MICROBES AND MICROBIOLOGICAL MEHTODS

PRACTICAL – I

(DMBL01)

(MSC MICROBIOLOGY)

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Practical Manual

Paper – I : Microbes and Microbiological Methods

Experiment No. 1

ISOLATION OF BACTERIA BY STREAK PLATE METHOD

Aim: To perform the quadrant mode of streaking for the isolation of individual colonies.

Requirements: Mixed culture broth, Nutrient agar plates, Spirit lamp, Inoculation chamber, Inoculating loop, Wax-marking pencil.

Principle: Dilution of inoculum during the streaking and development of separate colonies from each isolated cells on agar plates during incubation.

Composition of Nutrient agar medium:

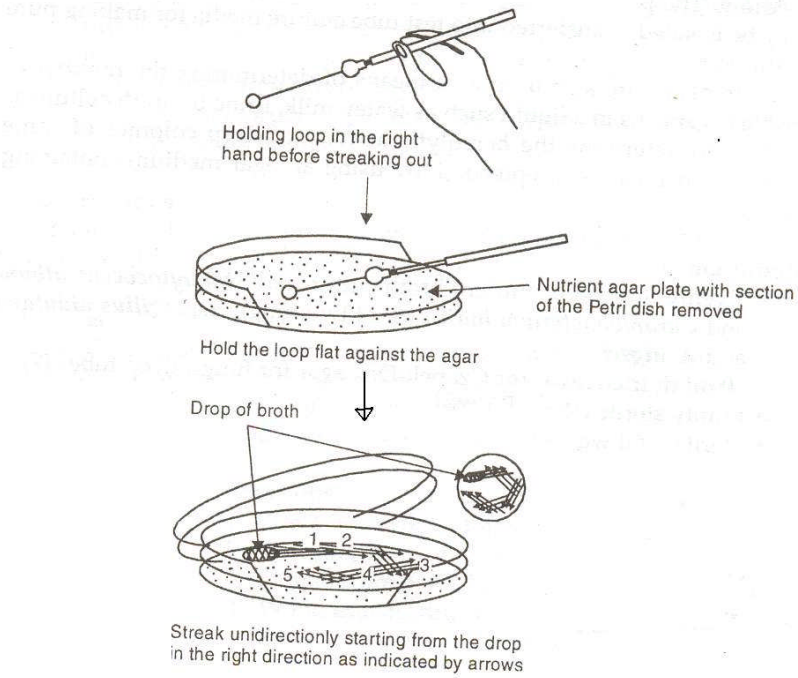
Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

Procedure:

1. Prepare the nutrient agar medium as per the composition and sterilize the medium in autoclave at 15 lbs pressure and 121^{o} C temperature for 20 minutes. Then cool the sterilized medium to molten state of temperature around 42^{o}C.
2. Sterilize the clean petri plates in hot-air oven at 160^{o}C temperature for 2 hrs. After sterilization transfer them to the inoculation chamber.
3. Pour about 20 ml aliquots of molten state sterilized agar medium into petri plates in inoculation chamber. Allow the plates for the solidification of the medium.
4. Sterilize the loop holding in the right hand, introduce the loop into the mixed culture broth and withdraw one loopful of culture.
5. Lift the upper lid of the agar petri plate with left hand and hold it at an angle of 60^{o}.
6. Place the inoculum of the loop on the agar surface at the edge farthest from you and streak the inoculum from side to side in parallel lines across the surface of area.

7. Reflame and cool the loop and turn the petri plate to 90^{o}. Touch the loop to a corner of the parallel lines in area 1 and streak the drawn inoculum across the agar in area 2 as in the previous step. The loop should never enter into area 1 again.
8. Repeat the same steps to streak the parallel lines in 3^{rd} and 4^{th} areas of the petri plate.
9. Replace the lid of the petri plate, after completing the streaking, and sterilize the loop by flaming.
10. Incubate the plates in an inverted position for 24 hrs. at room temperature.
11. Observe the plates for the presence of isolated and individual colonies on the streaked lines.

Result: During incubation, isolated colonies may develop on streaked lines of area 3 or area 4 of the petri plate, with an usual confluent growth in 1 and 2 areas.



Streak Plate Technique

Experiment No. 2

ISOLATION OF BACTERIA BY SPREAD PLATE METHOD

Aim: To isolate pure cultures of bacteria in the form of discrete colonies on solid nutrient agar medium.

Requirements: Nutrient agar medium, Petri plates, Mixed bacterial culture, Hot-air oven, Autoclave, Spreader or bent glass rod.

