THE NEOTIA UNIVERSITY

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ज्ञानम् आत्म प्रदीपाय

Agricultural Microbiology
Practical Manual
Course No, CC-AGL209
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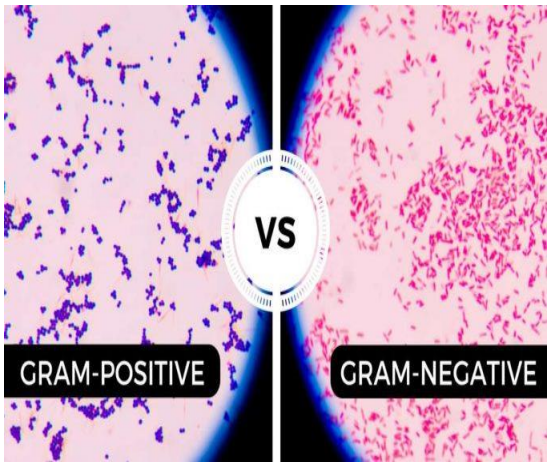

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PRACTICAL 1: INTRODUCTION TO MICROBIOLOGY LABORATORY AND ITS EQUIPMENTS-I

Objectives of the Microbiology Laboratory:

Welcome to Microbiology! In this portion of the course, we will explore the central roles of microorganisms in nature and in our daily lives. In this process of discovery, we will become adept with standard microbiological techniques that will allow us to investigate the structure and physiology of microorganisms. The various sub-disciplines of Microbiology including Soil Microbiology, Bacteriology and Environmental and Applied Microbiology will be introduced.

This laboratory course introduced students to procedures for handling microbes, methods of identification of microorganisms (microscopic and by diagnostic media), preparation of stained slides and wet mounts, aseptic techniques, isolation of a single colony, preparation of a pure culture, inoculation and interpretation of select diagnostic tests.

Facilities Required for Microbiology lab: I. Rooms II. Basic Requirements

1. **Main Laboratory Room**
   - Most of the laboratory activities are performed in the main laboratory. It should have sufficient space inside. A concrete shelf with glazed tile or marble top should project from the wall for keeping the equipments and for performing routine works.
   - Few sun mica-top laboratory tables with shelves, sinks and gas connections should be kept in the lab.

2. **Instrument Room**
   - This room should be kept neat and clean, as most of the sophisticated instruments are kept in it. It should be air-conditioned to make it dust-free and to reduce humidity as well as to avoid high ambient temperatures. Otherwise, accumulation of dust particles on and inside the instruments as well as exposure to high room temperatures decreases the longevity of the instruments.
   - At the same time, high humid conditions lead to the rusting of their metallic components and fungal growth on optical parts, such as microscope lenses and phototubes of spectrophotometers. The instruments to be kept on the floor of the room include fridge, refrigerated centrifuge and ultracentrifuge.
   - Other equipments, such as single-pan precision balance, Quebec colony counter, electronic colony counter, particle counter, electrophoresis apparatus, UV-cum-visible double beam spectrophotometer, computer, gas chromatography (GC), high performance liquid chromatography (HPLC), pH meter, trin-ocular research microscope with photomicrography attachment, projection microscope, fluorescence microscope, dark-field microscope, phase-contrast microscope and PCR thermo cycler should be arranged on a continuous concrete shelf projecting from the wall.

3. **Chemical Storage Room**
   - The store room should be closed from all sides with entrance into the main lab. There should be no window; otherwise the chemicals may get spoiled. There should be a number of concrete shelves for storage of chemicals, reagents, glassware's and other such items. The room should not be opened unless needed. The chemicals should be arranged alphabetically on the shelves for easy location.
   - Principles of storage of chemicals such as Labelling, Compatibility, Maintain good stock, do not store chemicals under sinks, Store large breakable containers, particularly of liquids, below shoulder height.

   - **Storage Facilities:** Shelving, Acid Cabinets, Flammable solvent cabinets, Fridges & Freezers

and Cupboards.
- **Storage of Different Materials:** Acids, Alkalis, Flammable solvents, Chlorinated solvents, Noxious chemicals and Oxidizers.

4. Media Preparation Place
- Media Preparation area should be near to chemical storage room, So that we can access all chemicals very easily.
- Media preparation area should also have distilled water plant, weighing balance, and tap water.

5. Inoculation Room
- This room is meant for inoculation of bacteria i.e. Transfer of bacteria from one container to another. Sometimes, unwanted microbes, usually floating in air on dust particles, may enter into the containers and contaminate the pure stock culture as well as the inoculated ones. To overcome this, the room is kept extremely hygienic. The walls should be plastic-painted and the room should be air-conditioned.
- There should be a laminar flow chamber with gas connection, for inoculation of bacteria. A bottle of disinfectant solution, a sponge pad and a dispose jar should be kept beside the laminar flow chamber as in case of tables in the main lab.

