PRACTICAL MANUAL

on

INTRODUCTORY MICROBIOLOGY

Course No. ABB-161 Credit Hours 2(1+1)

For

B.Sc. (Horticulture) Year I Semester I

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Syllabus:

Examination of natural infusion and living bacteria; examination of stained cells by simple staining and Gram staining. Methods for sterilization and nutrient agar preparation. Broth culture, agar slopes, streak plates and pour plates, turbid metric estimation of microbial growth, mushroom culture- Spawn production, Culture and production techniques, harvesting, packing and storage.

Name of Students .....

Roll No. .....

Batch .....

Session .....

Semester .....

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Date: Course Teacher

CONTENTS

PRACTICAL NO. 1

OBJECTIVE: To know about different laboratory equipment/tools

ACTIVITY: List twenty important laboratory equipment and write their uses

S. No.	Name of Equipment/ tools	Uses
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PRACTICAL NO. 2

OBJECTIVE: To know the Compound Light Microscope

Activity: Draw well labeled diagram of compound light microscope and mention different parts and their use.

PRACTICAL NO. 3

OBJECTIVES: Acquaintance with different laboratory chemicals.

ACTIVITY: Describe mode of action and nature of the given chemicals

a) Formaldehyde: ……

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b) Hydrochloric Acid: ……

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c) Alcohol (70%): ……

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d) Iodine: ……..

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e) Phenol: ……..

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f) Sodium Hypochlorite: ……

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g) Acetone: ……

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a) Cotton Blue (Methyl Blue): ……..

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b) Crystal Violet (Gentian Violet): ……..

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c) Safranin: ……..

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d) Phenol red/Congo red: ……

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PRACTICAL NO. 4

OBJECTIVE: To know different methods of sterilization.

ACTIVITY: 1. Which is most suited method for sterilization of glassware?

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2. Mention the method employed for media, oil, wax, antibiotics, and plastic ware sterilization separately and why?

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3. How sterilization is differed from sanitization, disinfection and pasteurization?

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PRACTICAL NO. 5

OBJECTIVE: To prepare Potato dextrose agar medium for isolation of fungi.

ACTIVITY: Prepare 2 litres of Potato dextrose agar medium. Write the materials required and quantity of the components.

MATERIALS REQUIRED: ……

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Composition: ……

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Use: ……..

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Procedure: ……

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Precautionary measures: ……..

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PRACTICAL NO. 6

OBJECTIVE: To prepare Nutrient agar medium for isolation of bacteria.

ACTIVITY: Prepare 2 litres of Nutrient agar medium. Write the materials required and quantity of the components.

MATERIALS REQUIRED: ……

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Composition: ……

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Use: ……..

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Procedure: ……

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Precautionary measures: ……..

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PRACTICAL NO. 7.1

OBJECTIVE: Preparation of Basic liquid medium or broth for cultivation of fungi.

ACTIVITY: Prepare 2 liters of Potato dextrose broth. Write down the materials required and quantity of the components.

MATERIALS REQUIRED: ……

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Composition: ……

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Use: ……..

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Procedure: ……..

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Precautionary measures: ……..

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PRACTICAL NO. 7.2

OBJECTIVE: Preparation of Basic liquid medium or broth for cultivation of bacteria.

ACTIVITY: Prepare 2 liters of Nutrient broth. Write down the materials required and quantity of the components.

MATERIALS REQUIRED: ……

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Composition: ……

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Use: ……..

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Procedure: ……

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Precautionary measures: ……..

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Inoculate bacterial culture in nutrient broth medium and observe for growth.

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How rotation speed of incubator shaker influence the growth of culture?

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PRACTICAL NO. 8

OBJECTIVE: To prepare Agar slants.

ACTIVITY: Prepare PDA/Nutrient agar slants. Inoculate the given fungal/bacterial culture in agar slant.

MATERIALS REQUIRED: ……

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Composition: ……

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Use: ……..

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Procedure: ……

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Precautionary measures: ……..

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PRACTICAL NO. 9

Objective: To prepare PDA/ Nutrient agar plates.

ACTIVITY: Prepare PDA/Nutrient agar media for pouring. Inoculate the given fungal/ bacterial culture in agar plates.

MATERIALS REQUIRED: ……

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Composition: ……

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Use: ……..

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Procedure: ……

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Precautionary measures: ……..

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PRACTICAL NO. 10

OBJECTIVE: Isolation of Bacteria from diseased plant sample.

ACTIVITY: 1. Write down the steps involved in isolation. Identify and describe the colony growth based on the observations.

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Precautionary measures: ……

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Write the difference between Pour plate and Spread plate technique.

Pour Plate Technique	Spread Plate Technique
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PRACTICAL NO. 11

OBJECTIVE: Isolation of microorganism (Bacteria/ Fungi) form given soil sample by serial dilution technique and count the colony forming unit (cfu).

ACTIVITY: 1. Write the necessary materials including different types of media used for bacterial and fungal isolation.

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2. Important steps involved in isolation of fungi/bacteria:

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3. Calculate the colony forming units (cfu) separately for bacterial and fungal population:

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4. Describe different streaking techniques:

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5. Write the precautions taken during isolation of microorganisms: ……..

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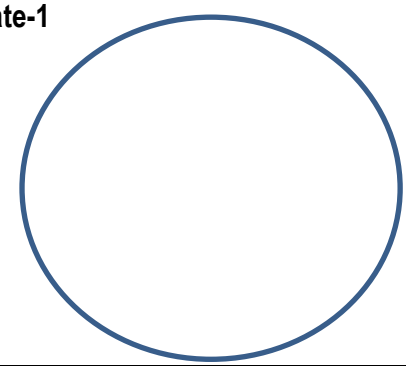
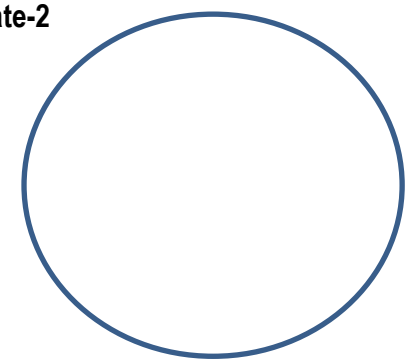
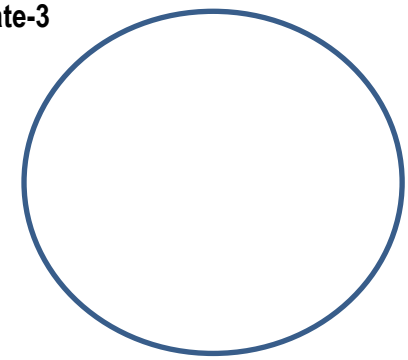
PRACTICAL NO. 12

Objective: To know the cultural characteristics of microorganisms

ACTIVITY: 1. Draw colony morphology seen in isolation plate and record observations.

Culture plates	Characteristics
Plate-1 	…… …… …… …… …… ……
Plate-2 	…… …… …… …… …… ……
Plate-3 	…… …… …… …… …… ……

2. Mention the morphological differences among bacteria, fungi and actinomycetes:

Bacteria	Fungi	Actinomycetes
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3. Describe growth of microorganisms under liquid medium (broth)

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PRACTICAL NO. 13

OBJECTIVE: Preparation of slide using simple staining technique.

ACTIVITY: 1. Write about the necessary dyes used in simple staining and their mode of action:

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2. Prepare a slide from the given culture and draw cell shape and arrangement as seen under the microscope

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3. Write about the precautions taken during simple staining: ……..

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PRACTICAL NO.14

OBJECTIVE: To perform gram staining technique.

ACTIVITY: 1. Write the steps involved in Gram staining technique and dyes used with their mode of action:

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2. Prepare a slide from the given bacterial culture using gram staining technique and observe under the microscope. Draw well labeled diagram. Write about the shape, colour and arrangement of the cells.

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3. List some Gram positive and Gram negative bacteria.

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4. Precautions taken during Gram staining procedure:

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PRACTICAL NO. 15

OBJECTIVE: To study different types of cell arrangement and flagellation in bacterial cell.

ACTIVITY: Prepare a slide from the given bacterial culture and observe under the microscope. Draw well labeled diagram and study different types of cell arrangement and flagellation in bacterial cell.

PRACTICAL NO. 16

OBJECTIVE: To know about Pure culture technique

ACTIVITY: Perform and write different methods of Pure culture technique.

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PRACTICAL NO. 17

OBJECTIVE: Turbid metric estimation of microbial growth.

ACTIVITY: Prepare Nutrient Broth. Inoculate the given bacterial culture. Take the observations at different time period.

OBSERVATIONS TO BE MADE:

Sample	OD at 30min.	OD at 60min.	OD at 90min.	OD at 120min.	OD at 3 150min.	OD at 180min.	OD at 210min.	OD at 360min.
E. coli								
Unknown bacterial culture								
Unknown bacterial culture								

Draw growth curve for test organisms:

Calculate the generation time of unknown bacterial strain: ……

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Precautionary measures: ……

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PRACTICAL NO. 18

OBJECTIVE: To isolate plant growth promoting rhizobia (PGPR) from root nodule

Activity: 1. Prepare Yeast extract mannitol agar medium. Isolate the PGPR from root nodule of the given plant sample.

Materials required: ……

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Procedure: ……..