Principle: During the spinning of petri plate meant for spreading of bacterial cells on solid medium surface, at some stage, single cells will be deposited by bent glass rod on the agar surface. These separated cells on agar surface develop into individual colonies on incubation.

Composition of Nutrient agar medium:

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

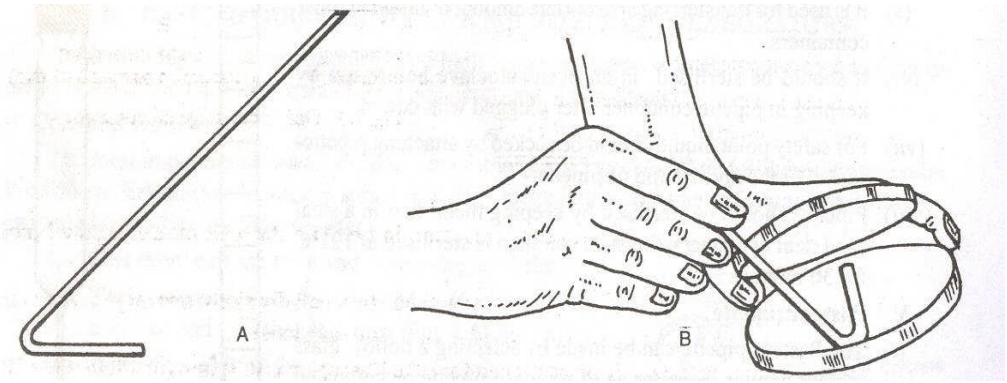
Procedure:

1. Prepare nutrient agar medium as per the composition and sterilize in an autoclave at 121^{o}C and 15 lbs pressure for 20 minutes.
2. After sterilization, cool the medium to molten state, pour 20 ml aliquots into sterilized petri plates and allow to solidify to prepare nutrient agar plates.
3. Prepare culture suspensions of different dilutions by employing ten-fold serial dilution technique as follows –
    (a) Take 1 ml of original broth culture into a test tube and add 9 ml of sterile distilled water to get 10^{-1} dilution.
    (b) Tale 1 ml of suspension from 10^{-1} dilution and add it to 9 ml of sterile distilled water to prepare 10^{-2} dilution.

(c) In the same manner, prepare the suspensions of 10^{-3},10^{-4},10^{-5},10^{-6} and 10^{-7} dilutions.

4. Place 0.1 ml of culture suspension of 10^{-7} dilution on the nutrient agar surface and spread it with a bent glass rod through spinning the petri plate on turn table.
5. Repeat the step 4 for different dilutions.
6. Incubate the plates in inverted position at room temperature for 24 hrs.
7. Observe the plates after incubation, for the distributed colonies.

Result: Well isolated and discrete colonies will be seen on the surface of solid agar medium. The number of colonies in the plates progressively decreases with increasing dilution.



A. Glass Spreader B. Method of Spreading

Experiment No. 3

ISOLATION OF BACTERIA BY POUR PLATE METHOD

Aim: To isolate pure bacterial species in the form of discrete colonies by using solid nutrient agar medium.

Requirements: Nutrient agar medium, Mixed bacterial culture, Petri plates, Hot-air oven, Autoclave.

Principle: The individual cells of bacterial inoculum in successive dilutions thoroughly mix with the molten agar medium during the rotation of petri plate and get fixed or seeded at different places in or on the medium on solidification of the medium. These fixed cells develop into individual colonies at that places on incubation.

Composition of Nutrient agar medium:

