Practical Manual

SOIL BIOLOGY AND BIOCHEMISTRY

APS 506 3(2+1)

For

M.Sc. (Ag.) Soil Science

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Department of Soil Science

College of Agriculture

Rani Lakshmi Bai Central Agricultural University
Jhansi-284003

Practical manual

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CONTENT

Exercise No. 1

Objective: Preparation of Nutrient Growth Media

Principle: Media is the substance which provides nutrients for the growth of micro organisms such as bacteria, fungi (yeasts and molds) and actinomycetes. The Nutrients on which micro organisms are cultivated are called Culture Media. The Culture Media of microbiology is a mixed nutrient for the growth, reproduction and metabolism. Because microorganisms have different types of nutrition, different requirements for nutrients and different research purposes there are many kinds of culture media and different raw materials. The Culture media of microbiology has appropriate pH value, Buffer capacity, Redox potential and Osmotic pressure. Preparation and sterilization is needed in order to meet the specific requirements of research.

As a part of the practical, preparation methods of some commonly used Media explained here as following:

Requirements: Sterile conical flask/ Erlenmeyer flask, spatula, measuring cylinder, 1N HCl, 1N NaOH, pH strip, weighing scale

1) Nutrient Agar Media (for number of bacteria calculation):

Place butter paper on the weighing scale and transfer the required quantity of chemicals using a spatula for 1000ml of media as described below:

S. No.	Ingredient	Amount (litre^{-1})	250 ml
1	Peptone	5g	1.25g
2	Beef extract	3g	0.75g
3	Sodium chloride	5g	1.25g
4	Agar	18g	4.5g

Take clean and dry conical flask and add weigh quantity of Peptone, Beef extract and Sodium chloride and pour 500 ml of distilled water and dissolve the chemicals.

Add agar to the solution and mix it well. When all the constituents are dissolved add more distilled water and make up volume 1000ml. Maintain pH 7.0-7.2 with microprocessor based pH meter. Divide media equally in three separate clean sterile conical flasks of 500 ml capacity. Put them in autoclave and sterilize.

2) Potato Dextrose Agar Media (For fungi):

S. No.	Ingredient	Amount (litre-1)
1	Potato extracts*	200ml
2	Dextrose	20g
3	Agar	18g
4	Distilled Water	1000ml
5	pH	7.0-7.2

*__Potato extract:__ Peel off 200 g potatoes, cut into thin slices, add 250 ml of distilled water, autoclave for 30 minutes. Then cool and filter.

3) Pikovskaya Media (pH7.0-7.2):

S. No.	Ingredient	Amount (litre^{-1})
1	Glucose	10g
2	Tri calcium phosphate $Ca_3(PO_4)_2$/Hydroxy- apatite	5g (0.5%)
3	Ammonium sulphate $(NH_4)_2.SO_4$	0.5g
4	Sodium chloride (NaCl)	0.2g
5	Potassium chloride (KCl)	0.2g
6	$MgSO_4.7H_2O$	0.1g
7	$MnSO_4$	Trace
8	$FeSO_4$.	Trace
9	Yeast extract	0.5g
10	Agar	18g

4) Ken-Knight and Munaier's medium (For Actinomycetes)

S. No.	Ingredient	Amount (litre^{-1})
1	Dextrose	1g
2	Potassium di hydrogen phosphate	0.1g
3	Sodium nitrate	0.1g
4	Ammonium sulphate $(NH_4)_2.SO_4$	0.5g
5	Potassium chloride (KCl)	0.1g
6	$MgSO_4.7H_2O$	0.1g
7	Agar	15g

5) Martin's Rose-Bengal medium (for Fungi)

S. No.	Ingredient	Amount (litre^{-1})
1	Dextrose	10g
2	Peptone	5g
3	Potassium di hydrogen phosphate	1g
4	MgSO4.7H2O	0.5g
5	Rose-Bengal (1% solution)	3.3ml
6	Agar	18g
7	pH	6.0
8	Streptomycin sulphate (to be added after autoclaving and just before plating)	2.7 ml of 1:100 solution (1% aq. Solution)

6) Composition for Soil extract nutrient agar medium (for bacteria)

S. No.	Ingredient	Amount
1	Glucose	10g
2	K2HPO4	1g
3	Yeast extract	0.5g
4	Agar	18g
5	Soil extract **	200 ml
6	Distilled water	800 ml
7	pH	6.8-7.0

****Soil extract:** To 1000 g of a fertile soil add 1000 ml of tap water and autoclave for 20 minute. Add approximately 0.5 g $CaCO_3$ to flocculate colloidal material and filter to clarify.

Agar: also called agar-agar, gelatin-like product made primarily from the red algae Gelidium and Gracilaria (division Rhodophyta). It is a complex polysaccharide. It is highly valuable to microbiologists, although it provides no nutrient support for bacteria. It is commonly used in the laboratory setting primarily because of the following reasons: It remains solid under normal conditions and only a few microbes can degrade it.

Agar Slants: Solid growth surface: easier to store and transport than plates.

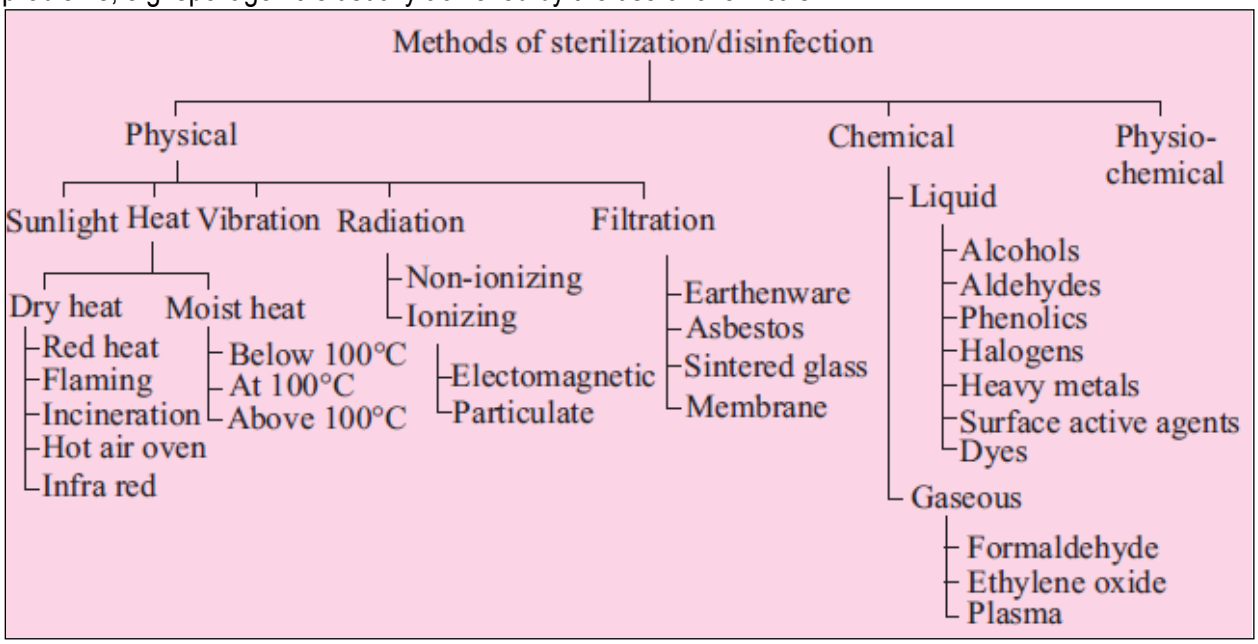
Exercise No. 2

Objective: Basic sterilization techniques

Sterilization: means the complete destruction of all the micro-organisms including spores, from an object or environment. Classical sterilization techniques using saturated steam under pressure or hot air are the most reliable and should be used whenever possible. Other sterilization methods include filtration, ionizing radiation (gamma and electron-beam radiation), and gas (ethylene oxide, formaldehyde). Specially, sterilization is a process of making an object free from all living organisms either by destroying or removing them from the object. This control of microorganisms is very important in microbiological research, preservation of food, prevention of diseases and in various industries.

Cleaning: is removal of visible soil (e.g., organic and inorganic material) from objects and surfaces. It is normally accomplished manually or mechanically using water with detergents or enzymatic products.

Disinfection: is the destruction, inhibition or removal of microbes that may cause disease or other problems, e.g. spoilage. It is usually achieved by the use of chemicals.