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2. Note the culture characteristics of appeared colony on YEMA plate.

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3. To confirm rhizobateria, transfer a loopful of the growing colony on fresh nitrogen free medium (YEMA medium).

PRACTICAL ACTIVITY

4. Prepare a slide and write microscopic observations in detail.

5. Precautionary measures: …….

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PRACTICAL NO. 18.1

OBJECTIVE: To isolate PGPR free living nitrogen fixer Azotobater from soil.

ACTIVITY 1: Prepare Ashby's medium. Isolate Azotobater from given soil sample.

Material required: ……..……..

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Procedure: ……..

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2. Note the culture characteristics of appeared colony on Ashby's medium plate.

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3. To confirm Azotobater transfer a loopful of the growing colony on fresh medium.

PRACTICAL ACTIVITY

4. Prepare a slide and write microscopic observations in detail.

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5. Precautionary measures: ……

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PRACTICAL NO. 18.2

OBJECTIVE: To isolate PGPR phosphate solubilizing bacteria (PSB) from soil sample

ACTIVITY 1: Prepare Pikovskaya's agar medium. Isolate phosphate solubilizing bacteria (PSB) from given soil sample.

Material required: ……..……..

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Procedure: ……..

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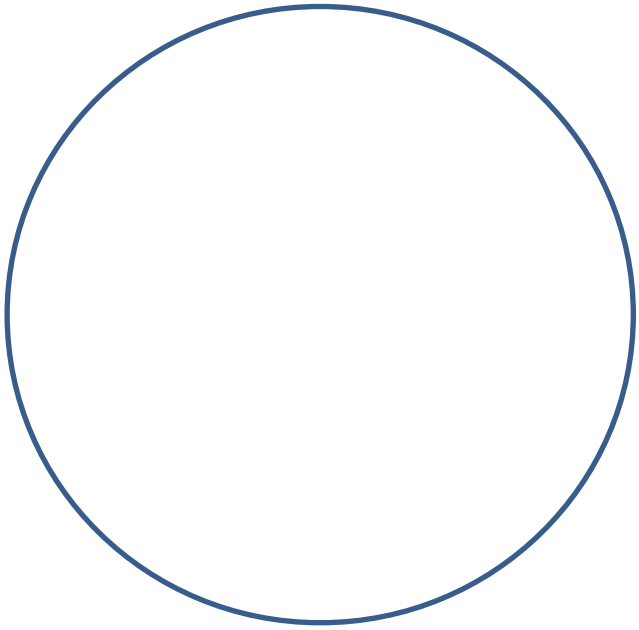
2. Note the culture characteristics of appeared colony on Pikovskaya's agar medium plate and draw the picture.



3. Calculate the Phosphate Solubilization Index (PSI) for quantitative analysis.

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4. Calculate the phosphate solubilizing activity in broth medium.

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5. Precautionary measures: ……

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PRACTICAL NO. 19

OBJECTIVE: To study about Mushroom- Spawn production, culture and production technique.

ACTIVITY: Identify and describe the morphology of the given mushroom. Draw well labeled diagram. Prepare PDA and maintain culture of the given mushroom.

Perform and describe different steps of mushroom cultivation

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APPENDICES

(A) Laboratory Equipments

1.	Autoclave	11.	Hot-air oven
2.	Refrigerator	12.	Centrifuge
3.	Hot Plate	13.	Rotary shaker
4.	Laminar Air flow Cabinet	14.	Microwave Oven
5.	Water bath	15.	Water Distillation Apparatus
6.	Haemocytometer	16.	Spectrophotometer
7.	Incubator	17.	Electrophoretic Apparatus
8.	pH meter	18.	Polymerase Chain Reaction (PCR) Unit
9.	Analytical balance	19.	Gel Documentation Unit
10.	Microscope	20.	Deep Freeze

(B) Glass-wares:

(i)	Conical flask	(ii)	Beaker
(iii)	Measuring cylinder	(iv)	Pippet (different volume)
(v)	Petri-dishes	(vi)	Slides
(vii)	Cover-slip	(viii)	Watch glass
(ix)	Culture tubes	(x)	Hand lens
(xi)	Reagent bottles	(xii)	Specimen bottles

(C) **Miscellaneous items:**

1.	Absorbant Cotton	2.	Thread
3.	Aluminium foil	4.	Rubber bands
5.	Trays	6.	Washing brush
7.	Sieves of different sizes	8.	Labolene
9.	Blotting paper	10.	Wire/ Plastic basket
11.	Wash bottle	12.	Mortar and pestle
13.	Knife / Blade	14.	Scissor
15.	Needle	16.	Cork-borer
17.	Scalpel	18.	Forcep
19.	Inoculation needle	20.	Inoculation loop
21.	Paraffin film	22.	Glass marker
23.	Autoclavable PP Bags	24.	Plastic O rings
25.	Micro- Pipette	26.	Micro-tips
27.	Spreader	28.	Stirring rod

(A) Chemicals:

1.	Acetic acid (glacial)	11.	Pottassium dichromate
2.	Acetone	12.	Sodium Chloride
3.	Lactic Acid	13.	Peptone
4.	Ethyl Alcohol (95%)	14.	Beef Extract
5.	Sodium Hydrochloride	15.	Agar-Agar
6.	Formalin (40% Formaldehyde)	16.	Hydrochloric Acid
7.	Mercuric Chloride	17.	Sulphuric Acid
8.	Pottassium Permanganate	18.	Dextrose (Anhydrous)
9.	Chromic acid	19.	Sucrose
10.	Methanol	20.	Iodine
21.	Glycerin	22.	Phenol

(B) Stains:

	Stains	Use
1.	Cotton Blue (Methyl Blue)	Used to stain Bacterial / Fungal cells
2.	Crystal Violet (Gentian Violet)	General histopathological stain
3.	Gram Stain	Used to test bacterial culture (Positive/Negative)
4.	Safranin	Used as counter stain for bacterial cell
5.	Acid Fuchsin	Used to stain fungal mycelium

(C) Mounting media/ Preservatives

	Mounting Media		Preservative/Fixative
1.	Lactophenol	1.	FAA solution
2.	Lactophenol-Cotton Blue	2.	Acid Alcohol
3.	Glycerin	3.	Formalin (5%)

AUTOCLAVE (VERTICAL): An autoclave provides a temperature of 121ºC. Saturated steam is more lethal in action and capable of penetrating of spores of microorganisms. It is most effective techniques for sterilization of material.

How to use:

- Open the lid of Autoclave and fill the water at marked
- Keep the items inside for sterilization
- Close the lid and tighten very strongly
- Power on the main switch and after run the autoclave continuous at ambient pressure.

HOT AIR OVEN: Hot Air oven is usually electrically operated with thermostat fitted in it besides a circulator fan, which facilitates free movement of hot air created within the chamber.

How to use:

- Power on the instrument and set the temperature by adjustment knob as per use
- Door of oven close during on position
- It is most widely used for sterilization of glasswares, scissor, scalpels, forceps, glass syringe, swabs etc.

Precaution:

- Check the temperature after half an hour interval so that temperature dose not rises at per set temperature.
- The door of oven open after 15 min. of rise ambient temperature

LAMINAR AIR FLOW CABINET (HORIZONTAL): Laminar Air Flow cabinet provides an aseptic working area for inoculation of microorganism and culture manipulation. The LAF cabinet provides a sterile environment for culture inoculation and protects the operator the potential infection risk from the culture. It has High efficiency particulate air filters (HEPA) with 99.99% efficiency for particles.

How to use:

- The working table should be sterile by 90% Ethanol before and after completion of work
- The LAF cabinet is highly sterile instrument. So it should be keep in separate room or partition that protect from direct dust, sunlight and air

BOD INCUBATOR: BOD incubator provides suitable environment for the growth of microbial cultures under aseptic conditions. It is also maintains Relative Humidity (RH) and temperature.

How to use:

- Switch on the main and instrument for power supply
- Set the temperature and RH by adjustment knob as per requirement
- Switch on the cooling switch

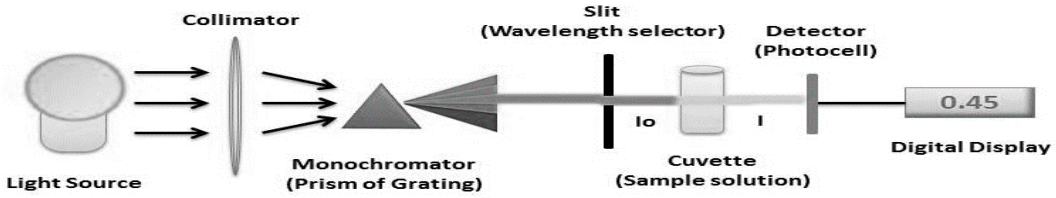
SPECTROPHOTOMETER OR COLORIMETER: In a Spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths. By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. The range of the wavelengths of the incident light can be as low as 1 to 2nm. The spectrophotometer is useful for measuring the absorption

spectrum of a compound, that is, the absorption of light by a solution at each wavelength. This is the basic Principle of spectrophotometry.