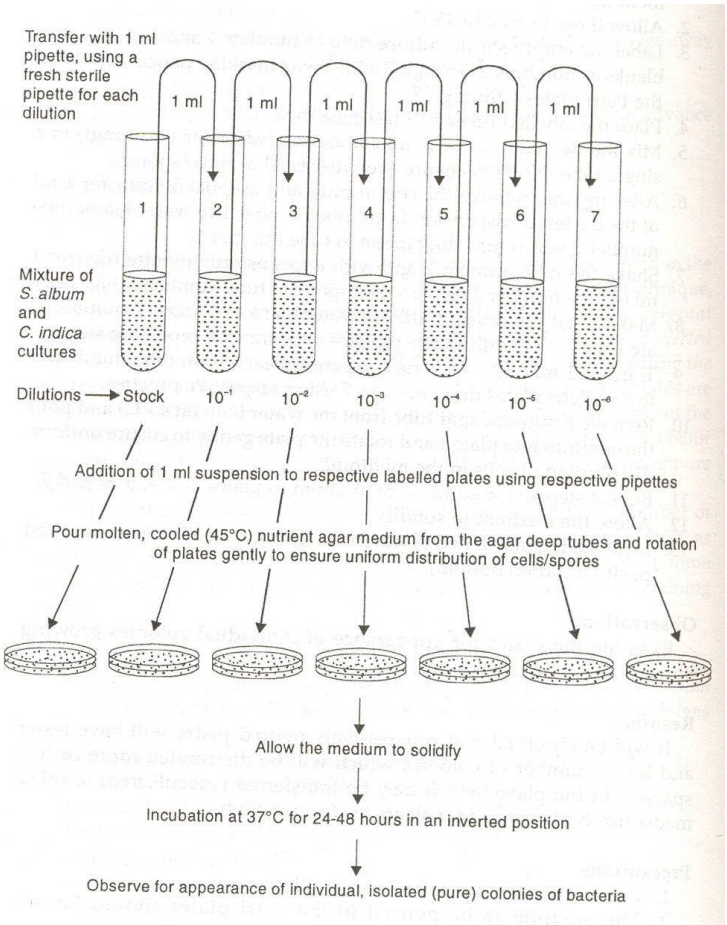
Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

Procedure:

1. Prepare the culture suspensions of different dilutions by using 10-fold serial dilution method as follows –
    (a) Add 1 ml of original broth culture of bacteria to 9 ml of sterile distilled water in a test tube to prepare suspension of 10^{-1} dilution.
    (b) Take 1 ml of suspension from 10^{-1} dilution and add to 9 ml of sterile distilled water to prepare the suspension of 10^{-2} dilution.
    (a) Prepare the suspensions of other higher dilutions viz., 10^{-3},10^{-4},10^{-5},10^{-6} and 10^{-7} dilutions in the same manner as above.
2. Prepare nutrient agar medium as per the composition and sterilize the medium in an autoclave at 121^{o}C temperature and 15 lbs pressure for 20 minutes.
3. Sterilize the clean glass petri plates in hot-air oven at 160^{o}C temperature for 2 hrs.

4. Pour 1.0 ml of bacterial suspension of different dilutions into separate petri plates labeled with the corresponding dilution factor.
5. Into each petri plate, then, pour about 20 ml aliquot of molten state agar medium and rotate the plate gently to ensure uniform distribution of cells in the medium.
6. Allow the plates for the solidification of the medium.
7. Incubate the plates at room temperature in inverted position for 24 hrs.
8. Observe the plates after incubation for the distribution of bacterial colonies.

Result: Well isolated and distributed colonies of surface, sub-surface and bottom surface nature will be seen. The number of colonies per plate progressively become lesser and lesser with increasing dilution of the bacterial inoculum.



Serial Dilution and Pour Plate Method

Experiment No. 4

ENUMERATION OF BACTERIA BY MPN METHOD

Aim: To enumerate the *Nitrosomonas* bacterial population from the soil by using Most-Probable-Number technique.

Requirements: Test tubes, Conical flasks, Soil sample, Ammonium-calcium carbonate medium, Griess-Illosvay reagent, Zn-Cu-MnO_2 mixture.

Principle: The MPN technique is based on the determination of the presence or absence of microorganisms in several individual aliquots of each of several consecutive dilutions of soil sample.

Composition of Ammonium-Calcium carbonate medium:

Ammonium sulphate	-- 0.5 g
Dipotassium hydrogen phosphate	-- 1.0 g
Ferrous sulphate	-- 0.03 g
Sodium chloride	-- 0.3 g
Magnesium sulphate	-- 0.3 g
Calcium carbonate	-- 7.5 g
Distilled water	-- 1000 ml

Preparation of Griess-Illosvay reagent:

(a) Dissolve 0.6 g of sulphanilic acid in 70 ml of hot distilled water. Cool the solution, add 20 ml of concentrated HCl and make the solution to 100 ml with distilled water.

(b) Dissolve 0.6 g of α-naphthylamine in 20 ml of distilled water containing 1 ml of concentrated HCl and make up the solution to 100 ml with distilled water.

(c) Dissolve 16.4 g of sodium acetate in 100 ml of distilled water.

Mix the solutions a, b and c in equal volumes before the use.

Preparation of Zn-Cu-MnO_2 mixture: Mix 1.0 g of powdered zinc with 1.0 g of powdered manganese dioxide and 0.1 g of powdered copper.