Autoclave: For sterilization, steam under pressure is generally employed using an instrument called autoclave. Autoclave can be used for sterilizing culture media, scalpel and other heat resistance instruments, glasswares, etc. but not for oils, powders and plastics. Autoclave was developed by *Chamberland* in 1884.

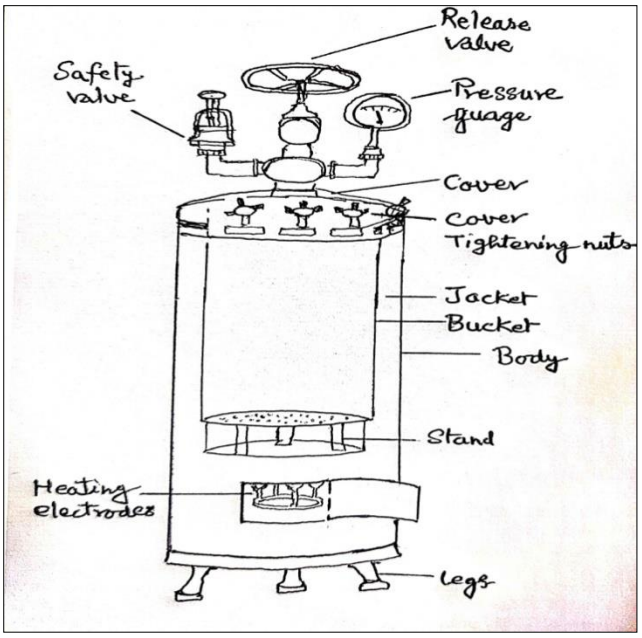


Figure : Autoclave

More the pressure, higher is the temperature and less is the time used for autoclaving. The autoclave cycle presented in Figure above illustrates this concept. Generally, pressure of 15 pounds with temperature at 121°C is employed for 15-20 minutes for autoclaving. Saturated steam heats an object about 2500 times more efficiently than dry heat at the same temperature. Steam condenses on the cooler surface of the object and transfers its heat energy to the object and sterilizes it.

Precautions:

1. Before using check is there enough/too much water at the bottom of the chamber?
2. Autoclave should not be packed tightly otherwise steam won't be able to come in contact with every object in the autoclave.
3. The air initially present in autoclave chamber should be removed before closing exhaust valve, otherwise temperature won't reach to 121°C though the pressure would be 15 pounds.
4. For larger sample of liquid load, autoclave time should be increased so that centre of the liquid should reach to 121°C.
5. After autoclaving, steam should be released slowly otherwise liquid media would come out.

Laminar Air Flow Cabinet:

This biological safety cabinet contains HEPA (high efficiency particulate air) filters which remove, 99.97% of the particles having size more than 0.3 µm. Air is forced through these HEPA filters and a vertical column of sterilize air gets formed across the cabinet opening. It prevents the contamination of room and the workers from microorganisms. The cabinet also has UV light, which is switched on about 15-20 minutes earlier before and after the work is finished to make the working surface sterile. Laminar flow hood is employed in the research labs for conducting assays, preparing media and culturing microbes.

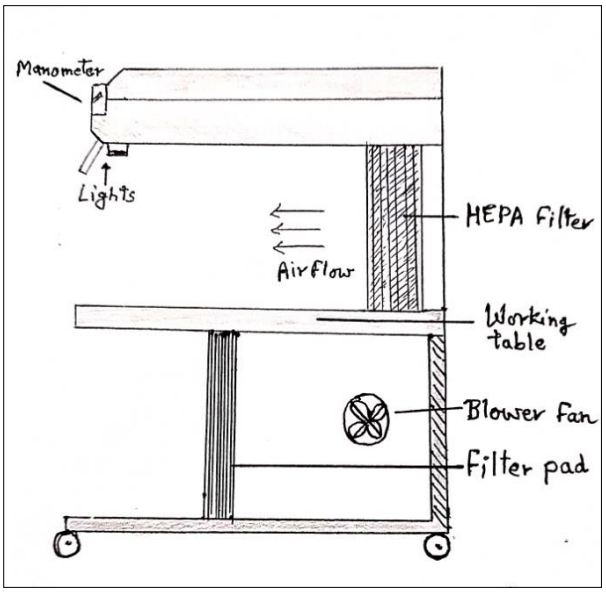


Figure : Laminar Air Flow Cabinet

During inoculation, inoculating wire is sterilized by heating in blue (hottest) portion of the flame till it becomes red hot followed by cooling either by touching to petri dish cover or inner surface of culture tube to avoid killing of microbial cells. Only a small amount of culture is used for inoculation. This can be accomplished by touching a single colony on agar surface by inoculating needle or by taking a loopful of culture from broth medium. After inoculation, inoculating wire is again sterilized to destroy remaining microorganisms.

Storage and incubation of petri plates should be done in an inverted position to prevent dropping down of water droplets formed by condensation during solidification of media on to the surface of the hardened agar surface. This can spread the microorganisms on the agar surface, resulting in confluent growth instead of discrete colonies. Petri dishes containing fungi do not need to be inverted.

Pipetting refers to the process of measuring and transferring small volumes of liquid using a device called a pipette.

Precautions:

1. Never do pipetting with mouth.
2. For culturing, sterilized pipettes should be used.
3. Never keep pipettes on working surfaces.

Exercise No. 3

Objective: Study of rhizosphere effect (R:S ratio): Isolation and enumeration of microbial population.

Procedure:

1. To analyze a plant's soil, uproot the target plant from its field and shake the soil particles closely adhering to its roots. The non-rhizosphere soil sample can come from anywhere outside of the target plant's field. Avoid storing the soil sample for a long period of time, and do not freeze it. If stored, keep it below room temperature in a plastic pouch to prevent it from drying out. Incubate it at room temperature for 24 hours prior to analysis.
2. Prepare serial dilutions for plating of the soil within the Laminar- flow assembly. Add 10 g soil sample in 95 ml sterile water blank in 500 mL conical flask fitted with rubber stopper. Vigorously shake to disperse soil thoroughly by giving vertical strokes. Striking the soil with vigor will disperse it thoroughly by making vertical strokes (at least 20 times). This establishes 1:10 or 10^{-1} dilution.
3. To get accurate diluted soil samples, it is important to shake all the soil samples evenly.
4. Transfer 1 ml soil suspension obtained in the previous step into 9 mL sterile water blank in the test tubes. Shake uniformly by rolling the tube between palms of your hands to provide horizontal shaking. Continue the series in similar manner to get up to 10^{-6} dilution level. Mark the dilutions properly on the test tubes.
5. Transfer 1 ml of required dilution (10^{-4} for fungi and actinomycetes and 10^{-5} to 10^{-6} for bacteria) into sterile Petri plates.
6. Pour the required media (at approx. 45°C) uniformly for specific organisms into petri-plates. Rotate clockwise and anti-clockwise to mix soil suspension with medium. Make the rotation uniform for all samples. Allow the medium to solidify. Mark details on the lid. (Sample no, Dilution, Date of plating etc.).
7. Incubate plates in inverted position at 28°C in incubator for 2 days for fungi; 4 days for bacteria and one week for actinomycetes. Take at least four replications for each sample.
8. Examine nature of colonies developing on plates after the required incubation period. Count the number of colonies. Discard plates showing large spreaders and mold colonies. Number of colonies multiplied by the respective dilution factor will give number of viable cells g^{1} soil. All the steps of serial dilution are schematically represented in next exercise.
9. Ascertain the per cent moisture in soil samples by gravimetric method. Express results on the basis of oven dry weight of soil sample.
10. Record number of colonies per plate in given tabular form.
11. Calculate standard deviation and standard error of mean. Statistically compare the number of organisms in the rhizosphere and non-rhizosphere soil samples.
12. Calculate rhizosphere effect in terms of R:S ratio (R/S).

Observation:

Organism:			Medium Used:			Sample no.
Soil sample		Dilution factor	Moisture Content (%)	No. per g dry soil	Average Population	R:S ratio
Rhizosphere						
	a.					
	b.					
	c.					
	d.					
Non-Rhizosphere						
	a.					
	b.					
	c.					
	d.					

Exercise No. 4

Objective: Isolation and enumeration of microorganisms from soil by the serial dilution–agar plating method (or viable plate count method).

Principle of Serial dilution method: It is a method of diluting a stock solution where concentration decreases by the same quantity in each successive step.