It is used for estimating population of bacteria, based on the principle of turbidity determination. Turbidity is the cloudiness of the suspension. The more turbid a suspension, less light will be transmitted through it. In other words, the amount of light absorbed and is scattered is proportional to the mass of cell. As bacteria grow in a broth, the clear broth becomes turbid. Since turbidity increases as the number of cells increases, this is used as an indicator of bacterial density in broth: The turbidity is expressed in unit of optical density (O. D.) which is expressed using Spectrophotometer.

Spectrophotometer Instrumentation: The essential components of spectrophotometer instrumentation include:

1. A Stable and cheap radiant energy source.
2. A monochromator, to break the polychromatic radiation into component wavelength (or) bands of wavelengths.
3. Transport vessels (cuvettes), to hold the sample.
4. A Photosensitive detector and an associated readout system.



Basic Instrumentation of a Spectrophotometer

ROTARY SHAKER: Rotary shaker platfrom is ideal for mixing and growing cultures. It is used in making ideal mixing of saturated gels, microbial cultures and other molecular solution.

How to use:

- Load the sample on shaker platform
- Switch on the power supply and press the ON/Off button of instruments as ON position
- Set the speed as per requirement
- Set the temperature as per requirement
- Set timer as per requirement
- Then, push the start button

Precautions:

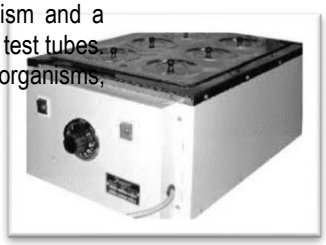
- Do not fill the sample vessel like flask, beaker, etc. more than half.
- Always fit sample vessel tightly by clamp because it can be broken during shaking
- Do not load extra sample.

ANALYTICAL BALANCE: Analytical balances are instruments used for precise determining mass of matter. Analytical balances are sensitive and expensive instruments, and upon their accuracy and precision the accuracy of analysis result depends. Various media components for culture media preparation and samples etc. are weighed on an electronic mono pan balance is recommended. As most of the media ingredients are highly hygroscopic, the balance should be cleaned immediately after use. The most widely used type of analytical balances are balances with a capacity of 100 g and a sensitivity of 0.1 mg.

REFRIGERATOR: It is a basic requirement in the microbiological laboratory and used for storing stock cultures of microorganism at 4°C to save sub-culturing every few days. The stored cultures at low temperature are fairly inactive and will not suffer damage due to evaporation of medium. It is also used to store sterilized media to prevent dehydration and to serve as a repository for thermo-labile solutions, serums, antibiotics and biochemical reagents.

Hot plate with stirrer: It is useful to stir the chemicals in water without heat to make suspension. It is fined with the stirrer and heat control. Stirring is done by creating magnetic field, which causes the bar magnet kept in the container to spin resulting in the stirring of the medium.

WATER BATH: It is an insulated metallic box fitted with an electric heating mechanism and a thermostat, which maintains the temperature at desired level. There are racks for holding test tubes. These are usually used for melting of media, testing enzymatic activities of various microorganisms, etc.

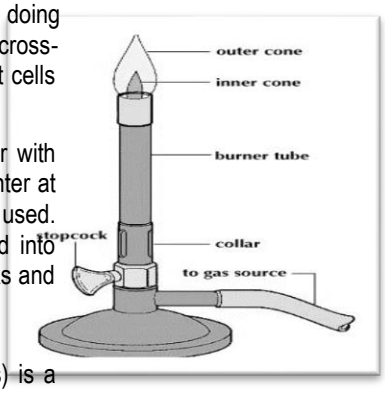


INOCULATION LOOP: An inoculation loop, also called a smear loop, inoculation wand or micro streaker. It is a simple tool used mainly by microbiologists to transfer microorganism from a culture of microorganism to fresh medium from. The wire forms a small loop with a diameter of about 5 mm. The loop of wire at the tip may be made of Platinum, Tungsten or Nichrome, the latter being inferior but less expensive. This loop removes a consistent amount of the liquid suspended inoculum by using the phenomenon of surface tension.

INOCULATION NEEDLE: An **inoculating needle** is a piece of Stainless steel, Nichrome, Platinum, or other wire mounted in a handle. The wire can be any size. Small diameter wires require less time to heat and cool. Most people use 26 guage platinum or 25 guage nichrome wires. I use 20 guage nichrome because that is the size I have. A 20 guage wire has a larger diameter than a 26 guage wire and takes longer to heat and cool.

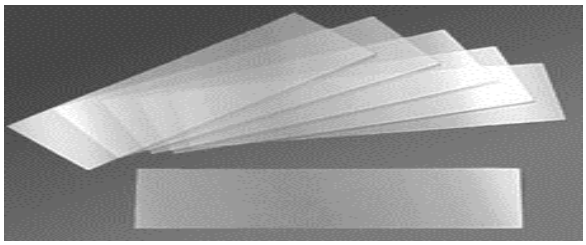
How to use? The inoculation loop is sterilized in a fire, until it becomes hot before and after each use. Heating for 5–7 seconds is enough to render the inoculation loop free from all forms of life. By doing this, the same tool can be reused in different experiments without fear of cross-contamination. After flame sterilization, the loop must be cooled so that the next cells the loop touches are not killed by the hot metal.

BUNSEN BURNER: It is named after R.W. Bunsen. It is a type of gas burner with which a very hot particularly non-luminous flame is obtained by allowing air to enter at the base and mix with gas. In the absence of Bunsen burner, alcoholic lamp is used. They are used to sterilize inoculation needles / loops before they are inserted into culture. It is also used for flaming the mouth of test tubes, media containing flasks and other glass apparatus to avoid contamination by other microorganisms.

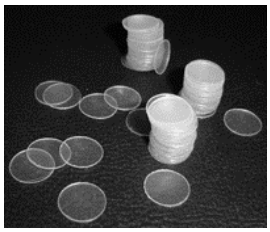


HAND LENS: A **magnifying glass** (called a **hand lens** in laboratory contexts) is a convex lens that is used to produce a magnified image of an object. The lens is usually mounted in a frame with a handle. **Sheet magnifier** consists of many very narrow concentric ring-shaped lenses, such that the combination acts as a single lens but is much thinner. This arrangement is known as a Fresnel lens. A magnifying glass can also be used to focus light, such as to concentrate the sun's radiation to create a hot spot at the focus for fire starting.

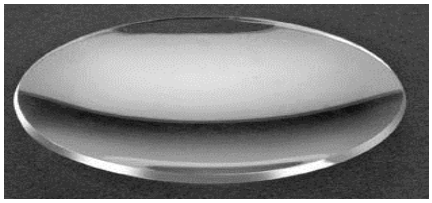
GLASS/ MICROSCOPIC SLIDE: A microscope slide is a thin flat piece of glass, typically 75 by 26 mm (3 by 1 inch) and about 1 mm thick, used to hold objects for examination under a microscope. Typically the object is placed or secured ("mounted") on the slide, and then both are inserted together in the microscope for viewing. This arrangement allows several slide-mounted objects to be quickly inserted and removed from the microscope, labeled, transported, and stored in appropriate slide cases or folders. Microscope slides are usually made of optical quality glass, such as soda



lime glass or borosilicate glass, but specialty plastics are also used. Fused quartz slides are often used when ultraviolet transparency is important, e.g. in fluorescence microscopy.



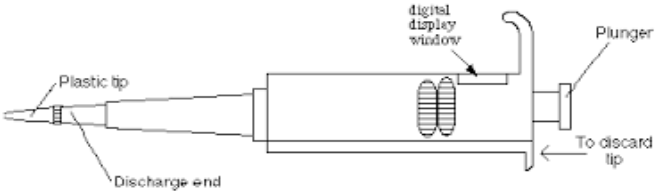
COVER SLIP: A **cover slip** or **cover glass** is a thin flat piece of transparent material, usually square or rectangular, about 20 mm (4/5 in) wide and a fraction of 1mm thick, that is placed over objects for viewing with a microscope.



WATCH GLASS: A watch glass is a circular concave piece of glass used in chemistry as a surface to evaporate a liquid, to hold solids while being weighed, for heating a small amount of substance and as a cover for a beaker. The latter use is generally applied to prevent dust or other particles entering the beaker; the watch glass does not completely seal the beaker, so gas exchanges still occur.

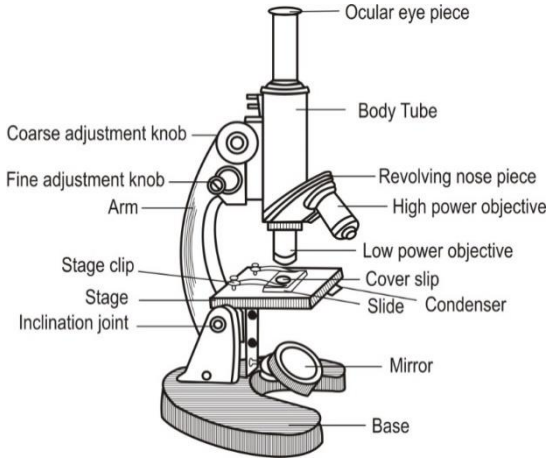
CORK BORER: A **cork borer** is a metal tool for cutting a hole in a cork or rubber stopper to insert glass tubing. Cork borers usually come in a set of nested sizes along with a solid pin for pushing the removed cork (or rubber) out of the borer. The individual borer is a hollow tube, tapered at the edge, generally with some kind of handle at the other end. A separate device is a *cork borer sharpener* used to hone the cutting edge to more easily slice the cork. Cork borer is used to punch holes on an agar plate, to perform Well Diffusion assays in microbiology.