6. Growth Chamber Room
- In routine microbiological analysis, very often it is required to isolate different types of bacteria in samples, to maintain the isolated bacteria as pure cultures and to identify them in subsequent days by performing several tests.
- Store all the cultures in BOD incubator for short time to grow and observe all cultures.

7. Wash Room
- Room with a toilet and a place to wash your hands in it.

Microbiology Lab Practices and Safety Rules:
- Wash your hands with disinfectants when you arrive at the lab and again before you leave.
- Wear laboratory coats in the lab. Students with long hair must put up the hair.
- At the start and end of each laboratory session, students should clean their assigned bench-top area with a disinfectant solution provided. That space should then be kept neat, clean, and uncluttered throughout each laboratory period.
- Eating or drinking in the laboratory is not permitted. No mouth pipetting.
- Label everything clearly. Sterilize equipment and materials.
- Avoid loose fitting items of clothing. Wear appropriate shoes in the laboratory.
- Report any breakage of equipment to the instructor.
- Report any personal accidents such as cuts to the instructor at once.
- Turn off Bunsen burner when not in use.
- Discard all cultures and used glassware into the container labeled CONTAMINATED. (This container will later be sterilized.) Plastic or other disposable items should be discarded separately from glassware in containers to be sterilized.
- Never place contaminated pipettes on the bench top.
- When you flame sterilize with alcohol, be sure that you do not have any papers under you.
- Before beginning your laboratory exercise, wash off the bench top with the disinfectant provided. When exercises are completed, wash off the bench top again. Always wash your hands with soap and water before leaving the laboratory.
- Before leaving the laboratory, see that all the equipments are in the proper location and gas

and water turned off.

- Purchase a fine point, waterproof marker and small roll of masking tape. Use them to clearly label your cultures.
- If you should spill or drop a culture or if any type of accident occurs, call the instructor immediately. Place a paper towel over any spill and pour disinfectant over the towel. Let the disinfectant stand for 15 minutes and then clean the spill with fresh paper towels. Remember to discard the paper towels in the proper receptacle and wash your hands carefully.
- Disinfect work areas before and after use with 70% alcohol or fresh 10% bleach. Laboratory equipment and work surfaces should be decontaminated with an appropriate disinfectant on a routine basis and especially after spills, splashes or other contamination.
- Replace caps on reagents, solution bottles and bacterial cultures. Do not open Petri dishes in the lab unless absolutely necessary.
- Cultures are not to be removed from the laboratory unless the instructor gives permission.
- Always place culture tubes (broth and slants) in the upright position in a rack or basket for incubation or disposal.
- Dispose off all solid waste materials in a biohazard bag and autoclave it before discarding in the regular trash.
- Treat all cultures as potentially pathogenic, *i.e.*, flood areas with disinfectant if cultures are spilled, wash hands after contact and notify your instructor at once.
- Read the instructions carefully before beginning an exercise. Also, make sure you have all the materials needed for the exercise at hand before you commence the experiment. Ask the instructor for clarification of any points about which you are in doubt.
- Flame the inoculating loop or needle immediately before and after use. If viscous material is present on the loop or needle, dry it at the side of the flame before placing it directly in the flame.
- Laboratory note books must be kept up-to-date. Illustrations should be done when requested.
- Make sure you consult the instructor to dispose of the cultures that are not needed any longer. Remove all labels and markings from the tubes before disposing of them; do not discard anything into the sinks.
- Please inform your instructor if you have any medical condition that could potentially affect your safety in the laboratory (eg: diabetes, epilepsy, immune suppression etc.). This information will help the instructor to deal with any emergency that would arise. The information will be treated confidentially and it will not affect their ability to participate in the laboratory activities.
- Be systematic and logical. Keep a faithful record of all the experiments and observations. Update it regularly and submit it for evaluation at the end of each exercise.
- Work either using laminar air flow chamber or light the burner at least five minutes prior to making any inoculations and work near the burner.

Handling Microbial Cultures :

• Before working with media and microbial cultures, wipe your work area with 70% ethanol or an appropriate disinfectant.

• Ensure you are wearing appropriate protective clothing. This will protect you from the culture as well as reduce accidental culture contamination.

• Only use sterile glassware, equipment, media, and reagents. Check media for contamination by observing for turbidity.

• Handle only one microbial culture at a time. The risk of cross contamination or misidentification increases when more than one strain is handled at a time.