Procedure:

1. Prepare the Ammonium-Calcium carbonate medium as per the composition and disperse 9 ml aliquots into the test tubes.

2. Sterilize the tubes by autoclaving at 121^{o}C temperature and 15 lbs pressure for 20 minutes and then cool to room temperature.
3. Prepare the soil suspensions up to 10^{-9} dilutions by using 10-fold serial dilution technique.
4. Add 1 ml of the soil suspensions of different dilutions into separate test tubes containing the sterilized medium.
5. Maintain five replicates for each dilution.
6. Incubate the inoculated tubes for 10 days.
7. After incubation, add few ml of Griess-Illosvay reagent to the incubated test tubes and observe for the reddish color development.
8. To the test tubes not developed the color, add a pinch of Zn-Cu-MnO_2 mixture and observe for the purplish color.
9. Mark the tubes that developed color after the addition of Griess-Illosvay reagent alone or both the reagents as positive tubes and remaining tubes as negative tubes.
10. Select P_1, the number of all or maximum positive tubes in least concentrated dilution, and P_2 and P_3 as the number of positive tubes in the next two higher dilutions.
11. Considering the P_1, P_2 and P_3 values from the data, select the table value from the 'Cochran' tables.
12. Multiply the table value with dilution value of P_2 to get the final populations of *Nitrosomonas* per gram soil.

Result: Purplish color development in inoculated test tubes after the addition of Griess-Illosvay reagent and after addition of Zn-Cu-MnO_2 mixtures indicates the presence of nitrite and nitrate in tubes, respectively. So, development of color in tubes is an indication for the positive presence of *Nitrosomonas* in that sample suspension added to tube.

Table of most probable numbers for use with 10-fold dilutions and 5 tubes per dilution (Cochran, 1950)

P_1	P_2	Most probable number for indicated values of P_3					
		0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.090
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.020	0.040	0.060	0.080	0.10	0.12
1	1	0.040	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.10	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.068	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.29	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.27	0.33	0.39	0.45	0.52	0.59
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.70	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.7	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16.00	-

Experiment No. 5

ISOLATION OF FUNGI BY SERIAL DILUTION METHOD

Aim: To isolate the fungi in discrete colonies on agar plates after serial dilution of the fungal inoculum.

Requirements: Mixed fungal culture, Czapek-Dox agar medium, Petri plates, Hot-air oven, Inoculation chamber, Test tubes.

Principle: The cells or spores of mixed inoculum will be diluted, during serial dilution process, to that extent which yields individual discrete fungal colonies on plating.

Composition of Czapek-Dox agar medium:

Sodium nitrate	-- 2.0 g
Dipotassium hydrogen phosphate	-- 1.0 g
Magnesium sulphate	-- 0.5 g
Potassium chloride	-- 0.5 g
Ferrous sulphate	-- 0.01 g
Sucrose	-- 30.0 g
Agar agar	-- 20.0 g
Distilled water	-- 1000 ml

Procedure:

1. Prepare the diluted suspensions of mixed fungal culture by using 10-fold serial dilution technique up to 10^{-5} dilution.
2. Sterilize the petri plates in hot-air oven at 160^{o}C temperature for 2 hrs.
3. Prepare the Czapek-Dox agar medium as per the composition and sterilize in autoclave at 121^{o}C temperature and 15 lbs pressure for 20 minutes.
4. Cool the sterilized medium to molten state and add small amount of antibacterial substance (streptomycin), mix well and pour 20 ml aliquots into petri plates.
5. Allow the plates for solidification of medium
6. Place 0.1 ml of suspension of fungal culture of 10^{-5} dilution on the Czapek-Dox agar plate and spread it with bent glass rod.
7. Incubate the inoculated plates in inverted position at room temperature for 48 hrs.
8. Observe the plates after incubation for fungal colonies.

Result: Discrete and well isolated colonies with cottony growth appearance will be seen.

Experiment No. 6

ISOLATION OF FUNGI BY BAITING TECHNIQUE

Aim: To isolate zoosporic fungi from soils by simple baiting procedure.

Requirements: Soil sample, Cockroach wings, Beakers, Distilled water, Microscope.

Principle: The zoospores of fungi swim through water and proliferate on reaching the substratum or bait.

Procedure:

1. Weigh 100 g of soil sample and place in glass beaker.
2. Flood the soil of beaker with sterile distilled water to provide a dept of 2 to 3 cm water level.
3. Add five 10 mm segments of cockroach wings to the beaker.
4. Cover the beaker with polythene sheet and pierce fine holes here and there.
5. Incubate the beaker for 3 weeks without any disturbance.
6. After incubation, remove the cockroach wings from the beaker and observe the lower surface under microscope.