PIPETTE: A **pipette** (sometimes spelled **pipet**) is a laboratory tool commonly used in chemistry, biology and medicine to transport a measured volume of liquid, often as a media dispenser. Pipettes come in several designs for various purposes with differing levels of accuracy and precision, from single piece glass pipettes to more complex adjustable or electronic pipettes. Measurement accuracy varies greatly depending on the style. Although specific descriptive names exist for each type of pipette. Sometimes, pipettes that dispense between 1 and 1000 µl are distinguished as **micropipettes**, while **macropipettes** dispense greater volumes.



COMPOUND LIGHT MICROSCOPE:

Parts of Microscope:



- ***a.*** *Eye piece (Ocular lens):* It is also known as the ocular. This is the part used to look through the microscope found at the top of the microscope. Its standard magnification is 10x.
- ***b.*** *Coarse adjustment knob:* The coarse adjustment knob is used to bring the objective down into position over any object on the stage.
- ***c.*** *Fine adjustment knob:* Used to focus on the specimen.
- ***d.*** *Objective lens*: Rotating the objective nosepiece allows different magnifications e.g. 10x, 20x, 40x and 100x, to be selected. The objective provides a magnified and inverted image.
- ***e.*** *Stage:* The stage has a hole in the center that permits light from below to pass upward into the lenses above. The object to be viewed is positioned on the stage over this opening.
- ***f.*** *The condenser:* Focuses light onto the specimen to give optimum illumination.
- ***g.*** *Illuminator:* Illuminator fit at the base is the source of light. Light is directed upward through the condenser.

STERILIZATION METHODS

Principle: 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 to prevent transmission of disease and infection, contamination by undesirable microorganisms and deterioration and spoilage of materials by microorganisms

Methods: 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.

Physical Methods: Sunlight, Drying, Filtration, Heat (Dry heat- Flaming, Incineration; Moist heat)

Chemical method: Ethylene oxide Alcohol, Nitrogen dioxide, Ozone, Bleach, Glutaraldehyde and Formaldehyde, Hydrogen Peroxide, Per-acetic Acid, Radiation sterilization : Ionizing radiation sterilization, Non-ionizing radiation sterilization

PREPARATION OF POTATO DEXTROSE AGAR MEDIUM

Materials required: Peeled potato slices (200g), Dextrose (20g); Agar- agar (20g), Distilled water (1000 ml)

Method:

(1) Potato slices are cooked in 500 ml of water.
(2) Then filtered with the help of muslin cloth.
(3) Agar-agar is melted in 500 ml of water.
(4) Potato juice is added to the melted agar.
(5) Volume is made 1000 ml by adding required water.
(6) Again lit is filtered through muslin cloth.
(7) Dextrose is added in this mixture and shaken well.
(8) Medium is sterilized in an autoclave at 1.1kg/cm^2 pressure for 20 minutes at temperature of 121.6°C. Thus the medium is ready for use.

PREPARATION OF NUTRIENT AGAR MEDIUM

Materials required: Peptone (10g), Beef extract (10g), Sodium Chloride (5g), Agar- agar (12g), Distilled water (1000 ml)

Procedure

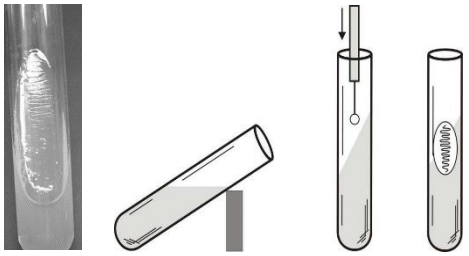
1. Dissolve the weighed amounts of peptone and beef extract into 500 ml of water.
2. Heat and dissolve the chemicals.
3. Weigh 20g agar and dissolve in 500 ml of distilled water in another beaker
4. Mix the dissolved agar with chemical solution and make the volume to 1lt.
5. Dispense 200 ml each into 5 conical flasks.
6. Plug the flask with non absorbent cotton and sterilize at 15 lbs pressure and 121^{0}C for 15-25 minutes in autoclave.

Precautions

1. Do not pour the media over 2/3 of flask capacity.
2. Do not pour media to Petri-plate when the medium is too hot since it produce condensation of water on underside of Petri plate lid and thus can fall on to agar surface and may lead to contamination and spreading of colonies.
3. Pour medium quickly to avoid contamination by air-pores and close lid down as soon as possible.
4. Perform the pouring of medium in inoculation chamber fitted with U. V. lamp with filtered air.
5. Pouring should be performed near the flame.

PREPARATION OF AGAR SLANTS FOR MAINTENANCE OF BACTERIAL CULTURE.

Required materials: Test tubes, Nutrient agar medium, Beaker, Conical flasks, Measuring cylinder, Non absorbent Cotton.



Procedure:

1. Place test tube in a test tube rack
2. Prepare Nutrient Agar medium.
3. Transfer 5ml medium in each test tube and place cotton plug in each test tube.
4. Sterilize the tubes at 121^{0}C for 15-25 minutes.
5. After sterilization tilt the test tubes onto the solid surface in upward position so that the medium in the tubes is slanted. Allow the medium to solidify in slanting position.
6. After cooling keep the tubes in refrigerator for further use

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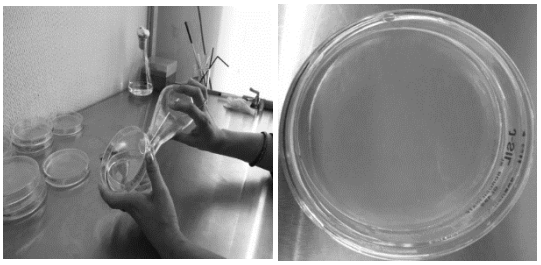
PREPARATION OF AGAR PLATES FOR MAINTENANCE OF BACTERIAL CULTURE.

Required materials: Nutrient agar medium, Petri plates, Beaker, Conical flasks, Measuring cylinder, Non absorbent Cotton.

Procedure:

1. Prepare Nutrient Agar medium and sterilize at 121^{0}C for 25 minutes in autoclave.



2. Sterilize Petri plates in Hot air oven at 160^0C for 60 minutes.
3. After sterilization pour 20ml lukewarm medium in each Petri plate in a Laminar air flow cabinet aseptically.
5. Allow the medium to solidify in Petri plates
6. After cooling keep Petri plates in refrigerator for further use.

PREPARATION OF BASIC LIQUID MEDIUM OR BROTH FOR CULTIVATION OF BACTERIA

Required material: Peptone (10g), Beef extract (10g), Sodium Chloride (5g), distilled water (1000ml), 0.1 N HCl , 0.1 N NaOH, Pan, beaker, measuring cylinder, non-absorbent cotton, test tube.
Required Instruments: Weighing balance, Hot plate or Induction plate, Autoclave.
Procedure:

1. Take the weighed amounts of peptone and beef extract and mix in 50 ml of distilled water.
2. Heat and dissolve the chemical and add more distilled water to make up the final volume 1 L.
3. Take the test rube and apply cotton plug.
4. Sterilize at 15 psi pressure for 20 minutes in an Autoclave.
5. Allow the Autoclave to cool, remove the nutrient broth tubes and store at room temp and cover with butter paper.

Precautions

1. Don't pour the media over 2/3 of flask capacity.
2. Cotton plug must not be loose while autoclaving.

ISOLATION OF BACTERIA FROM DISEASED PLANT SAMPLE

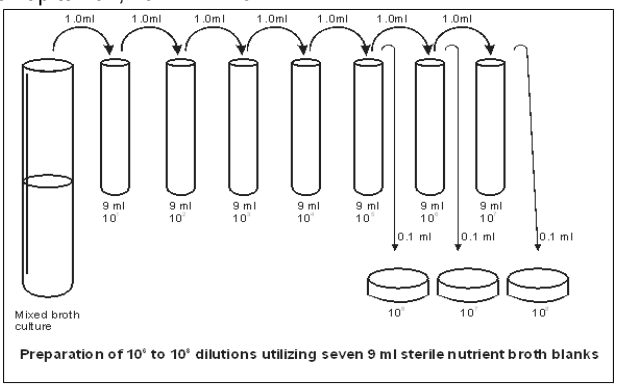
Required material: Infected young tissue of plant, sterile Petri -dishes, Watch glass, Nutrient Agar medium. NA slants, Sodium hypochlorite solution (1%v/v), sterile water, razor blade, forceps, inoculation loop, burner/spirit lamp, spirit, Tissue paper, Paraffin film, PDA medium.
Required instruments: Autoclave, Laminar Air, Flow cabinet, Weighing balance, BOD Incubator, Microscope.
Procedure:

1. Prepare Nutrient Agar medium.
2. Pour 20ml sterile Luke warm NA medium in Petri dish near the flame of spirit lamp under aseptic condition in Laminar Air Flow cabinet and keep for solidification.
3. Prepare the 1% v/v sodium hypochlorite solution with sterile water.
4. Take about 3-5 ml of 1% sodium hypochlorite in watch glass under aseptic condition of LAF cabinet.
5. Select infected host tissue with healthy tissue from infected plant material.
6. Cut plant material into small pieces (3-5mm) containing both diseased and healthy tissues and wash with sterilized water.
7. Dip the pieces into 1 % sodium hypochlorite solution for about one minute and wash with sterilized water. Repeat the process twice. Keep the pieces on sterilized filter paper for drying.
8. Transfer sterilized tissue pieces at different position in NA Plates near the flame of sprit lamp and wrap with Paraffin film.
9. Incubate the Petri dishes in inverted position at 30-35°C.
10. Observe the incubated plates after one day of inoculation for the growth of the bacterium.
11. Prepare the slants using Nutrient Agar medium.
12. Transfer aseptically growing bacterial colony on fresh NA medium and incubate at 30-35°C in BOD incubator for further study.
13. Prepare a slide from growing Bacterial colony and examine under the microscope.