• When working with test tube cultures, hold cultures at an angle after you remove the lid to avoid airborne particles from falling into the culture. Sterilize the outside of the culture tube using a Bunsen burner flame.
• When working with plated cultures, hold the Petri dish lid at an angle after you remove the lid to avoid airborne particles falling into the culture dish.
• When handling a microbial culture, work quickly and carefully in an environment that has minimal distractions. Do not leave the lid off your culture for extended periods of time.
• Never take cultures outside of the laboratory.
• Notify the laboratory supervisor immediately of any spills.

It is mandatory to have clear and accurate records of all experiments conducted in the laboratory.

Draw Microbiology Laboratory with all required Rooms?

You tube link: https://youtu.be/X8dhl28JfkI, https://youtu.be/2NlBq9Wa_DM, https://youtu.be/fOFqYm1t2iA

PRACTICAL 2: INTRODUCTION TO MICROBIOLOGY LABORATORY AND ITS EQUIPMENTS-II

Basic requirements of a microbiology laboratory:

A microbiology laboratory requires well-built rooms equipped with Chemicals, glassware, tools and equipments. Some of the most important items of equipment are the following.

I) Common Glassware

The most important glassware used in a microbiological laboratory are test tubes, culture tubes, Petri dishes, Measuring cylinder, pipettes, glass spreader, Flasks, screw-capped glass bottles, haemocytometer etc.

1) Test Tubes, Culture Tubes and Screw-Capped Tubes

2) Petri dish

3) Pipettes and Micropipettes

4) **Glass Spreader**

5) **Haemocytometer**

6) **Conical flasks and measuring cylinder**.

Tools and Equipments in Microbiology Laboratory:

The most common equipment are inoculation needle, inoculation/transfer loop, Bunsen burner, autoclave (or pressure cooker), incubator, hot air oven, refrigerator, centrifuge, spectrophotometer, magnetic stirrer, orbital shaker, hot plate, Distillation water still, UV- lamp, water-bath, carbon dioxide cylinder, single-pan balance with weights (for rough use), chemical balance, pH meter, colony counter, laminar air flow, electrophoretic apparatus, microscopes etc.

1) **Inoculation Needle & Inoculation Loop**

2) **Bunsen Burner**

3) **Water Bath**

4) **Autoclave**

Precautions

5) Laminar Air Flow Chamber

Precautions:

5. **Incubator**

Precautions:

6. **Colony Counter**

7. **Magnetic Stirrer**

8. **Hot Plate**

Conclusion;

You tube link: https://youtu.be/z3ptW4Y-9rk

PRACTICAL 3: MICROSCOPE-PARTS, PRINCIPLES OF MICROSCOPY, RESOLVING POWER AND NUMERICAL APERTURE-I

Introduction

A good microscope is an essential tool for any microbiology laboratory. There are many kinds of microscopes but the type most useful in diagnostic work is the compound microscope.

Principle

- A general biological microscope mainly consists of an objective lens, ocular lens, lens tube, stage, and reflector. An object placed on the stage is magnified through the objective lens. When the target is focused, a magnified image can be observed through the ocular lens. Microscope is designed to emit light onto or through objects and magnify the transmitted or reflected light with the objective and ocular lenses.

- A microscope is an optical instrument used to view small objects by enlarging them with two convex lenses. Optical microscopes, used for research, illuminate samples with visible or ultraviolet light. Depending on its structure, a biological microscope is categorized as an upright or inverted with a magnification ranging from 10x to 1500x.
- Different types of microscopes are used based on the desired level of magnification. Magnifying glasses or loupes are used for quick inspection with a low magnification; binocular microscopes are used to observe from 10x to 50x, and upright/inverted microscopes are used to observe from 50x to 1500x.

Care and Handling of the Microscope

- Always use both hands to carry the microscope, one holding the arm, other under the base.
- Before each use, examine the microscope carefully and report any unusual condition or damage.
- Keep the oculars, objectives, and condenser lens clean. Use dry lens paper only.
- At the end of each laboratory period in which the microscope is used, remove the slide from the stage, wipe away the oil on the oil-immersion objective, and place the low-power

objective in vertical position.

- Replace the dust cover, if available, and return the microscope to its box.

