose agar (PDA) for fungi.
   ◦ Actinomycete Isolation agar (AIA) for actinomycetes.
   ◦ selective media (e.g Macconkey agar, EMB agar) for specific bacterial groups.
3. **Sterile materials-** ◦ test tube containing sterile saline (0.85% Nacl) for dilutions.
   ◦ Petri dishes.
   ◦ sterile pipettes and inoculating loops.
4. **Equipments-** ◦ Incubator (set at 28-37°C)
   ◦ colony counter
   ◦ pH meter (optional).
   • sterile spreader or glass rod.

Teacher's Signature : .....

Expt. No. ..... Page No. 9

5. Reagents- • Gram staining reagents for bacterial identification
• Lactophenol cotton blue stain for fungal identification

6. personal protective Equipment (PPE)- Gloves, lab coat, goggles.

• Procedure-

1. Soil sample collection and prepration-
• collect soil from a depth of 5-10 cm using a sterile Spatula.
• Air dry the soil sample if it is moist, Then sieve to remove debris.
• weigh 1 gram of soil and suspend it in 9 ml of sterile saline to prepare a 10^{-1} dilution.
• Perform serial dilutions (e.g 10^{-2}, 10^{-3}, up to 10^{-6}) using sterile saline.

2. Plating on Media-
• Bacterial count- ○ pipette 0.1 ml of each dilution (10^{-3} to 10^{-6}) onto nutrient agar plates.
○ spread the incolum evenly using a sterile spreader.
• fungal count- ○ pipette 0.1 ml of each dilution onto potato dextrose agar plates.
○ spread evenly and Incubate at 28°C afer 3-5 days.

3. Actinomycete count- • pipette 0.1 ml of each dilution onto actinomycete Isolation Agar plates.
• spread Evenly and Incubate at 28°C for 5-7 days.

4. selective Isolation- • Use MacConkey or EMB agar to Isolate specific bacterial groups, such as coliforms.

3) Incubation- • Incubate nutrient agar plates at 37°C for 24-28 hours.
• Incubate PDA and AIA plates at 28°C for 5-7 days.

• observation and counting-
• count colonies on plates with 30-300 colonies.
• calculate the calculate colony forming unit. (CFU) per gram of soil using the formula.

CFU/g = number of colonies × dilution factor volume plated (ml) | text = {CFU/g} = \ frac {/ text {number of colonies}} | times \ text { dilution factor}} {\ text {volume plated (ml)}}.

• Identification-
1. Bacteria- • perform gram staining and observe cell morphology under a microscope.
• perform biochemical tests (e.g catalase, oxidase) for further identification.
2. fungi- • use lactophenol cotton blue stain to prepare fungal slides.
• observe fungal morphology (e.g. spores, hyphae) under a microscope.

Teacher's Signature : .....

Expt. No. ..... Page No. 11

3. Actinomycetes- • Identify actinomycetes colonies by their powdery appearance and characteristic earthy odor.

• Expected Result-

• Bacteria- colonies may appear smooth, round and opaque on nutrient agar.

• Fungal- fungal growth on PDA may show fuzzy, pigmented colonies.

• Actinomycetes- colonies on ISP are typically small, powdery and whitish - gray.

Expt. No. ..... Page No. 12

Practical-4 Production of Biogas from cow dung.

Objective-
To produce biogas from cow dung by setting up an anaerobic digestion system and observe gas production.

Materials required-

Organic Substrate- ◦ Fresh cow dung (approximately 1Kg)
◦ water.

Biogas digester setup- ◦ plastic or glass container with an airtight lid (capacity: 5-10 liters)
◦ gas collection system (e.g a gas bag, balloon, or tube connected to a water displacement setup).

Accessories- ◦ stirring rod
◦ funnel
◦ Rubber Tubing (for gas outlet).
◦ clamp to seal Tubing.

pH Indicator strips- for monitoring pH levels.
Thermometer- To monitor the Temperature of the Setup
Personal protective Equipment (PPE)- gloves, lab coat, goggles.

Procedure-

1. **Preparation of the Substrate-** ◦ collect fresh cow dung and mix it with water in a 1:1 ratio to create a slurry.
• stir thoroughly to obtain a homogeneous mixture.

Expt. No. .....

Page No. 13

5. Setup of the Biogas digester -
- Transfer the slurry into the container using a funnel. fill the container upto two-third of its capacity. leaving space for gas accumulation.
- Seal the container with an airtight lid. Ensure that the lid has an outlet for gas to escape.
- connect the outlet to a gas collection system using rubber tubing. Ensure the tubing is sealed properly to avoid gas leakage.

6. Incubation -
- place the setup in a warm location (optimal temperature : 30-40°C) to promote microbial activity.
- Monitor the setup daily for gas production.
- gently agitate the slurry every 2-3 days to ensure even microbial activity.

- Observation and Testing -

1. gas production -
- observe the inflammation of the gas collection system (e.g. a balloon or displacement of water in the gas collection apparatus).

2. Gas Testing -
- Release a small amount of collected gas and ignite it using a lighter or matchstick. A bluish flame indicates the presence of methane.