ISOLATION OF MICROORGANISM (FUNGI / BACTERIA) FORM GIVEN SOIL SAMPLE

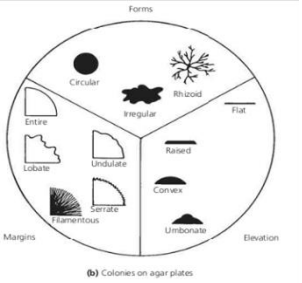
Required materials: Test tube, Petri dish, Conical Flask, Test tube Stand, Sterilized water, Measuring cylinder, Sprit, Sprit lamp, Micro pipette, Micro tips, Beaker, PDA/ Nutrient Agar medium, non absorbent Cotton, tissue paper, etc.
Required Instruments: Weighing balance, vertex mixer, LAF Cabinet, BOD Incubator, Colony counter.
Procedure:

1. Take 6 or 7 test tubes. Fill one test tube with 10ml sterile water and rest with 9 ml sterile water.
2. Weigh one gm of sample from original sample (Soil) and mix in 10 ml of sterile water in a test tube mix with the help of vertex mixer.
3. Take 1 ml from above suspension and transfer in second test tube fill with 9ml sterilized water. Then take the suspension from second test tube and transfer it into third test tube filled with 9ml water.
4. Repeat same process with all tubes. It will give the dilution up to 10^{-1}, 10^{-2} .....10^{-7}.
5. Finally pour 1ml suspension from final dilution in PDA/ NA Petri plates with the help of micropipette under aseptic conditions in LAF cabinet and spread with the help of L-shape spreader.
6. Seal the Petri plates with paraffin film and incubate at 25±1^{0}C in a BOD incubator.
7. Number of colonies can be counted with the help of colony counter.
8. Multiply total no. of colonies by dilution factor to find out the number of cfu (colony forming units per ml) in per gram sample.
9. The serial dilution method also used for obtaining pure culture of microorganisms.



CULTURAL CHARACTERISTICS OF MICROORGANISMS

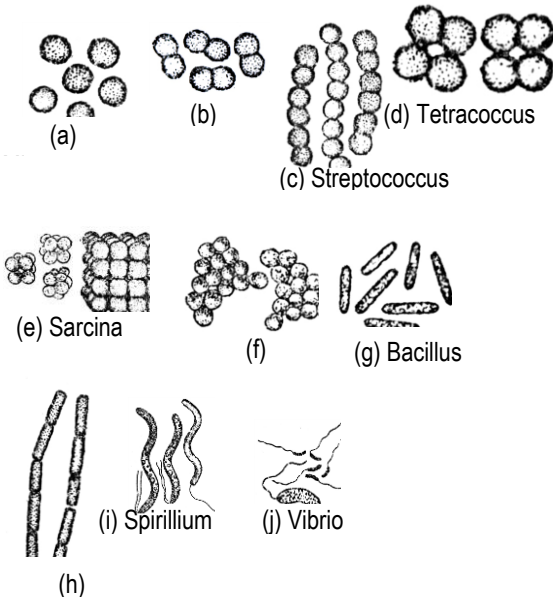
Principle: When grown on a variety of media, microorganisms will exhibit differences in the macroscopic appearance of their growth. These differences, called cultural characteristics, are used as a basis for separating microorganisms into taxonomic groups. The cultural characteristics for all known microorganisms are contained in Bergey's Manual of Systematic Bacteriology. They are determined by culturing the organisms on nutrient agar slants and plates, in nutrient broth, and in nutrient gelatin. The patterns of growth to be considered in each of these media are described below.
Materials required: Microorganisms growth plate, Light emission plate, marker
Observations: Size: Pinpoint, small, moderate, or large; **Pigmentation:** Color of colony; **Form:** The shape of the colony is (**Circular:** Unbroken, peripheral edge; **Irregular:** Indented, peripheral edge; **Rhizoid:** Root like, spreading growth); **Margin:** The appearance of the outer edge of the colony is (**Entire:** Sharply defined, even; **Lobate:** Marked indentations; **Undulate:** Wavy indentations; **Serrate:** Tooth like appearance; **Filamentous:** Threadlike, spreading edge); **Elevation:** The degree to which colony growth is raised on the agar surface is (**Flat:** Elevation not discernible; **Raised:** Slightly elevated; **Convex:** Dome-shaped elevation; **Umbonate:** Raised, with elevated convex central region).



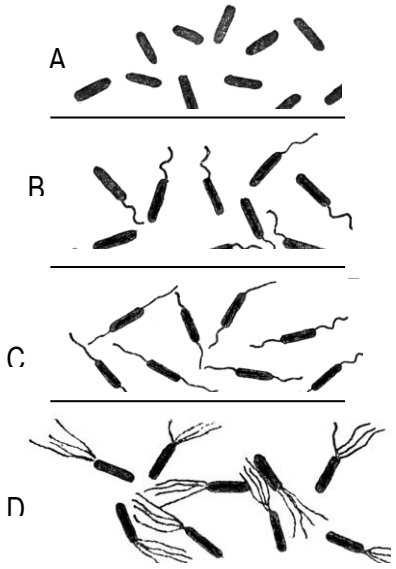
CELL ARRANGEMENT IN BACTERIA

(A) Coccus: Monococcus: Cells are found singly; **Diplococcoccus** : Cells remain attached in pair; **Streptococcus**: Due to division of cells in one plane, cells remain attached with each other in a chain or series in different length (chain may be short or comparatively long); **Tetracoccus**: Division of cells in two planes at right angle; **Sarcina**: Division in three planes, one horizontal and two vertical forming a cubical packet of 8 cells or further division of tetrad (back to back); **Staphylococcus**: Cell division in many irregular planes, which form like bunch of grapes.
(B) Bacillus: Monobacillus : The cells are found singly; **Diplobacillusccus** : Cells found in pair; **Streptobacillus**: Cells found in chain.
(C) Spiral: Spiral shaped bacteria are without any cell arrangement. **Vibrio**: (pl. vibrios or vibriones) "Comma" shaped bacteria; **Spirillum**: (pl. spirilla) They are twisted with one or two or even more turns (bends).

Flagellation in Bacteria: *Atrichous*: Cells without flagella; *Monotrichous*: a single flagellum at one end (pole) of the cell; *Lophotrichous*: two or more flagella (in tuft) at one or both ends of the cell; *Amphitrichous*: one flagellum at each end; *Peritricholus*: large number of flagella surrounding the cell, particularly at lateral side.



Cell arrangement in bacteria



Bacteria and their flagella

A. Atrichous B. Monotrichous C. Amphitrichous

PREPARATION OF PHYTO-BACTERIA SLIDE (SIMPLE STAINING)

Material Required: Glass slide, cover slip, Tissue paper, Sprit, Sprit lamp, inoculation loop, forceps, Non absorbent cotton, watch glass, dropper,
Instruments Required: Microscope.
Procedure:

1. Take a glass clean glass slide and wipe with 70% alcohol and Tissue paper.
2. Take a loop full culture and make a smear of culture on glass slide and heat fixing them.
3. Stain with Crystal Violet or Methylene blue.
4. Take a cover slip and keep on slide very smoothly that no air bubble formation on slide.
5. Examine the slide under microscope.