Result: A proliferated growth of mycelium will be observed on the lower surface of cockroach wings, if the soil consists the zoosporic fungi.

Experiment No. 7

OBSERVATION OF SOIL MICROBES BY CONTACT SLIDE METHOD

Aim: To study the relative preponderances of microorganisms in soil portions.

Requirements: Soil sample, 250 ml beakers, Microscopic slides, Microscope, Erythrosine.

Principle: The slides inserted into the soil serve as substratum for the growth of soil microbes in the same proportionality of their original distribution in the soil profile.

Procedure:

1. Weigh 200 g of soil sample and place in 250 ml glass beaker.
2. Add 20 ml of distilled water to the soil in beaker to maintain the moisture.
3. Insert two clean and dry microscopic slides in close contact up to ¾ of the slide length.
4. Incubate the set up for 2 weeks with intermittent wetting of the soil in beaker with few ml of distilled water.
5. After incubation, remove the slides carefully without disturbing the soil side of the slide.
6. Stain the slide on the side which is in contact with soil during incubation by using erythrosine.
7. Observe the stained slide for the occurrence and abundance of different microbes viz., bacteria, fungi and actinomycetes.

Result: Bacteria differ from fungi and actinomycetes by their uni-celled nature. The filamentous fungi and actinomycetes differ by their diameter of the mycelium. The fungal mycelium diameter will be greater than that of actinomycetes. Abundance of these microbes will be assessed by relative proportions on the slide.

Experiment No. 8

ANAEROBIC CULTURING OF BACTERIA BY PYROGALLIC ACID METHOD

Aim: To cultivate the anaerobic bacteria in O_2 free condition.

Requirements: 24-48 hr. old cultures of *Clostridium* and *Bacillus*, Cotton-plugged nutrient agar slants, Pyrogallic acid crystals, 4% sodium hydroxide, Rubber stopper, Glass rod, Pipette, Inoculating loop, Bunsen burner or Spirit lamp.

Principle: Pyrogallic acid and sodium hydroxide absorb the oxygen and thereby produce anaerobic environment in the tube in which organism is incubated.

Procedure:

1. Label the two nutrient slants with the name of the bacterium to be inoculated.
2. Inoculate each slant with one of the cultures, by streaking the agar surface aseptically.
3. Replace the cotton plug and ignite the plug by passing it through spirit lamp or bunsen burner.
4. By using a glass rod, push the burning cotton plug into the tube until it nearly touches the slant.
5. Add pyrogallic acid crystals to fill the space between the cotton and top of the slant tube.
6. Then, add 2 ml of 4% sodium hydroxide to the tube.
7. Place a rubber stopper to the tube immediately and tightly, and invert the tubes.
8. Incubate the tubes in inverted position at 37^oC temperature for 24-48 hours.
9. Examine the tubes for the presence or absence of bacterial growth.

Result: *Clostridium* growth will be observed on nutrient agar slant and no growth of *Bacillus* will be seen on slant.