3. pH monitoring -
- use pH indicator strips to check the pH of the slurry. optimal p.H for biogas production is 6.7 - 7.5

Expt. No. ..... Page No. 14

Expected Result-

- Biogas, primarily composed of methane and carbondioxide will be produced within 7-10 days.
- The gas collection system will inflate, indicating successful gas production.
- The slurry in the container will gradualy stabilize, indicating digestion of organic material.

Expt. No. .....

Practical-5 Ethanol production from Molasses-

- **objective-**
  To produce Ethanol from molasses using yeast fermentation.

- **Materials required-**

1. **Substrate and culture-** ◦ Molasses (50ml)
   ◦ Bakers yeast (saccharomyces cerevisiae) or Industrial-grade yeast.

2. **fermentation Setup-** ◦ conical flask (250ml or 500ml)
   ◦ Airlock or balloon for anaerobic conditions.
   ◦ cotton plug.

3. **Nutrient and Reagents-** ◦ distilled water.
   ◦ Ammonium Sulphate or ammonium phosphate (as a nitrogen source)
   ◦ dilute Sulfuric acid (for p·H adjustment).
   ◦ potassium dichromate and concentrated Sulfuric acid (for Ethanol Test).

4. **Acessories-** ◦ pH meter or pH paper.
   ◦ Measuring cylinder.
   ◦ Stirring rod.
   ◦ water bath or incubator.

5. **personal protective Equipment-** Gloves, lab coat, goggles.

Expt. No. .....

Procedure-

1. preparation of the Molasses Solution -
- dilute molasses with distilled water in ratio of 1:4 (50 ml molasses with 200 ml water).
- Add 1 g of ammonium sulfate or ammonium phosphate as a nitrogen source to promote yeast growth.
- Add the pH to 4.5-5.0 using dilute sulfuric acid.

2. Inoculation with yeast-
- dissolve 2.5 g of yeast in 10 ml of warm distilled water (35-40°C) and left it activate for 10 minutes.
- Add the activated yeast to the molasses solution.

3. fermentation Setup-
- Transfer the inoculated molasses solution into a sterile conical flask.
- Seal the flask with an airlock or a balloon to maintain anaerobic condition. Ensure the airlock / balloon is properly secured.
- place the flask in an incubator or water bath set at 30-35°C.

4. fermentation process- • Allow fermentation to proceed for 48-72 hours.
- observe signs of fermentation. ◦ Effervescence due to CO_2 production.
◦ sweet smell of molasses changing to a slightly alcholic odor.

Teacher's Signature : .....

Expt. No. ..... Page No. 17

5. Extraction and Testing of Ethanol-

1. Distillation- ◦ After fermentation, filter the solution to remove residual yeast and solid impurities.
◦ Transfer the filterate into a distillation apparatus and distill at 78°C to collect ethanol.

2. Ethanol Test- ◦ Add 2 ml of the distilled ethanol to a test tube
◦ Add a few drops of potassium dichromate solution and concentrated sulfuric acid.
◦ observe the color change from orange to green, indicating the presence of ethanol.

• Expected Result-
1. Fermentation produces ethanol and CO_2 observed as bubbling or ballon inflation.
2. Ethanol distillation yields a clear liquid with an alcoholic odor.
3. The potassium dichromate test confirms ethanol presence with a green colour change.

Practical-6 fermentation of cabbage To produce Sauerkraut

objective-

To ferment cabbage, measure changes in p.H and Total acidity and perform Total microbial count during fermentation.

Materials Required-

1. **fermentation Setup-**
   - fresh cabbage. (500g)
   - Non-iodized Salt (3% w/w of cabbage weight)
   - glass jar or fermenter with a tight-fitting Lid.
   - weighing scale.
2. **for pH. Measurement-** pH meter or pH strip.
3. **For Total Acidity Testing-**
   - phenolphtalein indicator
   - 0.1 N Sodium hydroxide (NaOH)
   - pipette and burette.
4. **for Microbial count-**
   - sterile Test Tubes.
   - nutrient agar (for Total bacterial count)
   - potato dextrose agar (PDA) for fungal count.

Fermentation Product	Analysis	0 th day	7 th day	14 th day	28 th day
cabbage	P.H	5.5	6.5	5	7
	Titration	8.26 g/L	3.75 g/L	10.5 g/L	7.125 g/L
	Total bacterial count	1.64×10^{-3} CFU/ml	2.06×10^{-3} CFU/ml	over growth	4.7×10^{-3} CFU/ml

Expt. No.

Page No. 19

Accessories - • sterile water
• Mortar and pestle or blender.
• Sterile spatula and forceps
• Inoculating Loop and spreader.

Personal protective Equipment- (PPE)- Gloves, Lab coat, goggles.

procedure-

Fermentation Setup-

shred fresh cabbage into thin strips.

weigh the shredded cabbage and add 2% non-iodized salt (e.g - 15 g for 500 g cabbage)

Mix the cabbage and salt thoroughly to draw out water allow it to sit for 15-20 minutes.

pack the cabbage tightly into the glass jar, ensuring it is submerged in its own brine.

seal the jar with a lid and store it at room Temperature (20-25°C) for fermentation.