PREPARATION OF PHYTO-BACTERIA SLIDE (GRAM STAINING)

Material Required Glass slide, cover slip, Tissue paper, Sprit, Sprit lamp, inoculation loop, forceps, non absorbent cotton, watch glass, dropper, Gram staining kit, 70% alcohol, Glycerin, fixing agent.
Instruments Required: Microscope,
Procedure:

1. Take a glass clean glass slide and wipe with 70% alcohol and Tissue paper.
2. Take a loop full culture and make a smear of culture on glass slide and heat fixing them.
3. Take some drop of Crystal blue solution and cover the smear with this solution and hold up to one minute.
4. After one minute the slides was washed with distilled water and dry it.
5. Take some drop of iodine solution and cover the smear with the solution and hold up to one minute.
6. After one minute the slides was washed with distilled water and dry it.
7. Take some drop of alcohol solution and cover the smear with the solution and hold up to 30 seconds.
8. After 30 seconds the slides were washed with distilled water and dry it.
9. Take some drop of Safranin solution and cover the smear with this solution and hold up to one minute.
10. After one minute the slides was washed with distilled water and dry it.
11. Take a very small amount of glycerin and keep on smear.
12. Take a cover slip and keep on slide very smoothly that no air bubble formation on slide.
13. Examine this slide under microscope.
14. There are two types of gram's reactions:

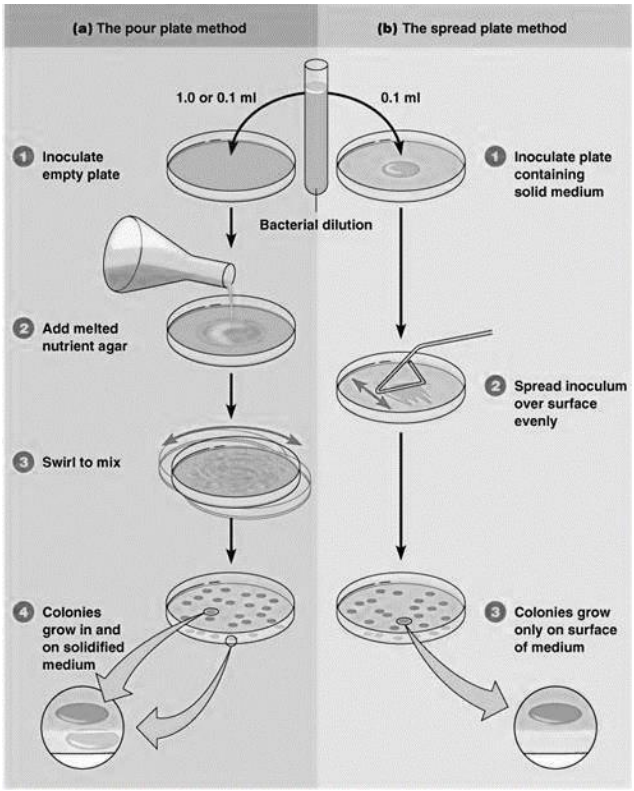
Gram (+) - Blue / violet colour
Gram (-) - Red colour

Examples of Gram-Positive Organisms: *Bacillus, Nocardia, Clostridium, Propionibacterium, Actinomyces, Enterococcus, Cornyebacterium, Listria, Lactobacillus, Gardnerella, Mycoplasma, Staphylococcus, Streptomyces, Streptococcus etc*
Examples of Gram-Negative Organisms: *Escherichia, Helicobcater, Hemophilus, Neisseria, Klebsiella, Enterobacter, Chlamydia, Vibrio, Pseudomonas, Salmonella, Shigella*

PURE CULTURE TECHNIQUES

A **pure culture** theoretically contains a single bacterial/ fungal species. A pure culture may be isolated by the use of special media with specific chemical or physical agents that allow the enrichment or selection of one organism over another. Simpler methods for isolation of a pure culture include **Pour plate or Spread plating** on solid agar medium with a glass spreader and **Streak plating** with a loop.
Material: Seven 9-ml dilution tubes of sterile saline; Seven nutrient agar plates; 1.0 ml and 0.1 ml pipettes; Spreader; 95% ethyl alcohol

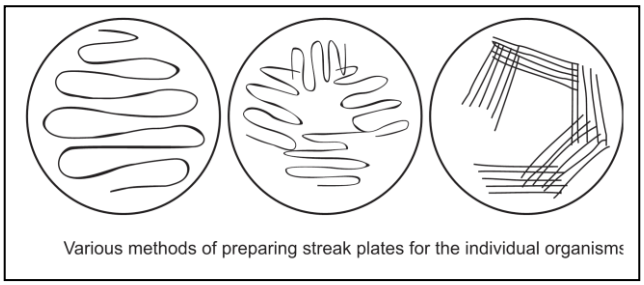
Pour Plate technique: Pour plate method is usually the method of choice for counting the number of colony-forming bacteria present in a liquid specimen. In this method, fixed amount of inoculum (generally 1 ml) from a broth/sample is placed in the center of sterile Petri dish using a sterile pipette. Molten cooled agar (approx. 15mL) is then poured into the Petri dish containing the inoculum and mixed well. After the solidification of the agar, the plate is inverted and incubated at 37°C for 24-48 hours. Microorganisms will grow both on the surface and within the medium. Colonies that grow within the medium generally are small in size and may be confluent; the few that grow on the agar surface are of the same size and appearance as those on a streak plate. Each (both large and small) colony is carefully counted (using magnifying colony counter). Each colony represents a "colony forming unit" (CFU). The pour plate technique can be used to determine the number of microbes/ml in a specimen. It has the advantage of not requiring previously prepared plates, and is often used to assay bacterial contamination of food stuffs.



Spread Plate Technique: In this technique, the number of bacteria per unit volume of sample is reduced by serial dilution *before* the sample is spread on the surface of an agar plate.

1. Prepare serial dilutions of the broth culture. Be sure to mix the nutrient broth tubes before each serial transfer. Transfer **0.1 ml** of the final three dilutions (10^{-5}, 10^{-6}, 10^{-7}) to each of three nutrient agar plates, and label the plates.
2. Rinse the spreader in alcohol and very carefully pass it over the flame just once. This will ignite the excess alcohol on the spreader and effectively sterilize it.
3. Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the medium no longer appears moist. Return the spreader to the alcohol.
4. Repeat the flaming and spreading for each of the remaining two plates.
5. Invert the three plates and incubate at room temperature until the next lab period.

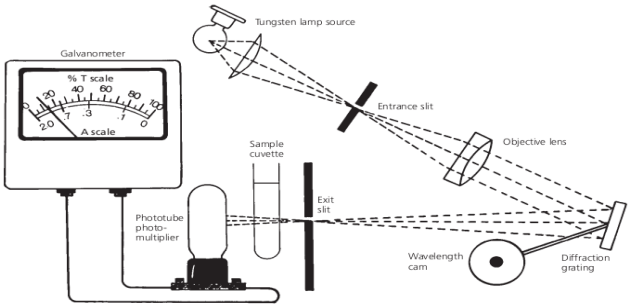
Streak Plate Technique: The streak plating technique isolates individual bacterial cells (colony-forming units) on the surface of an agar plate using a wire loop. The streaking patterns shown in the figure below result in continuous dilution of the inoculum to give well separated surface colonies. Once again, the idea is to obtain isolated colonies after incubation of the plate.


Various methods of preparing streak plates for the individual organisms

1. Label two nutrient agar plates No. 1 and No. 2.
2. Prepare two streak plates by following two of the 3 streaking patterns shown in the figure below. Use **10^{-1}** dilution as inoculum.
3. Invert the plates and incubate at room temperature.

TURBID METRIC ESTIMATION OF MICROBIAL GROWTH

Principle: Bacterial growth is measured in term of doubling of the cells and quantifies by turbidity in broth medium on incubation. Turbidity measurement is required spectrophotometer and its functioning based on Beer-Lambert law. It states that the transmission beam of light (T) at a single wavelength (mono-chromatic light) through a liquid culture. The cells suspended in the culture interrupt the passage of light, and the amount of light energy transmitted through the suspension is measured on a photoelectric cell and converted into electrical energy. The electrical energy is then recorded on a galvanometer as 0% to 100% T. In practice, the density of a cell suspension is expressed as absorbance (A) rather than percent T, since A is directly proportional to the concentration of cells, whereas percent T is inversely proportional to the concentration of suspended cells. Therefore, as the turbidity of a culture increases, the A increases and percent T decreases, indicating growth of the cell population in the culture.



Materials: 12-hours old *E. coli* broth cultures suspension, Saline suspension, Trypticase soy broth (TSB), Bunsen burner, sterile 1-ml pipettes, glassware marking pencil, test tube rack, and Spectrophotometer.

Procedure: (1st step) for initial preparation

1. Prepare TSB medium and autoclave at 121°C, 15psi for 30 minutes and allow cooling. Now, using a sterile 1-ml pipette, add 0.1 ml of the *E. coli* culture 12 hrs old to sterile TSB medium flask.

2. Incubate the flask at shaker incubator for 6 hours at 37 °C and 120rpm.

2nd step for turbidity measurement

After finish the incubation period, follow the instructions below and refer to Figure for the use of the spectrophotometer to obtain the absorbance readings of inoculated cultures.

1. Turn the instrument on 10 to 15 minutes prior to use.
2. Set wavelength at 600 nm.
3. Set percent transmittance to 0% (A to 2) by turning the knob on the left.
4. Read the four TSB cultures as follows:
   - **a.** Wipe clean the provided test tube of sterile TSB broth that will serve as the blank for the TSB broth culture readings.
   - **b.** Insert the STB broth blank into the tube holder, close the cover, and set the A to 0 (percent T = 100) by turning the knob on the right.
   - **c.** Shake lightly or tap one of the tubes of TSB culture to resuspend the bacteria, wipe the test tube clean, and allow it to sit for several seconds for the equilibration of the bacterial suspension.
   - **d.** Remove the TSB broth blank from the tube holder.
   - **e.** Insert a TSB broth culture into the tube holder, close the cover, and read and record the optical density reading on note book.
   - **f.** Remove the TSB culture from the tube holder.
   - **g.** Reset the spectrophotometer to an A of 2 with the tube holder empty and to an A of 0 with the TSB blank.
   - **h.** Repeat Steps (**c)** through (**g)** to read and record the absorbance (O.D.) of the remaining TSB broth cultures at different time interval e.g. 30 min., 60 min., 90 min., 120 min., 150 min., 180 min. 210 min., and 360 min. to draw a growth curve and note the absorbance.