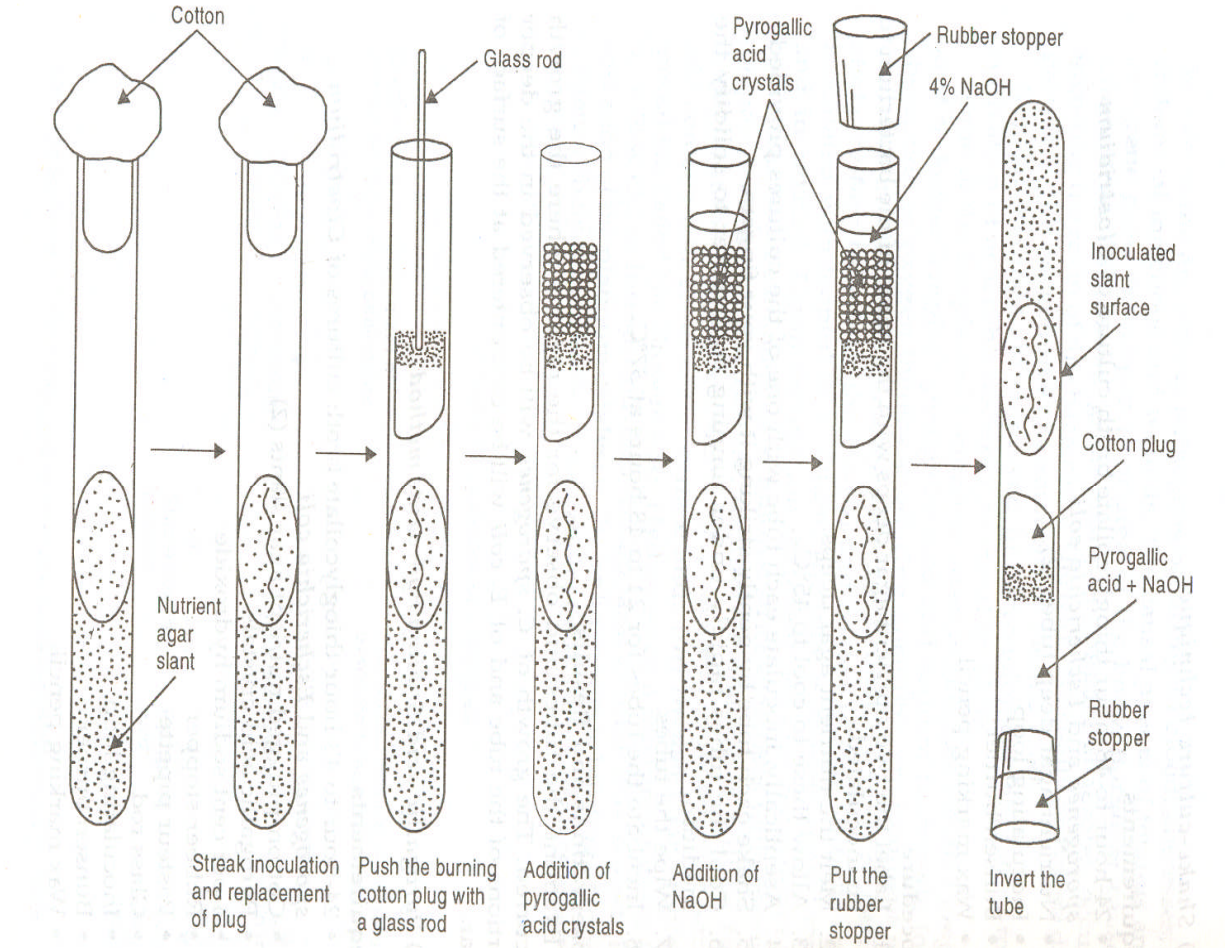


Fig. 12.6 : Pyrogallic acid-sodium hydroxide method for cultivation of anaerobic bacteria.

Pyrogallic acid method for cultivation of anaerobic bacteria

Experiment No. 9

DEMONSTRATION OF WINOGRADSKY COLUMN

Aim: To isolate purple and green phototrophic bacteria and other anaerobes.

Requirements: Soil, Plant roots and debris, Gypsum ($CaSO_4$), Water and Glass column.

Principle: The Winogradsky column set up works as a miniature anaerobic ecosystem and facilitates the isolation of purple and green phototrophic bacteria from soil.

Procedure:

1. Fill the 1/3 of glass column with organic rich soil mud mixed with gypsum, plant roots and debris.
2. Flood the mud with water to a depth of 6-7 cm.
3. Incubate the column for about 8 weeks, exposing the column to the diffused day light.
4. After incubation, observe the column for various regions.

Result: A black zone at the bottom of column representing anaerobic organisms like *Clostridium*, above which a green color zone representing photoautotrophs like *Chlorobium*. A red color zone appears above the green zone representing the photoautotrophs like *Chromatium*. Above this zone photoheterotrophs like purple non-sulphur bacteria develop as rust colored zone. On the top of water column, algae, cyanobacteria, *Beggiatoa, Thiobacillus* etc., will develop.

Experiment No. 10

SIMPLE STAINING OF BACTERIA

Aim: To stain the bacterial cells with simple basic stain and observe the morphology.

Requirements: 24-hr. old bacterial culture of Bacillus subtilis, 1% methylene blue solution, Glass slides, Inoculating loop, Spirit lamp, Blotting paper, Microscope.

Procedure:

1. Take clean glass slides, wash and dry them.
2. Place a loopful of broth of bacterial culture on the clean glass slide at the center.
3. Spread the bacterial suspension thinly on the slide with inoculating loop to prepare the smear.
4. Fix the smear by heating gently over the spirit lamp flame.
5. Stain the smear by adding few drops of methylene blue solution to the smear and allow it to stand for 1-2 minutes.
6. Remove the stain and wash the smear gently with slowly running tap water.
7. Blot-dry the slide using blotting paper but do not wipe the slide.
8. Examine the preparation under oil-immersion objective of microscope.