Basic Parts of Microscope

1. **Eye Piece Lens**

2. **Tube**

3. **Arm**

4. **Base**

5. **Coarse focus**

6. **Fine focus**

7. **Illuminator**

8. **Stage**

9. **Object lens**

10. **Rack Stop**

11. **Condenser lens**

12. **Diaphragm or Irish**

You tube link: https://youtu.be/rq1a7Pt8DCw

PRACTICAL 4: MICROSCOPE-PARTS, PRINCIPLES OF MICROSCOPY, RESOLVING POWER AND NUMERICAL APERTURE

PART II: USE

Using of Microscope

1. To carry the microscope grasp the microscopes arm with one hand. Place your other hand under the base.
2. Place the microscope on a table with the arm toward you.
3. Turn the coarse adjustment knob to raise the body tube.
4. Revolve the nosepiece until the low-power objective lens clicks into place.
5. Adjust the diaphragm. While looking through the eyepiece, also adjust the mirror until you see a bright white circle of light.
6. Place a slide on the stage. Center the specimen over the opening on the stage. Use the stage clips to hold the slide in place.
7. Look at the stage from the side. Carefully turn the coarse adjustment knob to lower the body tube until the low power objective almost touches the slide.
8. Looking through the eyepiece, VERY SLOWLY the coarse adjustment knob until the specimen comes into focus.
9. To switch to the high power objective lens, look at the microscope from the side. CAREFULLY revolve the nosepiece until the high-power objective lens clicks into place. Make sure the lens does not hit the slide.
10. Looking through the eyepiece, turn the fine adjustment knob until the specimen comes into focus.

Draw Microscope with Specimen;

You tube link: https://youtu.be/ckBMlVoAYeY, https://youtu.be/6jkK5fhZesA

PRACTICAL 5: METHODS OF STERILIZATION

Objectives

Sterilization is the process of destroying or physically removing all forms of microbial life including vegetative cells, spores and viruses from a surface, a medium or an article. The principal reasons for controlling microorganisms are:

1. To prevent transmission of disease and infection
2. To prevent contamination by undesirable microorganisms
3. To prevent deterioration and spoilage of materials by microorganisms

The methods of sterilization employed depend on the purpose for which sterilization is carried out, the material which has to be sterilized and the nature of the microorganisms that are to be removed or destroyed. The various agents used in sterilization can be grouped into physical and chemical agents

Procedure

I. <u>Physical Agents (Physical Methods)</u>

A. Dry Heat

Flaming

Hot Air Oven

B. Moist heat

Sterilization above 100°C (steam under pressure) - Autoclave

II. Chemical Methods

Surface Sterilization

- **Disinfectant**
- **Germicide**.
- **Bactericides**
- **Fungicides**
- **Algaecides**.

Conclusion;

You tube link: https://youtu.be/L5_6kAQBN5E

PRACTICAL 6: NUTRITIONAL MEDIA AND THEIR PREPARATIONS-I

Objectives

The food materials on which the organism is grown is known as culture medium and the growth of organism is known as culture. The culture is an *in vitro* technique of growing or cultivating microorganisms or only other cells in a suitable nutrients medium called culture medium.

Materials Required; Conical flask (250ml, Non absorbent cotton, Glass rods, weighing balance, Water, Peptone, Meat Extract, Yeast Extract, Electrolyte, Agar, Fermentable Compounds, Buffers.

Equipments: Autoclave, Mechanical Stirrer, Laminar air flow chamber, Hot air oven.

Procedure

There are different types of media which are Liquid Media, Semi Solid Media and Solid Media.

a) Liquid Media or Broth

- First weigh required amount of composition of nutrients by using weighing balance.
- Take 250 ml conical flask and add all those weighed nutrients in it.
- Add small amount of water into conical flask and mix it thoroughly until all nutrients are dissolved.
- Make up volume upto 250 ml by using measuring cylinder with distilled water.
- No solidifying agents (eg: agar) is added while preparing the medium (This one done for preparing to make liquid medium).
- Wrap the mouth of conical flask with newspaper after cotton plug is done.
- Now keep that conical flask in autoclave for sterilization at 121 degree C 15 psi for 15 to 20 minutes.
- Once autoclave is done. Take conical flask out from autoclave to cool it down with running tap water.
- Finally pour it in Petri dishes for further uses like for growing bacteria etc...

You tube link: https://youtu.be/ApLFFiCKBmo

PRACTICAL 7: NUTRITIONAL MEDIA AND THEIR PREPARATIONS -II

b) Semi-Solid Media

- The semi-solid medium remains in the semi-solid condition.
- Procedure is as same as Liquid medium preparation but here it is prepared by adding small amount of agar (0.5%).
- The semi-solid medium may be selective which promotes the growth of one organism and retards the growth of another organism. This type of medium can be used to study bacterial motility (semisolid media are useful for cultivation of microaerophilic bacteria).

c) Solid Media

- The solid medium is solid in consistency.
- Procedure is as same as Liquid medium preparation but here It is prepared by adding 2% or 1% agar is sometimes an inorganic solidifying agent for autotrophic bacteria.
- It's used for colony characterization, colony identification, etc.