Observations and Result: Record the optical density (O.D) of the sample on your note book. Plot a graph between O.D versus incubation time to prepare the growth curve and calculate the generation time for *E. coli* culture.

TO ISOLATE PLANT GROWTH PROMOTING RHIZOBIA (PGPR) FROM ROOT NODULE

Principle: First bacteria was isolated from the root nodule by the Beijernick in 1888 and named *Bacillus radicicola,* now it placed under the genus Rhizobium. They infect and live in symbiotic association with only the leguminous plant forming nodule in them and fix atmospheric nitrogen. Rhizobia are Gram-negative rods. Rhizobia are isolated from the root nodules by serial dilutions prepared from the root nodules or the fluid from crushed nodules is spread on the surface Yeast extract mannitol agar (YEMA) plates. Rhizobia luxuriously grow under at the temperature 26-27°C for 10 days.

Material: Root nodule, Yeast extract mannitol agar medium, 0.1% $HgCl_2$ or 3-5 % H_2O_2, 70% ethyl alcohol, sterile water blank (90ml, 10ml), Pipettes, Magnetic shaker, inoculating loop, Bunsen burner.

PREPARATION OF YEAST EXTRACT MANNITOL AGAR (YEMA) MEDIUM

For the preparation of Yeast Extract Mannitol Agar medium the following ingredients in different quantities are required-

Ingredients: K_2HPO_4 (0.5g); $K_2SO_4.7H_2O$; Yeast extract (1.0g); Agar (20g); Congo red (1%)-2.5ml; Distilled water (1000ml)
1. Dissolve the weighed amount of chemicals (except Congo red 1% solution) into 500 ml of water.
2. Heat and dissolve the chemicals.
3. Weigh 20g agar and dissolve in 500 ml of distilled water in another beaker
4. Mix the dissolved agar with chemical solution and make the volume to 1lt.
5. Dispense 200 ml each into 5 conical flasks.
6. Plug the flask with non absorbent cotton and sterilize at 15 lbs pressure and 121^0C for 15-25 minutes in autoclave.
***Note:** Congo red solution is to be sterilized separately and added to the medium at a time of pouring in plates
Procedure:
1. Prepare YEMA medium plates (see composition form Appendix 1)
2. Wash the root nodules in running tap water to remove adhering soil particles.
3. Immerse the nodules in 0.1 $HgCl_2$ or 3-5% H_2O_2 for 5 minutes to surface sterilizes these. Repeatedly wash the nodule in sterilized water for3-4 times to get rid of the sterilizing agents.
4. Place the nodules in 70% ethyl alcohol for 3 minutes (if treated with $HgCl_2$). Repeatedly wash the nodule in sterilized water.
5. Crush a nodule in 1ml of water with sterile glass rod and make a uniform suspension of rhizobia with water.
6. Make serial dilution of nodule extract**.**
7. Spread 1ml, each of suspension from various dilutions, on YEMA plates and incubate the plate at 26°C for 10 days
Observation: Observe the plates after 3-4 days incubation and regularly afterward for the development of rhizobia colonies.
Results: Large gummy colonies of rhizobia will appear on YEMA plates.

TO ISOLATE PGPR FREE LIVING NITROGEN FIXER AZOTOBATER FROM SOIL

Principal: Azotobacter is a free living aerobic, motile, nitrogen fixing bacterium and is known as non-symbiotic nitrogen fixer. Azotobacter can be isolate from soil by the serial dilution planting method using nitrogen free medium such as Ashby's, Jensen's medium, Burk's medium and Beijerinckia medium.
Materials: Rhizospheric fresh soil sample, Ashby's medium, Sterile water blank, Pipette, Magnetic shaker, Bunsen burner, Glass marker.

PREPARATION OF ASHBY'S AGAR MEDIUM

For the preparation of **Ashby's agar medium** the following ingredients in different quantities are required-
Ingredients: K_2HPO_4 (0.2g); K_2SO_4 (0.1g); NaCl (0.2g); Mannitol (20g); $MgSO_4.7H_2O$ (0.2g); $CaCO_3$ (5.0 g); Agar (15g); Distilled water (1000ml).
1. Dissolve the weighed amount of chemicals into 500 ml of water.
2. Heat and dissolve the chemicals.
3. Weigh 15g agar and dissolve in 500 ml of distilled water in another beaker
4. Mix the dissolved agar with chemical solution and make the volume to 1lt.
5. Dispense 200 ml each into 5 conical flasks.
6.Plug the flask with non absorbent cotton and sterilize at 15 lbs pressure and 121^0C for 15-25 minutes in autoclave.
Procedure:
1. Prepare Ashby's medium plate.
2. Collect fresh rhizospheric soil and 10g sieved (2mm) soil mix in 90 ml sterile water blank and shake it for 15 minutes on magnetic shaker.
3. Make serial dilution of diluted soil (mother suspension).
4. Spread 1ml, each of suspension from various dilutions, on Ashby's medium plates and incubate the plate at 28°C for 3 days
Observation: Observe the plates after 3 days incubation for appearance of colonies on to the medium plates.
Results: The Azotobacter colonies appears flat, soft, milky and mucoid.

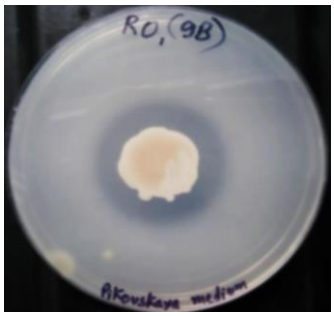
TO ISOLATE PHOSPHATE SOLUBILIZING BACTERIA FROM SOIL SAMPLE

Principal: Phosphate solubilizing bacteria (PSBs) are important categories of PGPRs which are used in the form of biofertilizers. Phosphorous is the major essential macronutrient for plant growth and development and hence is commonly added as fertilizer to optimize yield. "PSBs have been used to convert insoluble phosphate into soluble forms available for plant growth.
Materials: Rhizospheric fresh soil sample, Pikovskaya's agar media, Sterile water blank, Pipette, Magnetic shaker, Bunsen burner, Glass marker.

PREPARATION OF PIKOVSKAYA'S AGAR MEDIUM

For the preparation of **Pikovskaya's agar medium** the following ingredients in different quantities are required-
Ingredients: Yeast extract (0.5g); Calcium phosphate (5.0g); Ammonium sulphate (0.5g); Potassium chloride (0.2g); Magnesium sulphate (0.1g); Manganese sulphate (0.0001g); Ferrous sulphate (0.0001g; Bromophenol blue dye (0.5%); Dextrose (10g); Agar (20g); Distilled water (1000ml); pH (7.0).

1. Dissolve the weighed amount of chemicals into 500 ml of water.
2. Heat and dissolve the chemicals.
3. Weigh 20g agar and dissolve in 500 ml of distilled water in another beaker
4. Mix the dissolved agar with chemical solution and make the volume to 1lt.
5. Dispense 200 ml each into 5 conical flasks.
6.Plug the flask with non absorbent cotton and sterilize at 15 lbs pressure and 121^{0}C for 15-25 minutes in autoclave.



Procedure:

1. Prepare Pikovskaya's agar medium plate.

2. Collect fresh rhizospheric soil and 10g sieved (2mm) soil mix in 90 ml sterile water blank and shake it for 15 minutes on magnetic shaker.

3. Make serial dilution of diluted soil (mother suspension).

4. Spread 1ml, each of suspension from various dilutions, on Pikovskaya's agar medium plates and incubate the plate at 28°C for 7 days.

Observation: Observe the plates after 7 days incubation for clear (transparent) zone around the colonies on to the medium plates.

Results: The PSBs colonies with clear hollow zone appear.

MUSHROOM PRODUCTION

Mushroom is a saprophytic fungus that grows on dead and decaying organic matter. Due to the absence of chlorophyll, it is unable to synthesize its own food and hence is dependent upon the organic matter/substrate for food. Mushrooms can be defined as "a macro-fungus with distinctive fruiting bodies, epigeous or hypogeous, large enough to be seen with naked eyes and picked up by the hands". The mushroom fruiting body may be umbrella like or of various other shapes, size and colour. Commonly it consists of a cap or pileus and a stalk or stipe but others have additional structures like veil or annulus, a cup or volva. Cap or pileus is the expanded portion of the carpophore (fruit body) which may be thick, fleshy, membranous or corky. On the underside of the pileus, gills are situated. These gills bear spores on their surface and exhibit a change in colour corresponding to that of the spores. The attachment of the gills to the stipe helps in the identification of the mushroom.

Mushroom Culture:

Nucleus Culture: Tissue culture technique is used to bring the edible mushroom to pure culture so that the mushroom fungus can further be used to prepare spawn, which is an essential material for mushroom cultivation. This nucleus culture is grown on Potato Dextrose Agar medium in test tubes. A small tissue from a well-grown mushroom is aseptically transferred to agar medium in a test tube in a culture room. The test tubes are incubated under room temperature for 10 days for full white growth of fungal culture. This is further used for preparation of mother spawn.