Result: Rod shaped cells will be clear with a deep blue color of the stain.

Experiment No. 11

SIMPLE STAINING OF FUNGI

Aim: To stain and observe the fungal propagules.

Requirements: Young fungal cultures, Lactophenol-cotton blue mounting fluid, Glass slides, Cover slips, Needles.

Procedure:

1. Procure young cultures (5-7 days old) of fungi growing on culture medium.
2. Place a drop of lactophenol-cotton blue in the center of a glass slide.
3. Transfer a portion of mycelial mat from fungal colony into the drop of mounting fluid with the help of sterilized needle.
4. Spread the fungal propagules, with the help of two needles, for mixing the mycelia with stain.
5. Observe the slide under different objectives of the microscope.

Result: Stained fungal mycelium, conidia and conidiophores can be seen clearly.

Experiment No. 12

DIFFERENTIAL GRAM STAINING OF BACTERIA

Aim: To differentiate Gram-positive and Gram-negative bacteria by differential Gram staining technique.

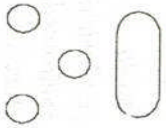
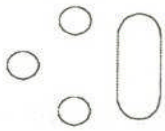
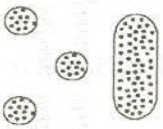
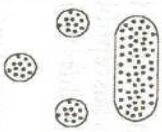
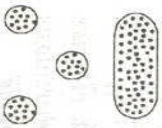
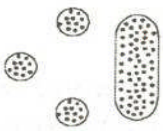
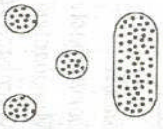
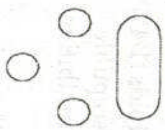
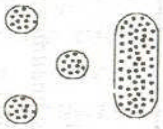
Requirements: 24 hr.-old cultures of *Bacillus subtilis* and *Escherichia coli*, Crystal violet stain, Gram's iodine solution, Ethyl alcohol, Safranin, Wash bottle, Inoculating loop, Glass slides, Blotting paper, Spirit lamp, Microscope.

Principle: The chemical and physical differences in cell walls of the bacteria respond differentially to the Gram stain in either retaining the primary stain (as in Gram-positive bacteria) or lose the primary stain and stained with counter stain (as in Gram-negative bacteria).

Procedure:

1. Make thin smears of *Bacillus subtilis* and *Escherichia coli* on separate glass slides.
2. Air-dry and heat fix the smears on the flame of spirit lamp.
3. Flood the smear with crystal violet stain for 30 seconds.
4. Wash the smear with distilled water for few seconds.
5. Flood the smear with Gram's iodine solution for 30 seconds.
6. Wash-off the iodine solution from the smear with 95% ethyl alcohol. During this step, add ethyl alcohol drop by drop keeping the slide in slant position until no more color of stain flows from the smear.
7. Wash the slides with distilled water and drain.
8. Apply the counter stain, safranin to the smear for 30 seconds.
9. Then, wash the smear with distilled water and blot-dry with absorbent paper.
10. Observe the preparation through microscope under oil-immersion objective.

Result: Bacteria that appear purple are referred to as Gram-positive and those appear pink are described as Gram-negative.

Reagent	Gram-positive	Gram-negative
None (Heat-fixed cells)	 Colourless	 Colourless
Crystal-violet (20 seconds)	 Purple	 Purple
Gram's-Iodine (1 minute)	 Purple	 Purple
Ethyl alcohol (10–20 seconds)	 Purple	 Colourless
Safranin (20 seconds)	 Purple	 Red (Pink)

Gram's Staining

Experiment No. 13

STAINING OF BACTERIAL ENDOSPORES

Aim: To observe the stained endospores of bacteria.

Requirements: 72 hr. old culture of *Bacillus cereus* or *Bacillus subtilis*, 5% malachite green, 0.5% safranin, Glass slides, Inoculating loop, Blotting paper, Spirit lamp, Microscope, Immersion oil.

Principle: Special stains give the color to endospore by penetrating through the spore wall.

Procedure:

1. Prepare the smear of *Bacillus cereus* on clean, dry glass slide.
2. Air-dry and heat fix the smear on spirit lamp flame.
3. Flood the smear with malachite green.
4. Heat the flooded smear intermittently on spirit lamp flame for 10 minutes by adding more stain to the smear from time to time.
5. Wash the slides slowly under running tap water, keeping the slide in slant position.
6. Counter-stain the smear with safranin for 30 seconds.
7. Wash the smear with distilled water and blot-dry with blotting paper.
8. Observe the slide under oil-immersion objective of microscope.