Media compositions

1. Nutrient agar (Bacteria)	**g/l**
Beef extract	3.00
Peptone	5.00
Sodium chloride (NaCl)	5.00
Agar	20.00
Distilled water	1000 ml
pH	7.0 ± 0.2

2. Glucose Yeast Extract Agar Medium (Fungi)	**g/l**
Glucose	10.00
Peptone	5.00
Yeast extract	5.00
Agar	20.00
Distilled water	1000 ml
pH	75.5-6.5

3. Kenknight's Medium (Actinomycetes)	**g/l**
Dextrose	1.00
Dihydrogen Potassium Phosphate (KH2PO4)	0.10
Sodium Nitrate	0.10
Potassium Chloride (KCl)	0.10
Magnesium Sulphate (MgSO4)	0.10
Agar	20.00
Distilled water	1000 ml

Observations;

Conclusion;

You tube link: https://youtu.be/h_DCpIRTCrc

PRACTICAL 8: ENUMERATION OF MICROBIAL POPULATION IN SOIL-BACTERIA, FUNGI AND ACTINOMYCETES-I

Objectives: Soil has great biological activity because of its high organic matter content in the form of decomposing biota. Micro-organisms can be isolated and enumerated from soil by serial dilution agar plate method, direct microscope examination and the enrichment culture technique. The number of cells/ml of suspension or per gram of soil is cultured as;

Number of cells/ml = Number of colonies X Dilution Factor

Materials Requirements:

Soil Sample, 10ml pipettes, Conical flasks, Non absorbent Cotton, Sterile water blanks (90 ml and 9ml), Sterile Petri plates, Measuring cylinder, Growth media (Nutrient Agar, Glucose Yeast Extract Agar and Kenknight's Agar)…

Equipments Requirements: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Precautions:

1. Isolation of microorganisms should be done from a composite sample collected randomly from a field.
2. Soil should be in a powdered form.
3. Each dilution must be thoroughly shaken before removing an aliquot for subsequent dilution.
4. Use separate sterile pipettes for each dilution
5. After pouring of medium, plates should be swirled gently so that inoculum gets mixed in the medium.

Procedure;

1. Collect soil samples at random (minimum five places), from a field, mix thoroughly to make a composite sample for microbiological analysis.
2. Airs dry the soil sample and pack it tightly, keeps it in dark place.
3. Prepare different media by already given composition rates in different conical flasks (250 ml) (Take conical flask add required amount of composition of nutrients in it and add 1 lit of water, mix it thoroughly. After this use cotton plug the head of flask and cover it with paper for sterilization)
4. Sterilize the all the required glasswares and chemicals such as 100 ml conical flask (add 90 ml water before sterilization), 10 ml pipettes, 10 No's test tubes. After sterilization keep all the sterilized things inside laminar air flow chamber to prevent things from contamination.

Media compositions

4. Nutrient agar (Bacteria)	**g/l**
Beef extract	3.00
Peptone	5.00
Sodium chloride (NaCl)	5.00
Agar	20.00
Distilled water	1000 ml

Ph	8.0 ± 0.2

5. Glucose Yeast Extract Agar Medium (Fungi)	**g/l**
Glucose	10.00
Peptone	5.00
Yeast extract	5.00
Agar	20.00
Distilled water	1000 ml
pH	75.5-6.5

6. Kenknight's Medium (Actinomycetes)	**g/l**
Dextrose	1.00
Dihydrogen Potassium Phosphate (KH2PO4)	0.10
Sodium Nitrate	0.10
Potassium Chloride (KCl)	0.10
Magnesium Sulphate (MgSO4)	0.10
Agar	20.00
Distilled water	1000 ml

You tube link: https://youtu.be/gQQXzRv3RUA

PRACTICAL 9: ENUMERATION OF MICROBIAL POPULATION IN SOIL- BACTERIA, FUNGI AND ACTINOMYCETES -II

Methodology:

1. Add 10g of it into 90 ml sterile water and shake it. This is 10^{-1} dilution.
2. Serially dilute the 10^{-1} dilution by successive transfer of dilution into 9ml water blanks. In this way, prepare 10^{-2} to 10^{-3} dilutions. Out of these, use 10^{-4} to 10^{-7}, 10^{-2} to 10^{-4} and 10^{-2} to 10^{-4} dilutions for isolating bacteria, actinomycetes and fungi, respectively.
3. Transfer 1 ml aliquots from 10^{-2} dilution blank into 3 Petri plates, from 10^{-3} to 6 sterile Petri plates, 10^{-4} to 9 Petri plates, 10^{-5} to 6 Petri plates and 10^{-6} Petri plates.
4. Add about 20 ml of cooled specific media to these petriplates and mix the inoculum by gentle rotation of Petri plates, such that 10-4 to 10-7, 10-2 to 10-5 and 10-3 to 10-6 dilutions are used for Nutrient agar, Glucose yeast extract and Ken knight agar respectively.
5. Upon solidification of media, incubate plates in an inverted position at 28 degree C for 2—7 days.
6.