It is one of the commonly followed procedures for isolation and counting of fungi, bacteria and actinomycetes which are the most prevalent microorganisms. This method is based upon the principle that when material containing microorganisms cultured each viable microorganism will develop into a colony, hence the number of colonies appearing on the plate represent the number of living organisms present in the sample.

In serial dilution agar-plate method a known amount (10 ml or 10 g) of material is suspended or agitated in a known volume of sterile water blank(90 ml or so to make the total volume to 100 ml)to make a microbial suspension. Serial dilutions 10^{-2},10^{-3},....10^{-7} are made by pipetting measured volumes(usually 1ml or 10ml)into a additional dilution blanks(having 99 ml or 90 ml sterile water). Finally 1 ml aliquot of various dilutions are added to sterile petri dishes(triplicate for each dilution) to which are added 15 ml (approximately) of the sterile, cool molten (45ºC) media(Nutrient agar for bacteria, Glycerol yeast agar for actinomycetes and Czapek-Dox agar or Sabouraud agar medium, supplemented with antibiotics (like chlorotetracycline or streptopenillin, 10µg/ml, for fungi).

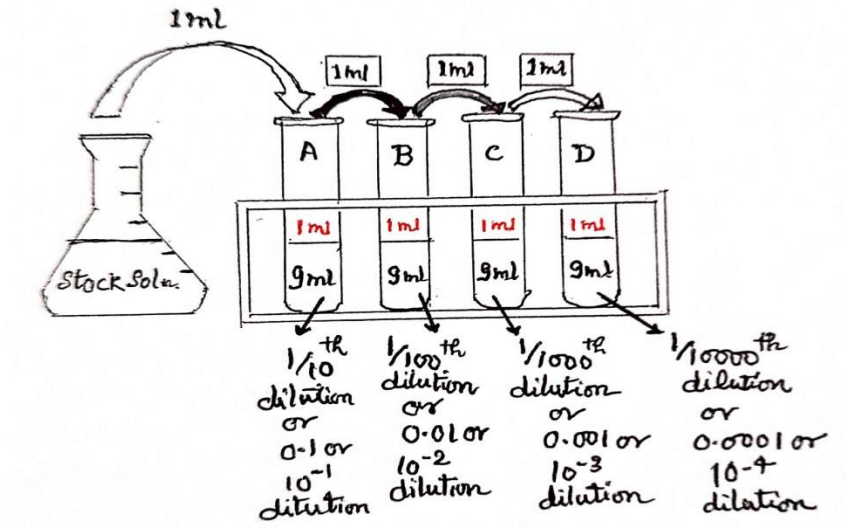


Figure: Serial Dilution Preparation

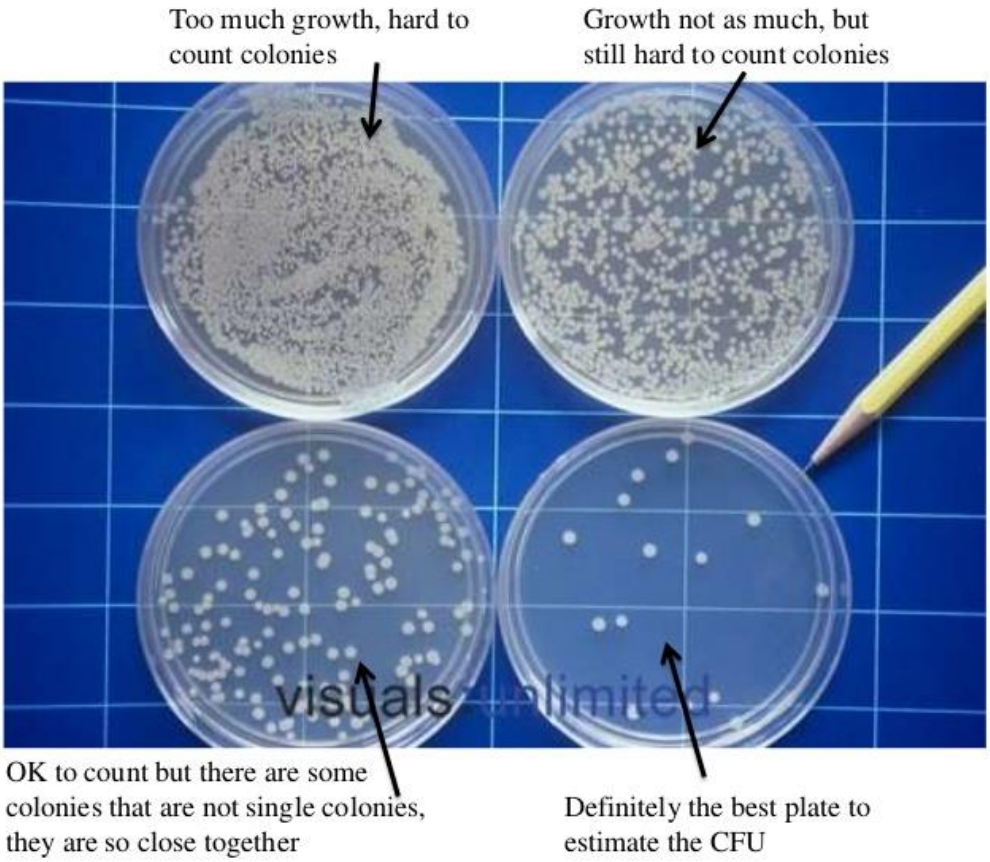
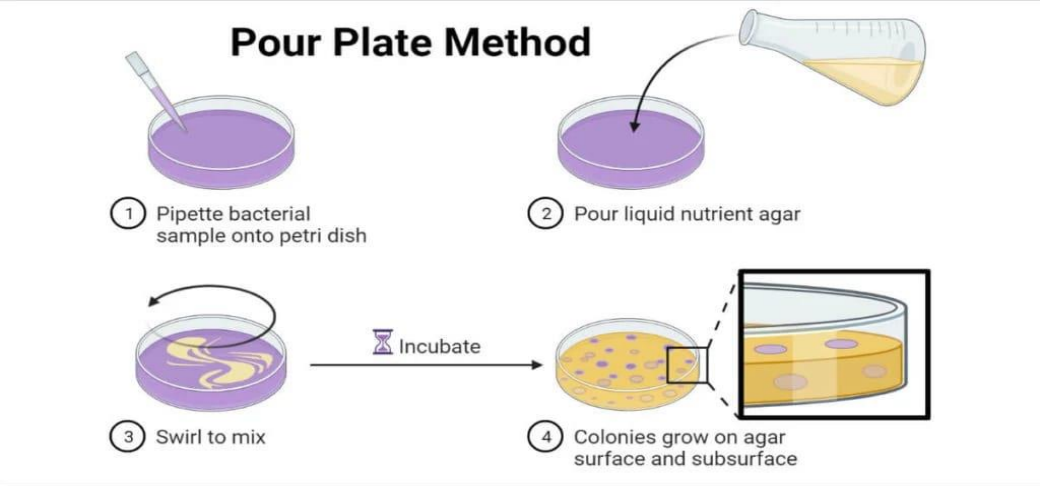


Figure: Colonies count

POUR PLATE: A pour plate is prepared by mixing a small amount of bacterial culture with molten nutrient medium. Pour plates allow microorganisms to grow both on the surface and within the medium.



The purpose of a Streak Plate is to dilute the small amount of bacterial culture put on the plate down to individual cells; each of which will form an isolated pure colony during incubation.

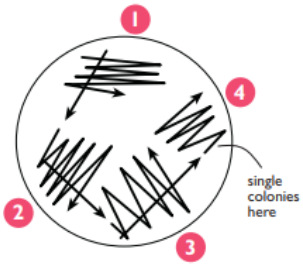


Figure: Streak Plate

After inoculation, use self-adhesive tape to seal the Petri dishes as shown below. Do not seal the plate entirely, as this will create anaerobic conditions within the dish.