Sampling and Test during fermentation-

Sampling schedule - • Takes samples on days 0 (start) 3, 7 & 14.

pH measurement - • Blend or crush a small portion of fermented cabbage with sterile water.
• Measure the pH using a pH paper or strips.

Teacher's Signature :

Expt. No. ..... Page No. 20

Total acidity measurement-
- Take 10 ml of the brine and add 2 drops of phenolphthalein.
- Titrate with 0.1 N (NaOH) until the solution turns pale pink.
- calculate Total acidity as % lactic acid using the formula:

Total acidity (% lactic acid) = volume of NaOH (ml) x 0.09 / sample volume (ml) \Text{ Total acidity (\% lactic acid)} = \frac{\Text{volume of NaOH (ml)} \Times 0.09}{\Text{sample volume (ml)}}.

Total microbial count-
- prepare serial dilutions of the fermented cabbage extract (10^{-1} to 10^{-5}).
- spread 0.1 ml of each dilution onto nutrient agar, PDA and lactobacilli specific media.
- Incubate plates at 28-27°C for 24-28 hours
- count colonies and calculate CFU/ml using the formula:

CFU/ml = number of colonies x dilution factor / volume plated (ml) \Text{CFU/ml} = \frac{\Text{number of colonies} \Times \Text{dilution factor}}{\Text{volume plated (ml)}}.

Observation & Recording-
- pH - Record the decrease in pH over time as fermentation progre
- Total acidity - Record the increase in Total acidity, which correlates with lactic acid accumulates./ production

Expt. No. ..... Page No. 21

3. Microbial count-
- LAB count increase significantly during the early stages of fermentation.
- Total bacterial and fungal count stablize as LAB dominate.

Expected Results-

1. pH- decreases from neutral (6.5-7.0) to acidic (3.5-4.0) over the fermentation period.
2. Total activity- Increase progressively as lactic acid accumulates.
3. Microbial counts- - LAB count increases significantly during the early stages of fermentation
- Total bacterial and fungal counts stablize as LAB dominate.

Expt. No.

Practical-7 Production of Amylases from Microbes-

Objective-

To produce amylases from microbes and Test their Enzymatic activity on starch.

Materials Required-

1. **Microbial source/growth** - • Bacillus subtillis (bacterial source) or aspergillus niger (fungal source)
   • Sterile inoculating loop.
2. **Culture Media-** • Stock agar media: Nutrient agar Supplemented with 1% Soluble starch.
   • Submerged fermentation media: Starch-based Liquid media (e.g peptone, starch and mineral shorts).
3. **Reagents-** • Iodine solution for starch hydrolysis Test
   • distilled water.
4. **Equipment-** • conical Flask (250 ml)
   • Petri dishes
   • Incubator (set at 30-37°C for bacteria and 25-30°C for fungi.
   • centrifuge (optional for submerged fermentation)

Expt. No. ..... Page No. 23

Accessories- ◦ sterile Test Tubes
◦ pipettes and measuring cylinders.
◦ pH meter of pH paper.
◦ glass spreader or sterile swabs.

Personal protective Equipment (PPE)- Gloves, lab coat, and goggles.

Procedure-

preparation of media-

starch agar media- ◦ prepare nutrient agar and add 1% Soluble Starch.
◦ sterile the media by autoclaving at 121°C for 15 minutes.
◦ pour the media into sterile petri dishes and allow it to solidify.

submerged fermentation media-
◦ prepare a liquid media containing starch (1%), peptone (0.5%) and mineral Salts
◦ sterile the media by autoclaving.

2. Inoculation and Incubation -

• Solid - state fermentation : ◦ sterile bacillus subtillis or aspergillus niger onto starch agar plates.
◦ Incubate the plates at the optimal Temperature (30-37°C), 25-30°C for fungi) for 24-48 hours.

• Submerged fermentation - ◦ Inoculate the liquid medium with the microbial culture.
◦ Incubate the flask at 120 rpm in a shaker incubator for 48-72 hours.

3. Testing for Amylase production -

• Solid - state fermentation - ◦ After inculeation, flood the starch agar plate with idoine solution.
◦ observe the formation of a clear zone around the microbial growth, indicating starch hydrolysis and amylase production.

• Submerged fermention - ◦ centrifuge the culture broth to seprate the supernatant (containing amylase)
• add 2ml of supernatant to 2ml of 1% starch solution and Incubate at 37°C for 30 minutes.
◦ Add Idine solution to Test for residual starch. The disappearance of the black - blue color indicates amylase activity.

Teacher's Signature : .....

Expt. No. ..... Page No. 25

1. Observation-

Solid-state fermentation- • A clear halo around the microbial colonies on the starch agar plate confirms amylase production.

Submerged Fermentation- • The intensity of the blue-black colour decreases with effective starch hydrolysis, indicating enzymatic activity.

2. Expected Result-

1. Microbial strains like Bacillus subtillis or Aspergillus niger produce amylase, as evidence by starch hydrolysis.
2. Submerged fermentation yields an active enzyme extract that hydrolyzes starch in a liquid medium.