Observations:

Observe the plates for umber of colonies of bacteria, fungi and actinomycetes from each dilution and select the plates for enumeration which contain colonies in the range of 30-300.

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		
Fungi	10^{-3}		
	10^{-4}		
	10^{-5}		
	10^{-6}		
Actinomycetes	10^{-3}		
	10^{-4}		
	10^{-5}		
	10^{-6}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil (dry wt.)}}$$

Conclusion;

You tube Link: https://youtu.be/smqTEP7u-9A

PRACTCIAL 10: METHODS OF ISOLATION AND PURIFICATION OF MICROBIAL CULTURES-I

Objective:

A culture that contains only one kind of microorganisms is called a pure culture. A culture which contains more than one kind of microorganisms is called mixed culture. Most of the cultures obtained in nature are mixed cultures. Pure cultures are essential to study the cultural, morphological and physiological characters of an individual species.

Materials Required:

Mother Culture, Conical flasks, Non absorbent Cotton, Sterile test tubes with 10 ml water blank, Sterile Petri plates, Measuring cylinder, Growth media (Nutrient Agar, Glucose Yeast Extract Agar and Kenknight's Agar).

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure:

- First choose the mother culture for isolation of pure colonies from it.
- Prepare the 250 ml of different media (Nutrient Agar, Glucose yeast extract and Ken knight medium) in 250 ml conical flask for isolation of pure colonies from mother culture.
- Once all medium are prepared in conical flask sterilize it by using autoclave at 121 degree C in 15 psi for 20 minutes. After autoclaving cool the conical flasks under tap water.
- After few minutes pour the required amount of medium on sterilized petriplates for streak plate and spread plate method.
- But for pour plate method, first put a drop of isolated microbial culture on petriplates then pour medium on it and rotate those petriplates properly for mixing of culture with medium. After some time keep those petriplates in BOD incubator in inverted position at 36 degree C for 2 to 3 days for Bacteria, 3 to 5 days for Fungi and 5 to 7 days for Actinomycetes.
- Finally observe the colonies after these days.

Methodology:

Methods of Isolation of Pure Culture

The common plating techniques employed in microbiology are Streak Plate Method, Spread Plate Method and Pour Plate Method.

1) Streak Plate Method

This method was developed by two bacteriologists, Leoffler and Gaffkey in the laboratory of Robert Koch. This method is routinely employed for the isolation of bacteria in pure culture. In this method a sterilized inoculating loop or transfer needle is dipped into a suitable diluted suspension of microorganisms which is then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks. The process is known as streaking and the plate so prepared is called a streak plate. The main objective of the streak plate method is to produce well

separated colonies of bacteria from concentrated suspensions of cells.

PRACTCIAL 11: METHODS OF ISOLATION AND PURIFICATION OF MICROBIAL CULTURES -II

2) Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position. The dispersed cells will develop into isolated colonies. Because the number of colonies will be equal to the number of viable organisms in the sample spread plates can be used to count the microbial population.

3) Pour Plate Method

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplates to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify. After incubation, the plates are examined for the presence of individual colonies. The pure colonies may be isolated and transferred into test tube culture media for making pure cultures. This technique is employed to estimate the viable bacterial count in a suspension.

Observations:

Conclusions

You tube link: https://youtu.be/ZZdIXCZtdZQ

PRACTICAL 12: ISOLATION OF *RHIZOBIUM* FROM LEGUME ROOTS NODULE:

Introduction

The Most important system by which molecular nitrogen is fixed biologically results from a symbiotic association between a soil bacterium and a legume plant. Rhizobium, infect legume seedlings through root hairs and stimulate the formation of tumor like nodules on the roots. The nodules comprised of both plant and microbial tissue, are capable of using atmospheric nitrogen.

Root Nodules

Objective

The Objective of this method is to provide a procedure for the isolation of Rhizobium species from nodules found on the roots of legume plants and study about their morphology.