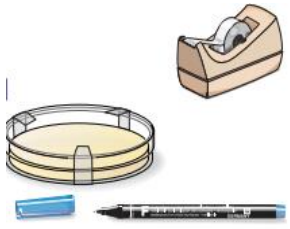


Figure: Adhesive tape sealing

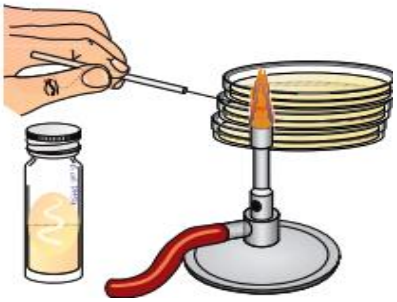


Figure: Sterilization of inoculating loop

Dip a glass spreader in alcohol and pass it through a Bunsen flame. The alcohol will burn and sterilize the glass. Keep the alcohol beaker away from the Bunsen flame. Place the spreader onto the surface of the inoculated agar and, rotating the dish with the left hand move the spreader in a top-to-bottom or a side-to-side motion to spread the inoculum over the surface of the agar. This will result in a culture spread evenly over the surface of the growth medium.

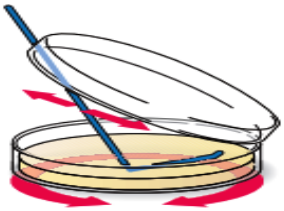


Figure: Use of Spreader

Exercise No. 5

Objective: Isolation of Rhizobium from Root nodules.

Protocol:

1. Dissolve weighed amounts of all the constituents of YEMA medium, constituents of which are as follows: K_2HPO_4 0.5 g, $K_2SO_4.7H_2O$ 0.2 g, NaCl 0.1 g, mannitol 10.0 g, yeast extract 1.0 g, Agar 20.0g, Congo red 2.5 ml, Distilled water 1000.00 ml (except K_2HPO_4 which is to be dissolved separately) in distilled water and mix in the agar medium. Make the volume to 1000 ml and autoclave it. Congo red solution is to be sterilized separately and added to the medium at the time of pouring in Petri plates.
2. Uproot roots of leguminous plants and bring to the laboratory.
3. Wash the root system in running tap water to remove adhering soil particles.
4. Select healthy pink, unbroken and firm root nodules and wash in water.
5. Immerse the nodules in 0.1%$HgCl_2$ or 3-5% H_2O_2 for 5 minutes to surface sterilize these.
6. Repeatedly wash the nodules in sterile water for 3-5 times to get rid off the sterilizing agent.
7. Place the nodules in 70% ethyl alcohol for 3 minutes (if treated with $HgCl_2$).
8. Repeatedly wash the nodules in sterile water.
9. Crush a nodule in 1 ml of water with a sterile glass rod.
10. Make a uniform suspension of Rhizobium with water.
11. Make sterile dilutions of the nodules extract as outlined in the figure shown below.
12. Spread 1 ml, each of suspension from various dilutions, on YEMA plates.
13. Incubate the plates at 26ºC for a week.

Precautions during Rhizobium isolation:

1. Pinkish nodules should be selected for isolation.
2. Nodules should be surface sterilized properly and should be free from all the surface borne microorganisms.
3. Sterilizing agent must be got rid off before nodules are used for isolation.
4. Suspension of bacterium be poured on pre plate YEMA medium.

Observations: observe the plates after 3-4 days incubation and regularly afterwards for the development of Rhizobium colonies.

Results: Large gummy colonies of Rhizobium will appear on YEMA plates within 4-5 days. The characteristics β-hydroxybutyrate granules are seen as stained objects within the cell when smears stained to carbon fuchsin stain are examined.

Wash root system in running tap water

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Selection and washing of healthy root nodules

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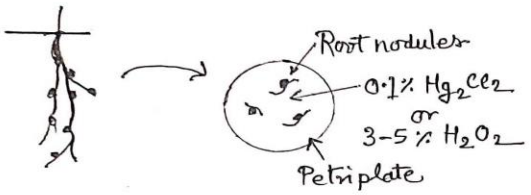
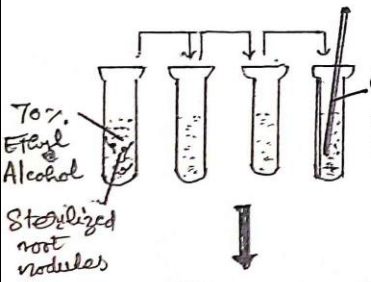
Surface sterilization of healthy root nodules



Washing and crushing of root nodules



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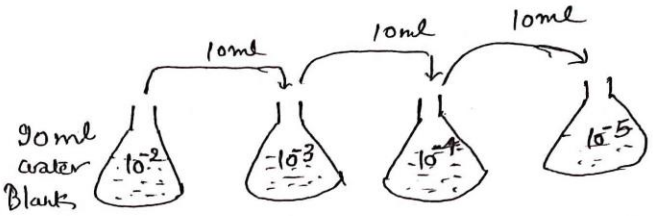
Preparation of Serial dilutions



Transfer of 1ml suspension into each yeast extract Mannitol Agar (YEMA plates)

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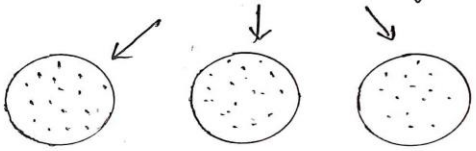
Incubation at 26°C for 7 days



Observe for appearance of Colonies of rhizobia on YEMA plates

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Appearance of large gummy Colonies of rhizobia after 4-5 days

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Transfer large gummy colonies onto Agar slants

Exercise No. 6

Objective: Isolation of Azotobacter from soil.

Principle/Protocol:

1. Preparation of Ashby's medium** of the following constituents
   Mannitol 20.0g, K_2HPO_4 0.2 g, $MgSO_4.7H_2O$ 0.2 g, NaCl 0.2 g, K_2SO_4 0.1 g, $CaCO_3$ 5.0 g, Agar 15.0g, Distilled water 1000.0 ml
2. Dissolved the weighed amount of mannitol, $MgSO_4.7H_2O$, NaCl, K_2SO_4 and $CaCO_3$ in 200-ml of distilled water in a 250 ml flask. In another flask dissolve K_2HPO_4 in 100 ml of distilled water. Mix all the constituents and make the volume to 1000.0 ml by the addition of more distilled water. Pour it into flasks and autoclave the medium at 121ºC for 15 minutes.
3. Pour the autoclaved, cooled (45 ºC) medium into sterile Petri plates.
4. Allow the medium to solidify.
5. Add 10 g of sieved (2mm) soil to 90 ml sterile water blank and shake it for 15-20 minutes on a magnetic shaker.
6. Prepare serial dilutions 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} as outlined in the earlier experiment.
7. Add 1 ml aliquots of various dilutions over cooled and solidified agar medium in Petri plates.
8. Rotates the plates for uniform distribution of spores.
9. Incubate the plates at 28 ºC for 3 days.

Observations: Observe the plates after 3 days of incubation for appearance colonies onto the agar surface.

Result: The Azotobacter colonies appear flat, soft, milky and mucoid.

****Another medium for Azotobacter : Jensen's medium** (sucrose 10 g, dipotassium hydrogen phosphate 1 g, magnesium sulfate 0.5 g, sodium chloride 0.5 g, ferrous sulfate 0.1 g, sodium molybdate 0.005 g, agar 20 g, for 1 liter, pH 7.0-7.2).

Precautions:

1. Always use cooled and set agar medium for pouring of suspensions.
2. Evenly spread the suspension over the medium.

Exercise No. 7a

ENZYME ACTIVITY DETERMINATION

Objective: Determination of Fluorescein-diacetate enzyme activity

Fluorescein-diacetate activity (FDA) hydrolysis is widely accepted as an accurate and simple method for measuring total microbial activity in a range of soils. FDA hydrolysis activity measurement was made following the method of Adam and Duncan (2001).

Principle: The Fluorescein-diacetate (FDA) hydrolysis assay measures the enzyme activity of microbial populations and can provide an estimate of overall microbial activity in an environment sample. The assay is considered non specific because it is sensitive to the activity of several enzyme classes including lipases, esterases and proteases. Activity of these enzymes results in the hydrolytic cleavage of FDA (colorless) into fluorescein (fluorescent yellow-green). In the FDA assay, the environmental sample is mixed with FDA and buffer an incubated with shaking for 1-2h. The intensity of the resulting yellow green colour is indicative of the amount of enzymatic cleavage of the FDA molecule and the overall enzymatic activity in the sample. Quantification of enzyme activity is performed by assessing the intensity of colour formation using spectrometry.