Procedure:

1. Select well grown, disease free button mushroom early in the morning and keep it on a clean paper for 2-3 hr, to get certain amount of moisture present in the mushroom to get evaporated.
2. Clean the culture room/ laminar flow chamber with antiseptic solution.
3. Keep the sterilized PDA slants, razor blades, forceps etc. inside the chamber and put on the UV light.
4. After 20 minutes put off the UV light and start working after 5 minutes.
5. Sterilize all the instruments to be used by exposing to Bunsen burner.
6. Take in the mushroom and split open the mushroom longitudinally into two halves.
7. Using a blade cut a small piece of tissue from the centre of the spilt mushroom at the junction of pileus and stipe.
8. Remove the cotton plug of the agar slant and transfer tissue aseptically inside the slant by using a sterilized forceps and closes it immediately.
9. After transferring tissues from the mushroom, the tube are arranged in a wire basket and kept in a clean room at room temperature for the growth of the fungus
10. Observe the tube at periodical intervals and remove the contaminated ones. The tubes will be ready for further use within another ten days. The base spawn is used for preparation of mother spawns.

Precautionary measures

- Wash the hands with antiseptic lotion before start working inside the chamber. If possible, it is better to use hand gloves during inoculation.
- While separating the tissue from the centre of the mushroom it should not touch the bottom or sides of the mushroom.

Mother Spawn: Mother spawn is nothing but the mushroom fungus grown on a grain based medium Well-filled, disease-free Wheat grains are used as substrate for growing the spawn materials. The various steps involving in preparation of mother spawn are listed below-

SPAWN PREPARATION

⇩

Wheat grains

⇩

Boiled with water (1:1.5 w/v)

⇩

Drain off excess water

⇩

12g/kg grains $CaSO_4$ and 3g/kg grains $CaCO_3$

⇩

Filled in bottle

⇩

Sterilized at 22psi for 90 minutes

⇩

Inoculation with pure culture

⇩

Master spawn

⇩

Commercial spawn

Mushroom cultivation process involves four major steps

a. Preparation of compost
b. Spawning of compost
c. Casing (Covering the spawned compost)
d. Cropping and crop management

Preparation of compost: Unlike other traditional crops soil is not the appropriate substrate for mushroom cultivation. Rather, the substrate for mushroom called compost, is prepared from agro wastes like straw, stem, shoot, apices etc. with organic manure. Mushroom substrate may be simply defined as a lingo-cellulosic material that supports the growth, development and fruiting of mushroom mycelium. This compost is pasteurized by various micro-organisms and at appropriate temperature range. Essential supplement are also added/ supplemented to the compost. The whole process is termed as composting. Generally composting refers to the piling of substrates for a certain period of time and the changes due to the activities of various micro-organisms, which result in a composted substrate that is chemically and physically different from the starting material. The compost provides nutrients, minerals, vitamins and ions required for proper growth of mushroom. This compost supports the growth of only the mycelium of button mushroom and prevents that of other competitive moulds.

Methodology for compost preparation: Compost is an artificially prepared growth medium from which mushroom is able to derive important nutrients required for growth and fructification. Cemented floors are required for making good quality compost. There are two main methods for compost preparation (i) Long method of composting (ii) Short method of composting. Here we discuss about Long method of composting.

1. **Long method of composting:** This is an outdoor process and takes around 28 days in its completion with a total of seven turnings. The following materials are required for long method of compost:

Wheat straw	**300 Kg**
Wheat bran	**15 Kg**
Ammonium sulphate or calcium ammonium nitrate	**9 Kg**
Super phosphate	**3 Kg**
Muriate of Potash	**3 Kg**
Urea	**3 Kg**
Gypsum	**30 Kg**

Furadan	**150 g**
B.H.C.	**150 g**

This method is accomplished in two phases:

Phase I- Outdoor composting: Wheat straw mixed with chicken manure is sprayed with water and a 45cm high pile is made on the fourth day and first turning is made. On 7th day, wheat bran, gypsum and urea is mixed thoroughly and piled up to 1.25-1.50 m height with a width ranging from 1.25 -1.5 m. The internal temperature of the compost should be maintained at 70-75^{0}C within 24hr. Second turning is done on this day where as third turning is done on 8th day with subsequent mixing of gypsum. On the 10th day, the compost is transferred to the pasteurization tunnel. Compost is filled in the pasteurization tunnel to a height of 7'. Filling height depends upon the size of the tunnel.

Phase II- Indoor composting: This is the pasteurization procedure which is done in a closed environment. Pasteurization has got many purposes.

i) If the temperature during composting has been low and the compost is heterogeneous, many parasites (nematodes, pathogens, flies and mites etc.) will survive in the compost mass, therefore, pasteurization is the best means with which these parasites can be destroyed.
ii) To end fermentation and to convert compost into a chemical and biological state favourable to the development of the mycelium and unfavourable to moulds.
iii) Conversion of ammonia into microbial protein.

Compost is filled in the pasteurization tunnel and as soon as the compost in the tunnel is completely filled the doors and fresh air damper are properly closed and blower is put on for recirculation of air @ 150-250 cubic metre/ 1000 kg compost/ hour. The phase II process is completed in three stages:

i) **Pre-peak heat stage:** After about 12-15 hours of compost filling, the temperature of compost starts rising and once 48-50^{0}C is obtained, it should be maintained for 36-40 hours with ventilation system. Normally such temperature is achieved by self generation of heat by the compost mass without steam injection.

ii) **Peak heat stage:** raise the temperature of compost to 57-58^{0}C by self generation of heat from microbial activity if it is not obtained. Injecting the live steam in the bulk chamber and maintain for 8 hours in order to ensure effective pasteurization. Fresh air introduced by opening of the fresh air damper to 1/6 or 1/4 of its capacity and air outlet too is opened to the same extent.

iii) **Post- peak heat stage:** lower down the temperature gradually to 48-52^{0}C and maintain till no traces of ammonia are detected in compost. This may take 3-4 days in a balanced formulation. When the compost is free from ammonia, full fresh air is introduced by opening the damper to its maximum capacity and cool down the compost to around 25^{0}C which is considered as the favourable temperature for spawning. Compost when ready for spawning should possess the following characteristics:

Moisture	About 68%
Ammonia	Below 0.006%
pH	7.2-7.5
Nitrogen	Around 2.5%
Fire fangs (Actinomycetes)	Excellent growth

Spawning: The process of mixing of the spawn in the compost is known as spawning. Spawn is thoroughly mixed in the compost at the rate of 600-750 gm per 100 kg of compost (0.6 - 0.75%). The spawned compost is filled in tray or polypropylene bags covered with formalin treated news papers. In case of bags, they should be folded at the top and covered up. After spawning, temperature and humidity of crop room should be maintained at 18-22^{0}C and 85-90%, respectively. Water should be sprayed over the covered news papers, walls and floors of the crop room. After 12-14 days of spawning white mycelial growth is seen running the entire length of the tray/bag. This is then covered with casing soil on the surface.

Qualities of a good spawn

- The spawn should be fast growing in the compost
- It should give early cropping after casing
- It should be high yielding
- It should produce better quality of mushroom

Casing: The significance of casing soil is to maintain the moisture content and exchange of gases within the surface of the compost which helps in the proper growth of the mycelium. The pH of the casing soil should be 7.5-7.8 and must be free from any infection or disease. In our country casing soil is prepared from the following ingredients.

Two years old manure + garden soil 3:1
Two year old manure + garden soil 2:1
Two year old manure + spent compost 1:1
Two year old manure + spent compost 2:1
Two year old manure + spent compost 1:2

Pasteurization of casing Soil: The casing soil is piled on cemented floor and is treated with 4% formalin solution. Thorough turning of the soil is done and it is covered with polythene sheet for the next 3-4 days. Pasteurization of casing soil at 65^0C for 6-8 hours is found to be much more effective

Using the casing soil: 3-4cm thick layer of casing soil is being spread uniformly on the compost when the surface has been covered by white mycelium of the fungus. Formalin solution (0.5%) is then being sprayed. Temperature and humidity of the crop room should be maintained at 14-18^0C and 80-85%, respectively. Proper ventilation should be arranged with water being sprayed once or twice a day

Harvesting of crop: Pin head initiation takes place after 10-12 days of casing and the fruiting bodies of the mushroom can be harvested for around 50-60 days. The crops should be harvested before the gills open as this may decrease its quality and market value.

Productivity: From 100 kg compost prepared by long method of composting 14-18 kg of mushroom can be obtained.

2. Short method of composting: Compost prepared by short method composting is superior in production quality and the chances of infection and disease is quite low.

Ingredients:

Wheat straw	1000 kg
Chicken manure	600 kg
Urea	15 kg
Wheat bran	60 kg
Gypsum	50 kg

This method is accomplished in two phases:

Phase I- Outdoor composting: Wheat straw mixed with chicken manure is sprayed with water and a 45cm high pile is made on the fourth day and first turning is made. On 7^{th} day, wheat bran, gypsum and urea is mixed thoroughly and piled up to 1.25-1.50 m height with a width ranging from 1.25 -1.5 m. The internal temperature of the compost should be maintained at 70-75^0C within 24hr. Second turning is done on this day where as third turning is done on 8^{th} day with subsequent mixing of gypsum. On the 10^{th} day, the compost is transferred to the pasteurization tunnel. Compost is filled in the pasteurization tunnel to a height of 7'. Filling height depends upon the size of the tunnel.

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Productivity: From 100 kg compost prepared by long method of composting 14-18 kg of mushroom can be obtained. Similarly, 18-20 kg mushroom can be obtained from pasteurized compost (Short Method Compost).