Materials Required:

Root systems of nodule-bearing legume plants freshly collected ones, Sterilized water, Sterilized Petri dishes, Ethanol (70%), Sterilized forceps, Scalpel or Razor blade, Inoculating loop, YEMA medium

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. Wash the soil from the roots and nodules of legume plant chosen for study. Note the appearance, distributions and abundance of nodules.
2. Carefully out, do not tear a nodule from the roots so that small portions of the root remains attached. If possible, select a plum firm nodule preferably pinkish in colour. Wash under running water to insure the removal of all soil particles.
3. Put the nodule into a Petri dish containing 0.1 % $HgCl_2$ and leave it immersed for 5 minutes.
4. Transfer the nodule to a sterile Petri dish containing sterile water. Use sterile forceps to make the transfer. Wash the nodule in the sterile water.
5. Transfer the nodule to another Petri dish containing 70% ethanol for 3 minutes. Remove to another Petri dish of sterile water and rinse thoroughly, and then transfer to a second plate of sterile water for final rinsing.
6. Add 0.1 ml of sterile water to each of six sterile Petri dishes. Remove the nodule from rinse water to Petri dish No.1 and Crush it with flamed forceps. Mix the nodule tissue with the water.
7. Transfer two loops full of the suspension in the No.1 Petri dish to Petri dish No.2 and mix it with the sterile water previously added. Repeat this loop dilution progressively for plate nos.3, 4, 5 and 6.
8. Add 15 ml of YEMA medium (melted in a boiling water bath and held at 42 degree C for pouring) to plates Nos. 2 to 6 inclusive. Mix the agar and dilutions thoroughly by swirling, and incubate at 28 degree C for 7 days.

9. At the end of one week representative colonies of Rhizobium should have developed and isolation can be made for further use or storage. Select the mucoid, or flat, watery colonies that are most abundant in the loop dilution plates. Choose an isolated colony among the domain, Rhizobium-like colonies derived from a single nodule, and transfer aseptically to several slants of the same medium. Label, incubate, and store for further testing.

YEMA medium Composition; (Yeast Extract Mannitol Agar)

Mannitol	10 g/l
K2HPO4	0.5 g/l
MgSo4.7H2O	0.2 g/l
NaCl	0.1g/l
Yeast Extract	0.5 g//l
Agar	20 g/l
Dis.Water.	1000ml

Methodology:

Pour Plate Method

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplates to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify.

Observations:

Observe the plates for umber of colonies of ***Rhizobium*** bacteria from each dilution and select the plates for enumeration which contain colonies in the range of 30-300.

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Rhizobium Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Root}}$$

Conclusion:

You tube link: https://youtu.be/7cCqriEFsmQ

PRACTCIAL 13: ISOLATION OF *AZOTOBACTER* FROM SOIL:

Objective

- The most widely studied of the free living, or non-symbiotic, biological agents of bacteria fixation is undoubtedly the gram negative soil bacterium (*Azotobacter*).
- The objective is isolation of *Azotobacter* from soil and study about its morphology.

Materials Required:

Soil Samples, recently collected and not air dried one, Autoclave, Laminar Air flow chamber, BOD Incubator, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare Jensen medium as per requirements.
2. Pour Jensen medium into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 150 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
5. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
6. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.
7. Azotobacter colonies appear as flat, soft, mucoid and milky colonies.

Jensen's nitrogen free medium (*Azotobacter*)	**g/l**
Sucrose	20.00
Dipotassium hydrogen ortho-phosphate (K_2HPO_4)	1.00
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.10
Sodium chloride (NaCl)	0.50
Sodium molybdate di hydrate	0.001
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.01
Calcium carbonate ($CaCO_3$)	2.00
Agar	20.00
Distilled water	1000 ml
pH	7.0 ± 0.2

Methodology

Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position

Observations:

Observe the plates for umber of colonies of ***Azotobacter*** bacteria from each dilution and select the plates for enumeration which contain colonies in the range of 30-300.

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Azotobacter Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil}}$$

Conclusion;

You tube link: https://youtu.be/FCIE1masG5A

PRACTCIAL 14: ISOLATION OF *AZOSPIRILLUM* FROM ROOTS:

Objective

- *Azospirillum* is an aerobic or microaerophilic, motile, helical, or fibroid, gram negative bacterium. Being an associative symbiotic, this bacterium brings many benefits to many non leguminous crop like cereals, millets, forage crops, and vegetable crops.
- Objective of this experiment is isolation of *Azospirillum* from root surface of crops and study about its morphology.
-

Materials Required:

1. Rhizospheric Soil Samples Recently collected and not air dried one.
2. Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.
3.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare **Dobereiner's Medium** as per requirements.
2. Pour **Dobereiner's Medium** into sterile Petri plates and allow them to solidify.
3. Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
4. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
5. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

Dobereiner's medium (*Azospirillum*)	**g/l**
Malic acid	5.00
KOH	4.00
Yeast Extract	5.00
Mn SO_4H_2O (1%)	1.00
$MgSO_47H_2O$ (10%)	1.00
NaCl (10%)	2.00
K_2HPO_4 (10%)	4.00
$NaMoO_4$ (0.1%)	0.20
$CaCl_2$ (10%)	1.00
$FeSO_4.7H_2O$ (5%)	1.00
1 m NH_4Cl	5.00
Bromthymol Blue	3.00 ml

Methodology

Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a

small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

Observations:

Observe the plates for umber of colonies of ***Azotobacter*** bacteria from each dilution and select the plates for enumeration which contain colonies in the range of 30-300.