Apparatus: Volumetric flasks, conical flask, centrifuge tubes (50 ml), centrifuge machine, incubator, spectrophotometer

Reagents:

1. Potassium phosphate buffer: (60 mM), pH 7.6: Dissolve 8.7 g K_2HPO_4 and 1.3 g KH_2PO_4 in 800mL double distilled water and makeup volume to 1 litre. store the buffer in fridge at 4^oC and check the pH on the day of use.
2. Chloroform + Metanol (2:1): Add 666mL chloroform to 1litre volumetric flask, makeup the volume to 1L with methanol and mix thoroughly.
3. Fluorescein diacetate (FDA) stock solution (1000 µg mL^{-1}): Dissolve 0.1 g 3'6'-diacetyl-fluorescein in 80 ml acetone and makeup the volume o 100 ml with acetone. Store the solution at -20^oC.
4. Fluorescein working solution (50 µg ml^{-1}): Add 2.5 ml of stock solution (2000 µg fluorescein ml^{-1}) to 100 ml with 60mM potassium phosphate buffer (pH 7.6).
5. Calibration standards: Pipette 0 (reagent blank), 0.5,1.0, 1.5, 2.0 and 2.5 mL of fluorescen working standard (50 µg mL^{-1}) solution into 25 mL volumetric flasks and makeup the volume with 60mM potassium phosphate buffer. These solutions correspond to 0, 1, 2, 3, 4 and 5 µg fluorescein ml^{-1}.

Procedure:

1. Take 2 g of field moist soil in 50 mL conical flask. Take another conical flask without soil to prepare a control.
2. Simultaneously, place the fiel moist soil in aluminium box and keep it in oven at 105^oC for 24-48 hrs to determine the dry weight of soil.
3. Add 15 mL of 60 mM potassium phosphate buffer (pH 7.6) and 0.2 mL of stock solution of FDA (1000 µg mL^{-1}) as substrate in the conical flasks to start the reaction.

4. Stopper the flasks and shake the contents well then incubate the flasks in an incubator at 30 ^{o}C for 20 minutes.
5. Immediately after incubation, add 15 mL of chloroform/methanol (2:1 v/v) solution to terminate the reaction.
6. Then transfer the contents of the conical flasks to 50 ml centrifuge tubes and centrifuge at 2000rpm for 3 minutes.
7. Filter the supernatant with Whatman No 41 filter paper into 50 ml conical flasks.
8. Measure the intensity of yellow- green colour of the filtrates at 490 nm in a spectrophotometer.

Calculation: FDA activity (µg fluorescein g^{-1} h^{-1}) = (S-C)*60/20*w

S = Concentration of FDA in sample as calculated from the calibration curve

C= Concentration of FDA in control as calculated from the calibration curve

w = Dry weight of soil (g) obtained from oven dying of the fresh soil

Exercise No. 7b

Objective: Dehydrogenase Activity determination

Dehydrogenase is ubiquitous in all intact viable microbial cells and is thus widely used as the total microbial activity in soil. The dehydrogenase activity is commonly used as an indicator of biological activity in soils. The activity of dehydrogenase enzyme in soil is important to determine as it may give indications of the potential of the soil to support biochemical processes which are essential for maintaining soil fertility. Biological oxidation of organic compounds is generally a dehydrogenation process.

Principle:

Dehydrogenase activity was measured by the method given by Casida *et al.,* (1964). The method involves colorimetric determination of 2,3,5-triphenyl formazan (TPF) produced by the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) by soil microorganisms. Tetrazolium salts are quaternary NH_4^+ salts and as such possess a high degree of water solubility. The solubility of TTC is sufficiently great to allow the salt to be used in a water solution. This colorless and pale colored tetrazolium salt possess the property of being easily transferred into colored, water insoluble, methanol soluble formazan, which makes this compound act as an electron acceptor.

Apparatus: Some test tubes with 2 cm diameter and a minimum volume of 30 ml, incubator, Spectrophotometer

Reagents:

1. Calcium carbonate (CacO3, reagent grade)
2. 2,3,5 Triphenyltetrazolium chloride (TTC 3%): Dissolve 3 g of TTC in about 80 mL of distilled water and adjust the volume to 100 mL with distilled water.
3. Methanol (analytical grade)
4. Triphenyl formazan (TPF) standard solution (1000 µg mL^{-1}): Dissolve 100 mg of TPF in about 80 mL of methanol,and makeup the volume to 100 mL with methanol, Mix thoroughly.
5. Calibration standards: Pipette 0 (reagent blank), 2.5, 5.0, 12.5 and 25mL of TPF standard solution into 25 mL volumetric flask and make up the volume with acetone. These solutions correspond to 0,100,200,500 and 1000 µg TPF mL^{-1}.

Procedure:

1. Take 1 g of field moist soil in test tubes. Take another test tube without soil to prepare a control.
2. Simultaneously, place the field moist soil in aluminium box and keep in oven at 105°C for 24-48 h to determine the dry weight of soil.
3. Add 0.2 g of $CaCO_3$, 1 ml of 3% 2,3,5-triphenyltetrazolium chloride (TTC) and 2.5 ml of distilled water in the test tubes.
4. Swirl the test tubes content and place them in an incubator with stopper at 37°C for 24 h.
5. After 24 h remove the stoppers, add 10 ml of methanol and shake it for few seconds.
6. A red color will appear due to the production of 2,3,5-triphenyl tetrazolium chloride in to triphenyl formazan (TPF).

7. Filter the suspension into a 100 mL of volumetric flask. Wash the tubes repeatedly with methanol and quantitatively transfer the supernatant until reddish color disappears.
8. Dilute the filterate with methanol 25 mL.
9. Measure the intensity of the red color in a spectrophotometer at 485 nm.

Calculation:

$$\text{Dehydrogenase activity } (\mu g \text{ TPF } g^{-1} d^{-1}) = (S-C)/w * 100 \text{ (dilution factor)}$$

S= Concentration of TPF in samples as calculated from the calibration curve.

C= Concentration of TPF in control as calculated from the calibration curve.

w= Dry weight of soil (g) obtained from oven drying of the fresh soil.

Exercise No. 7c

Objective: β glucosidase Activity determination

β Glucosidase is a glucosidase enzyme that acts upon β 1-4 bonds linking two glucose or glucose-substituted molecules (i.e., the disaccharide cellobiose) and hydrolyze carbohydrates with a β-D- glycoside bond by splitting off the terminal β-D- glucose. This group of enzymes includes, among others, the cellobiase, gentobiase, aryl-β glucosidase and salicinase. β Glucosidases play an important role in the total degradation of cellulose to glucose. Eno-and exo- β-1, 4-glucanases hydrolyze cellulose to cellobiose, which competitively inhibits this process. Cellobiose is converted to glucose through the activity of β-glucosidases. Cellulose degradation is thus promoted and the organisms are simultaneously supplied with an important energy source.

Principle: β glucosidase activity was measured by the method given by Eivazi and Tabatabai (1988). The method of β glucosidaseassay is based on colorimetric determination of p-nitrophenol released by β glucosidase when soil is incubated with buffered (pH 6.0) p-nitrophenyl β-D-glucoside (pNG) solution and toluene.

Apparatus: Volumetric flasks (1L, 100 mL and 50 mL); some conical flasks (50 mL); Some beaker (500 mL), magnetic stirrer, incubator, spectrophotometer

Reagents:

1. Toluene
2. Hydrochloric acid solution (0.1 N) : Take 8.33 mL of Conc HCl (12 N) in 1 litre volumetric flask and dilute to 1 litre with distilled water.
3. Sodium hydroxide solution (NaOH, 0.5 M): Dissolve 20g of sodium hydroxide in water and make final volume to 1litre.
4. Modified universal buffer (MUB) stock solution: Dissolve 12.1 g of Tris hdroxymethyl aminomethane (THAM), 11.6 g of Maleic acid, 14.0 g of Citric acid, 6.3 g of Boric acid (H_3BO_3) in 488 mL of 1 N sodium hydroxide (NaOH) and makeup the volume to 1 litre with distilled water. Store it in a refrigerator.
5. Modfied universal buffer (MUB) wrkin solution ph 6.0: Take 200 mL of MUB stock solution in a 500 mL beaker containing a magnetic stirring bar and place the beaker on a magnetic stirrer. Titrate the solution to pH 6.0 with 0.1 N HCL and adjust the volum to 1 litre with distilled water.
6. Standard p nitrophenol solution (1 mg p-nitrophenol mL^{-1}): Dissolve 1.0 g of p-nitrophenol in about 700 mL of water and dilute the solution to 1 litre with distilled water. Store the solution at 4^oC in a refrigerator.

Procedure:

1. Take 1 g of field moist soil in a 50 ml conical flask. Take another conical flask without soil to prepare a control.
2. Simultaneously, place the field moist soil in aluminium box and keep in oven at 105^oC for 24-48hrs to determine the dry weight of soil.

3. Add 0.25 ml toluene, 4 ml of modified universal buffer (MUB; pH 6.0) and 1 ml of p-nitrophenol – β-D-glucoside (pNG; substrate of the reaction). Do not add pNG solution to the control.
4. Swirl the flask to mix the contents properly.
5. Stopper the flask and place it in incubator at 37^{o}C for 1 h.
6. After 1 h remove the stopper and add 1 mL of 0.5 M $CaCl_2$, swirl and then add 2 mL of 0.1 M THAM (pH 12) to the contents to stop the reaction.
7. Swirl the flasks for few seconds and then filter the soil suspension through Whatman No. 41 filter paper.
8. For controls, follow procedure but make the addition of 0.5 mL $CaCl_2$ and 2 ml of TAM buffer pH 12 (i.e., just before filtration of the soil suspension).
9. For making calibration curve, dilute 1 ml of standard p-nitrophenol solution to 100 ml in a volumetric flask using distilled water (10 µg ml^{-1} solution). Pipette 0,1,2,3,4 and 5 ml by addition of distilled water (this is equivalent to 0,2,4,6,8 and 10 µg of p-nitropenol). Now, add 1 ml of 0.5 M $CaCl_2$ and 4 ml of THAM buffer pH 12.Filter the suspension.
10. Measure the intensity of the yellow color of filterate at 420 nm in a spectrophotometer.

Calculation:

$$\beta\text{- glucosidase activity } (\mu g \text{ pNP } g^{-1}g^{-1}) = (S-C)/w$$

S=Concentration of pNP in samples as calculated from the calibration curve
C= Concentration of pNP in control as calculated from the calibration curve
w= Dry weight of soil (g) obtained from oven dying of the fresh soil

Points to watch:

If color intensity for a sample> 10 µg of p-nitrophenol standard, dilute sample to within the limits of standard curve using THAM pH 10.

Exercise No. 7d

Objective: Urease Activity determination

Urea is one of the most widely used nitrogenous fertilizers in agriculture throughout the world today. The conversion of organic nitrogen through hydrolysis of urea to ammonia and CO_2 is an essential part of the nitrogen cycle and represents an import pathway in soils receiving application of urine and dung. Rapid hydrolysis of urea in soils due to activity of urease leads to accumulation of ammonium which undergoes volatilization under alkaline conditions.

Principle: The method is designed for estimation of the rate of urea hydrolysis in soil. It involves colorimetric determination of the urea remaining after incubation at 37°C for 5h of soils with urea solution (Tabatabai Bremner, 1972). The amount of urea hydrolyzed g^{-1} of soil 5 h^{-1} is estimated from the difference between the initial amount of urea added and that recovered after incubation.

Apparatus: Some volumetric flasks (1l, 500ml, 100 ml and 50 ml), incubator, water bath, spectrophotometer

Reagents:

1. Urea substrate solution: Dissolve 2 g of urea in about 700 mL of distilled water and adjust the volume to 1 L with distilled water. This solution contains 2 mg of urea mL^1. Store this solution in a refrigerator.
2. Phenylmercuric acetate (PMA) solution: Dissolve 50 mg of PMA in 1L of distilled water.
3. Potassium chloride-phenylmercuric acetate solution (2M KCl-PMA): Dissolve 1490 g of reagent grade KCl in 7 L of distilled water, add 1L of PMA solution and make up the volume to 10L using distilled water.
4. Diacetylmonoxime (DAM) solution: Dissolve 2.5 g of DAM in 100 mL of distilled water.
5. Thiosemicarbazide (TSC) solution: Dissolve 0.25 of TSC in 100 mL of distilled water.
6. Acid reagent: Add 300 mL of 85% phosphoric acid (H3PO4) and 10 mL of concentrated sulfuric acid (H2SO4) to 100 mL of water and makeup the volume to 500 mL with distilled water.
7. Color reagent: Prepare this reagent immediately before use by adding 25 mL of Dam solution and 10 mL of TSC solution to 500 mL of acid reagent.
8. Standard urea stock solution for standard curve: Dissolve 0.5g of urea in about 1500 mL of 2 M KCl-PMA solution and dilute to 2L with the same solution. If pure, dry urea is used, this solution will contain 250 µg of urea mL^{-1}. Store this solution in a refrigerator.
9. Calibration standard solutions: Take 0 (reagent blank), 10,20, 40, 60 and 80 mL of standard urea stock solution in 100 mL volumetric flasks and make up the volume with distilled water. This will give 0, 25, 50, 100, 150, and 200 µg mL^{-1} solutions.

Procedure:

Take 20g field moist soil in 100 ml conical flasks and add 20 mL of urea solution (40 mg of urea).

1. Stopper the flasks and incubate at 37 ^oC. After 5 h remove the stopper and add 200 ml of 2 M KCl-PMA solution.

2. Stopper the flasks and shake it for 1 h.
3. Filter the soil suspension.
4. Take 1-2 ml of extract (contain upto 200 µg of urea). Add 10 ml of 2M KCl-PMA solution and add 30 ml of colour reagent.
5. Shake the flask for ten seconds to mix. Place it in a boiling water bath for 30 min.
6. Cool the flask immediately in running water for 15 min.(this can be accomplished by placing the flask in a deep tray containing cold water 12-20^{o}C).
7. Then make up the volume to 50 ml with distilled water and mix thoroughly.
8. To prepare the calibration curve, treat 1 ml of calibration standard like the soil filtrates. Calibration standards contain 0, 25, 50, 100, 150 and 200 µg urea ml^{-1}.
9. Measure the intensity of used color produced in spectrophotometer at 527 nm.

Calculation

$$\text{Urease activity (µg urea hydrolysed } g^{-1}h^{-1}) = (U_i - U_f)/T_i * w$$

U_i= Initial concentration of urea added in samples

U_f = Final concentration of urea left in the sample as calculated from the calibration curve

Ti= Time of incubation (h)

w= Dry weight of soil (g) obtained from oven drying of the fresh soil.

Exercise No. 7e

Objective: Acid and Alkaline Phosphatases activities determination.

In most soils, the organically bound P-fraction is higher than the inorganic. Among the organic phosphoric acid esters, the largest fraction in the soil is phytanic acid or phytin. Phosphorus uptake by plants requires mineralization of the organic P-component by phosphatases to orthophosphate. Extracellular phosphatases are produced by microorganisms and roots (microbial phosphatases dominate in soils) and contribute to the mineralization of organic P. Phosphatases are inducible enzymes that are produced predominantly under conditions of low phosphorus availability.

Principle: The activity of acid–and alkaline phosphatase is measured by the method of Tabatabai and Bremner (1969). The procedures described for assay of phosphomonoesterases (acid &alkaline Phosphatase) activities are based on colorimetric estimation of the p-nitrophenol released by phosphatase activities when soil is incubated with buffered sodium p-nitrophenyl phosphate solution and toluene (pH 6.5 for acid phosphatase activity and pH 11 for alkaline phosphate activity). The colorimetric procedure used for estimation of p-nitrphenol based on the fact that alkaline solutions of this phenol have a yellow color (acid solution of p-nitrophenol and acid and alkaline solution of p-nitrophenyl phosphate are colorless). The $CaCl_2$-NaOH treatment described for extraction of p-nitrophenol after incubation for assay of acid and alkaline phosphatases serves. There are 3 steps in this test:

1. To stop phosphatase activity
2. To develop the yellow color acid to estimate the phenol
3. To give quantitative recovery of p-nitrophenol from soils.

Apparatus: Some volumetric flasks (1litre, 500 ml, 100ml and 50 ml), some conical flasks (50ml capacity), incubator, water bath, spectrophotometer.