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Azospirillum **Bacteria**	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil (g)}}$$

Conclusion;

You tube link: https://youtu.be/Ho2LeKrAwJs

PRACTICAL 15: STAINING AND MICROSCOPIC EXAMINATION OF MICROBES:

I) SIMPLE STAINING

Aim

To compare the morphological shapes and arrangements of bacterial cells

Principle

Bacterial smear is stained with a single reagent, which produces a distinctive contrast between the organism and its background. The purpose of simple staining technique is to determine cell shape, size arrangement of bacterial cells. Simple staining is performed by using basic stains which have different exposure time (Crystal Violet 20-60 s, Carbol fuschin 15-30 s and Methylene blue 1-2 minutes).

Procedure

- Clean glass slide was taken and was washed and dried.
- Bacterial smears were prepared from the bacterial cultures.
- The slide was kept on the staining tray and 5 drops of stain was added for a designed period.
- The extra stain was poured off and the smear was washed gently under slow running tap water.
- The slide was then blot dried using blotting paper.
- The slide was then examined under 10X, 45X and oil immersions objects respectively.

Observation

On the basis of microscopic observation, bacteria appeared blue, violet and red respectively depending on the stain taken.

II) DIFFERENTIAL STAINING

Differential staining requires the use of at least 3 chemical reagents that are applied sequentially to a heat fixed smear. Its function is to impart its colour to all cells

GRAM STAINING

Aim

To differentiate two principal groups of bacteria

Principle

Gram staining classifies bacteria into 2 major groups, Gram positive and Gram negative bacteria. The Gram stain reaction is based on the difference in the chemical and physical composition of bacterial cell wall. The bacteria which retain the primary stain appear dark blue or violet and not decolorized when stained with Gram's method are called Gram positive, where as those that lose the crystal violet used counter stain, Saffranin appear red are called as Gram negative.

The Gram stain uses different reagents in the order, **crystal violet, iodine solution, alcohol** and **Saffranin.**

Procedure

- Thin smear was prepared of the given bacterial species on a clean glass slide.
- Let the smear dry.
- Heat fixed smear.
- Hold the smear using the slide rack.
- Covered each smear with crystal violet for 1 minute.
- Washed each slide with distilled water for few seconds using wash bottles.
- Covered each smear with Gram's iodine solution for 1 minute.
- Gently washed with distilled water.
- Decolorized with 95%.
- Washed the slide with distilled water and drained.
- Counter stain was applied Saffranin for 30 seconds.
- Washed with distilled water and blot dried with absorbent paper.
- The stained slides were air dried and observed under the microscope.

Observation

- Examined the slides microscopically using oil immersion objective.
- Identified the Gram reaction of the given cultures and classified it and described the morphology and arrangement of cells.

Conclusion;

You tube link: https://youtu.be/sxa46xKfIOY

PRACTICAL 16: ISOLATION OF *CLOSTRIDIUM* FROM SOIL

Objective

- The most widely studied of the free living, or non-symbiotic, biological agents of bacteria fixation is undoubtedly the gram positive soil bacterium (*Clostridium*), Rod shaped, Anaerobic, produce endospores to survive extreme conditions.
- Found in soil, mud, plants, animal faeces and soured milk.
- The objective is isolation of *Clostridium (C. botulinum, C. difficile, C. tetanus)* from soil and study about its morphology.

Materials Required:

Soil Samples, recently collected and not air dried one, Autoclave, Laminar Air flow chamber, BOD Incubator, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare Yeast Extract in Minimal Salts (YEMS) medium as per requirements.
2. Pour Yeast Extract in Minimal Salts (YEMS) medium into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 45 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
5. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
6. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.
7. Clostridium colonies appear as flat, soft, mucoid and milky colonies.

Yeast Extract in Minimal Salts (YEMS) medium	**g/l**
Mannitol	10
K2HPO4	0.5
MgSO4.7H2O	0.2
NaCl	0.1
Yeast Extract	0.5
Agar	20.00
Distilled water	1000 ml
pH	7.0 ± 0.1

Methodology

Pour Plate Method

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplates to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify.

Observations:

Observe the plates for umber of colonies of ***Clostridium*** bacteria from each dilution and select the plates for enumeration which contain colonies in the range of 30-300.

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Clostridium Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil}}$$

Conclusion;

You tube link: https://youtu.be/alTgt0h5jOE