Reagents:

1. Toluene
2. Modified universal buffer (MUB) stock solution: Dissolve 12.1 g of tris hydroxymethyl aminomethane (THAM), 11.6 g of maleic acid, 14.0 g citric acid 6.3 g of boric acid (H_3BO_3) in 488mL of 1N sodium hydroxide (NaOH) and makeup the volume to 1litre with distilled water. Store it at 4^oC in a refrigerator.
3. Modified universal buffer (MUB) pH 6.5 and pH 11.0: Place 200 ml of MUB stock solution in a 500ml beaker containing a magnetic stirring bar and place the beaker on a magnetic stirrer. Titrate the solution to pH 6.5 with 0.1NHCl or to pH 11 by 0.1 N NaOH. Make up final volume to 1 litre.
4. Hydrochloric acid solution (0.1N): Take 8.33 ml of conc. HCl (12N) in 1L volumetric flask and dilute to 1 litre with distilled water.
5. Calcium chloride solution ($CaCl_2$, 0.5N): Dissolve 73.5 g of $CaCl_2.2H_2O$ in about 700 ml of distilled water and make final volume to 1 litre.
6. Sodium hydroxide solution (NaOH, 0.5 M): Dissolve 20g of NaOH in bout 700mL of distilled water and dilute the volume to 1 litre.

7. Standard p-nitrophenol solution (1000ppm): Dissolve 1g p-nitrophenol in about 70 ml of water and dilute solution to 1 litre.

Procedure:

1. For each soil take two sets of 1g oven dry soil (<2mm) in 50ml conical flasks. Out of there two sets, one will be used as control.
2. Add 0.2ml touline and 4 ml of MUB (pH 6.5 or 11) to all flasks.
3. Add 1ml p-nitrophenyl, phosphate solution to only oneset of samples.
4. Stir the flasks of both the sets for few seconds to mix the contents. Stopper them and place in a incubator at 37°C for 1 hour.
5. After incubation, remove stopper and add 1ml of 0.5M $CaCl_2$ and 4 ml of 0.5M NaOH. Swirl the flasks for few seconds.
6. Add 1 ml of p-nitrophenyl phosphate to remaining set (control) of samples.
7. Filter sample/suspension quickly through Whatman no 2 filter paper. Measure yellow color intensity of filtrate with a blue filter at 440nm.

Preparation of standard curve

1. Dilute 100 ml of standard p-nitrophenyl to 100ml (10 ppm solution): Pipette 0, 1, 2, 3, 4 and 5ml aliquots into 50ml conical flasks. Adjust volume to 5ml distilled water.
2. Add 1ml of 0.5M $CaCl_2$ and 4ml of 0.5M NaOH. Swirl the flask for few seconds.
3. Filter all the suspensions, quickly through Whatman No-2 filter paper. Measure the yellow color intensity of the filtrate with the filter or at 440 nm.

Conclusion:

Phosphomonoesterases activity is expressed on µg p-nitrophenyl released g^{-1} soil hr^{-1}.

Exercise No. 8

Objective: Study of compound microscope / bright field microscope

A microscope may be defined as an optical instrument consisting of lens or combination of lenses for enlarging or magnifying images of minute objects. Compound microscope differs from a simple one. In that it consists of two sets of lenses – one known as objectives and other as ocular or eye – piece.

A. Study of parts of microscope

Mechanical parts:

1. **The body with its draw tube**: It is a metallic part and draw tube is provided with a millimeter scale, which shows the total length of the working tube.
2. **Stage**: It is a platform which accommodates the microscopic glass slide on which the object to be examined is mounted. It has an aperture in the center to permit light. The stage may be fitted with two metal clips, which are of mechanical type where by the slides can be moved by rack and pinion adjustment.
3. **Coarse adjustment**: It is a metallic part of the microscope, which is big circular screw, it moves the nose - piece up and down rapidly for approximate focusing.
4. **Fine adjustment**: It is the metallic part of the microscope (small screw) which move the nose - piece very slowly for definite sharp focusing.
5. **Nose-piece**: It bears different objective lenses and can be rotated to change from one objective to another according to the requirement.
6. **Arm**: It resembles English letter 'C', supports the upper half of the microscope.
7. **Base:** This is horse – shoe shaped metallic part and supports the entire microscope.
8. **Irish diaphragm**: This is made of metal and controls the amount of light striking the object. It can be opened and closed with the lever.
9. **Eyepiece**: It is an optical part of the microscope and also called a ocular. It has two lenses.

1) Field lens (upper small lens)
2) Eye lens (lower larger lens)

10. **Objectives:** This is an optical part of the microscope, which is nearest to the stage.

Originally microscopes have three objectives, which are fixed in a revolving nosepiece.

1) **Low power objective (or 10x):** It is an objective where the working distance is more. If the ocular is 10x, then the magnification is 100 times.
2) **High – power objective (40x or 45x):** Here the working distance is less as compared to low power. If the ocular is 10x, then the magnification is 400 or 450 times.
3) **Oil immersion objective**: (100x): This requires the use of a drop of either cedar wood oil or liquid paraffin between the lens and the object. The oil serves to prevent loss of light rays due to refraction. It has a refractive index (R.I.) 1.51. Here the working distance is very small. If the ocular is 10x, then the magnification is 1000 times.

11. **Condenser:** It is an optical part, which is made of lenses. It condenses the light rays there by preventing the escape of light rays. It also controls light intensity.

12. **Mirror:** It has two reflecting surfaces (1) plane and (2) concave. The plane mirror reflects the light rays parallel to one another and the light source may be artificial like tube lights, while the concave mirror concentrates the light and the light source may be visible natural diffused light rays.

Principle of microscope:

The light rays reflected from mirror pass through the diaphragm then condenser were the rays are condensed pass through the specimen and reach the objective. The first real and inverted image is formed within body tube, which again serves for further magnification, by ocular system, which forms virtual image.

Definitions:

1. **Resolving power**: The resolving power of a lens is its ability to show two closely adjacent points as distinct and separate

$$\text{Resolving power (RP)} = \frac{Wavelength}{2\ x\ Numerical\ aperture}$$

2. **Numerical aperture**: It is the function of the effective diameter of an objective in relation to its focal length and refractive index of the medium between the specimen and the objective.
3. **Magnification**: It is the ratio between size of the image and size of the object.
4. **Working distance**. It is distance between the specimen and the objective lens.

B. Examination of animate and inanimate objects

Materials required:

Pond water sample, cover slips, slides, filter paper strips, distilled water and microscope.

Procedure:

1. Place a small piece of filter paper on clean grease free slide and pond water on another slide.
2. Use a drop of distilled water to fix the filter paper (small pieces) to slide and if necessary use the cover slip.
3. Mount the slide on stage, adjust and focus with low power and high power objectives.

Observations:

1. Observe cellulose fibers in low power and high power objectives. Take neat diagram of fibers.
2. Observe different microorganisms namely algae, protozoa from pond water sample in low power and high power objectives. Take neat diagram of the specimens.

Conclusion…….

WORKING OF A MICROSCOPE:

Materials required:

A slide with a smear, lens cleaning paper, cedar wood oil or liquid paraffin and microscope.

1. Place the clean grease free slide on the stage, specimen side up and center the specimen as accurately as possible over the hole in the center of the stage.

2. Adjust the light source until it posse's maximum amount of light through the specimen. With the low power objective in position, lower the body tube by means of coarse adjustment until the objective is about ¼ inch from the slide.
3. Look through the eye-piece; adjust with coarse adjustment knob until the specimen is the approximate focus. Bring the specimen to sharp focus with the fine adjustment knob.
4. After examining with low-power objective change to the thigh power objective by rotating the nose-piece without changing the position of the slide.
5. Look though eye-piece and bring the image into final accurate focus by using fine adjustment.
6. Focus the specimen by the oil immersion objective with a small drop of cedar wood oil on the object/specimen. Raise the condenser, open the Irish diaphragm fully and turn the mirror to get maximum amount of light. Use fine adjustment knob to get clear sharp image of the specimen.

Precautions to be taken while using a microscope.
1. Open both the eyes while looking into a microscope.
2. Never touch the lenses. If the lenses are dirty, wipe them gently with lens cleaning paper
3. Always remove oil from the oil- immersion objective after use.
4. Keep the stage of the microscope clean and dry,
5. Never remove any parts of the microscope.
6. Always use both hands while carrying the microscope
7. When the microscope is not is use keep it covered in the microscope compartment.

Exercise No. 9

Objective: Determination of Soil Microbial Biomass -C by Fumigation-Extraction Method

As soil microorganisms play an important role in the retention and release of nutrients and energy; any attempt to assess nutrient and energy flow in soil systems must take into account the role of soil microbial biomass. To ascertain the same through direct observation methods (like colony counting method) have certain disadvantages like more time consuming, data difficult to interpret as variation is large and often erratic, distinction between live and dead organisms is often difficult and uncertainty that all organisms actually present have been counted.

Principle: A non-subjective and replicable method which can be carried out easily is described here for total microbial biomass carbon determination in soil at any point of time. In the fumigation-extraction method, a direct measurement of C and other nutrients contained therein in microbial biomass is carried out. Overnight fumigation with chloroform is carried out to kill all the organisms in soil samples. The microbial biomass constituents released by $CHCl_3$ fumigation treatment can be extracted directly through chemical extractants. The readily oxidizable C contained in the extractant can be measured through standard chemical procedures.

The method is based on some underlying assumptions as follows:

a) Carbon in dead organisms is more living organisms.
b) Fumigation leads to a complete kill.
c) Death of organisms in the non-fumigated soil is negligible compared to that in fumigated soil.
d) The only effect of soil fumigation is to kill the living biomass.
e) The fraction of dead biomass C mineralized over a given time period does not differ in different soils.

Materials required:

1. Moisture box
2. Separating funnel
3. Glass beads and other glassware
4. Whatman No. 1 filter paper

Reagents:

1. Distilled chloroform,
2. Conc. H_2SO_4,
3. 0.5M K_2SO_4: Dissolve 43.563 g K_2SO_4 in distilled water and dilute to 500ml.
4. 0.2N $K_2Cr_2O_7$: Dissolve 0.9808 g $K_2Cr_2O_7$ in 100 mL distilled water.
5. Orthophosphoric acid
6. 0.005N Ferrous Ammonium Sulphate (FAS): Dissolve 3.92 g FAS and 0.15 mL H_2SO_4 in distilled water and dilute to 2 L.
7. Ferroin / Diphenyl amine Indicator

Equipments/Instruments

1. Vacuum desiccator and vacuum pump
2. Rotary shaker
3. Hot plate

Procedure

1. Put soil sample in plastic bag soon after collection to prevent drying due to evaporation. Sieve the soil through 2 nm mesh. Do not dry. Soil samples are to be analyzed preferably on the same day.
2. Weigh five sets of 10g sol for each sample. In one set determine the moisture content of the soil gravimetrically.
3. Of the remaining four sets of the soil, keep two sets in 50 ml beakers for fumigation and pack remaining two sets to keep in refrigerator for extraction on the next day.
4. Take 20 ml chloroform for each 10g soil in a separating funnel. Wash the chloroform two times with conc. H_2SO_4 (each with half the volume of chloroform) and discard the acid (bottom phase) carefully after phase separation. Take precaution to open the stop-cock after each shaking to release the pressure inside.
5. Wash twice with the same volume of distilled water and collect the bottom whitish phase. These washings are given to make the chloroform free of ethanol.
6. Keep the ethanol free chloroform in 100 ml beakers. Do not keep more than 40 ml in each beaker to provide space for boiling Place some glass beads in the beaker to reduce bumping.
7. Place all the beakers containing soil and chloroform in a vacuum desiccator. Line the inner surface of the desiccator with moistened filter paper. Don't use plastic desiccator. Use high- density vacuum grease at the lid-joint to ensure proper scaling. Use a rubber tube to direct the exhaust through water.
8. Put on the vacuum pump and keep it on until the chloroform boils for about five minutes. Close the outlet, switch off the pump and put the desiccator in dark for 24 h.
9. Next day release the vacuum, take out the beaker containing chloroform, and the inner paper lining. Perform back suction for five to six times to ensure removal of any excess/adhered chloroform vapor.
10. Take the non-fumigated soil sample from refrigerator and thaw it.
11. Transfer both the fumigated and non-fumigated soils in 250 ml conical flasks. Add 25 ml of 0.5M K_2SO_4 and shake for half hr. After shaking, filter the suspension through Whatman No 1 filter paper.
12. Transfer 10 ml of the filtrate in 500 ml conical flask. Add 2 ml $K_2Cr_2O_7$, (0.2N), 10 ml of conc. H_2SO_4 and 5 ml of orthophosphoric acid to each flask. Run at least two blanks with 10 ml distilled water each, along with the acids mentioned above.
13. Keep the flasks on hot plate at 100°C for ½h under refluxing condition. Take out the flasks and add about 250 ml of distilled water immediately. Allow the contents to cool to room temperature.
14. Add two to three drops of ferroin indicator and titrate the contents against 0.005N ferrous ammonium sulphate to get a brick-red end point. Alternatively use diphenyleamine indicator.

Calculations

1. Soil water content (WS):

$$WS\ (\%) = \frac{\text{Wt. of wet soil (g) - Wt. of oven dry soil (g)}}{\text{Wt. of oven dry soil (g)}} \times 100$$

2. Weight of soil sample (oven dry weight equivalent) taken for microbial biomass measurement (MS):

$$WS\ (\%) = \frac{\text{Wt. of wet soil (g)}}{\{100 + WS\ (\%)\}} \times 100$$

3. Total volume of solution in the extracted soil (VS):

$$VS(ml) = [\text{Wt. of wet soil (g) -Wt. of oven dry soil (g)}] + \text{extractant volume (ml)} \times 100$$

4. Determination of extractable carbon (EC in µg ml):
   a) Standardization of FAS solution:

$$\text{Normality of FAS (XN)} = \frac{\text{Volume of } K_2Cr_2O_7 \text{ (2ml) x Strength of } K_2Cr_2O_7 \text{ (0.2N)}}{\text{Average titre value for the blank (ml)}\{100 + WS\ (\%)\}} \times 100$$

   b) Determination of volume of $K_2Cr_2O_7$ solution consumed by FAS in any sample (Y) ml

$$Y = \frac{\text{Normality of FAS (XN) x Titre value (ml)}}{\text{Normality of } K_2Cr_2O_7 \text{ (0.2N)}}$$

   c) Volume of $K_2Cr_2O_7$ consumed for oxidizing easily mineralizable C in 10 ml of extractant =2-Y ml

   d) Extractable C (EC) in µg ml:

   1 mL of 1N $K_2Cr_2O_7$ oxidizes =0.003 g of C
   1 mL of 0.2N $K_2Cr_2O_7$oxidizes =0.0006 g of C ie. 600 µg of C
   (2-Y) mL of 0.2N $K_2Cr_2O_7$ oxidizes = 600 x (2-Y) µg of C

   So, the amount of extractable C (EC)

$$EC\ (\mu g\ m^{-1}) = \frac{600 \times (2-Y)}{10}$$

5. Total weight of extractable C in the fumigated (EC_F) and non-fumigated (EC_{NF}) soil samples:

$$EC_F \text{ or } EC_{NF}\ (\mu g\ g^{-1}\ soil) = EC(\mu g\ g^{-1}\ soil) \quad EC(\mu g\ ml^{-1}\ soil) \times \frac{Vs\ (ml)}{MS\ (g)}$$

6. Microbial biomass Carbon in soil (MBC)

$$MBC\ (\mu g\ g^{-1}\ soil) = \frac{(EC_F - EC_{NF})}{K_{EC}}$$

K_{EC} = 0.25 ± 0.05 and it represents the efficiency of extraction of microbial biomass carbon

Exercise No. 10

Objective: To determine the type of bacteria with Gram staining.

Principle:
When the bacteria is stained with primary stain Crystal violet and fixed by the mordant, some of bacteria are able to retain the primary stain & some are decolorized by alcohol. This technique is used to differentiate the bacteria into Gram positive & Gram negative bacteria. This technique was introduced by Danish Bacteriologist Hans Christian Gram in 1884.

The cell walls of Gram positive bacteria have a thick layer of protein-sugar complexes called Peptidoglycan and lipid content is low, decolorizing the cell causes this thick cell wall to dehydrate and shrink, which closes the pores in the cell wall & prevent the stain from exiting the cell. So ethanol cannot remove Crystal violet-iodine complex that is bound to thick layer of peptidoglycan of gram positive bacteria and appearsblue/purple in color.

Reagents Required:
1. Crystal violet, the primary stain,
2. Iodine the mordant,
3. A decolorizer made of acetone and alcohol (95%),
4. Saffranin the counter stain

Procedure:
1. Take a clean, grease free slide
2. Prepare the smear of suspension on clean slide with loopful of sample.
3. Air day and heat fix.
4. Pour Crystal violet and kept for about 30seconds to 1 minute and rinse with water.
5. Flood the Gram's Iodine for 1 minute & wash with water.
6. Then, wash with 95%. alcohol or acetone for about 10-20 seconds and rinse with water.
7. Add Saffranin for about 1 minutes and wash with water
8. Air dry, observe under microscope.