Result: The rod shaped vegetative cells stain red and elliptical or spherical or oval spores stain green and appear as refractile bodies.

Experiment No.14

NEGATIVE STAINING OF BACTERIA

Aim: To stain the bacteria negatively by using nigrosin stain

Requirements: 24 hr. old bacterial culture of *Bacillus subtilis*, Nigrosin solution, Clean glass slides, Inoculating loop, Microscope.

Principle: Acidic stain with negative charge stains only background but not the bacterial cell that possess negative charge on the surface. Due to negative charge-negative charge repulsion the stain will deposit or accumulate around the cell surface but not penetrate into the cell.

Procedure:

1. Place one drop of nigrosin at one end of a clean glass slide.
2. With the help of a sterile inoculating loop, transfer a loopful of inoculum to the drop of stain and mix gently with the loop.
3. Take another clean slide, place it against the drop of suspended organism at an angle of 30^{o} and allow the droplet to spread across the edge of the top slide.
4. Spread the mixture of the stained inoculum into a thin and wide smear by pushing the top slide to the left along the entire surface of the bottom slide.
5. Allow the smear to air-dry and examine under oil-immersion objective of the microscope.

Result: Rod shaped cells appear transparent or colorless against a blue-black background.

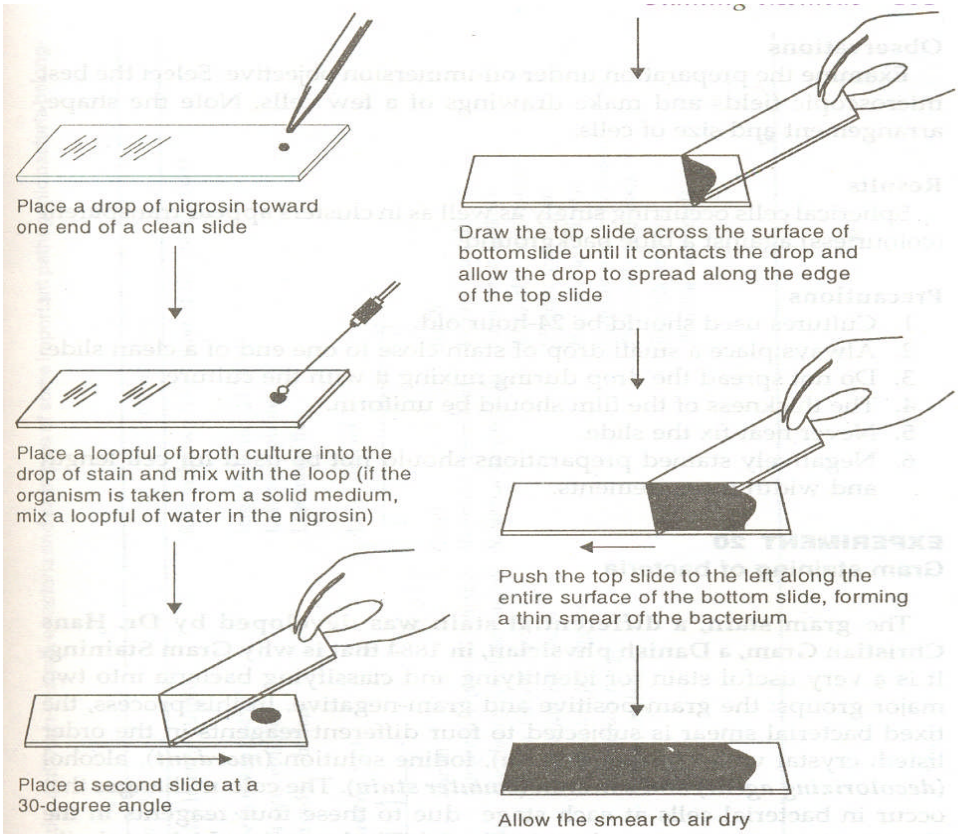


Fig. 8.3: Procedure for negative staining in bacteria.

Procedure for negative staining

Experiment No. 15

DETERMINATION OF SIZE OF THE BACTERIAL CELL AND FUNGAL SPORE BY MICROMETRY

Aim: To measure the size of the bacterial cell and fungal spore with the help of ocular micrometer.

Requirements: Slide of bacterial cells, Slide of fungal spores, Ocular micrometer, State micrometer, Microscope.

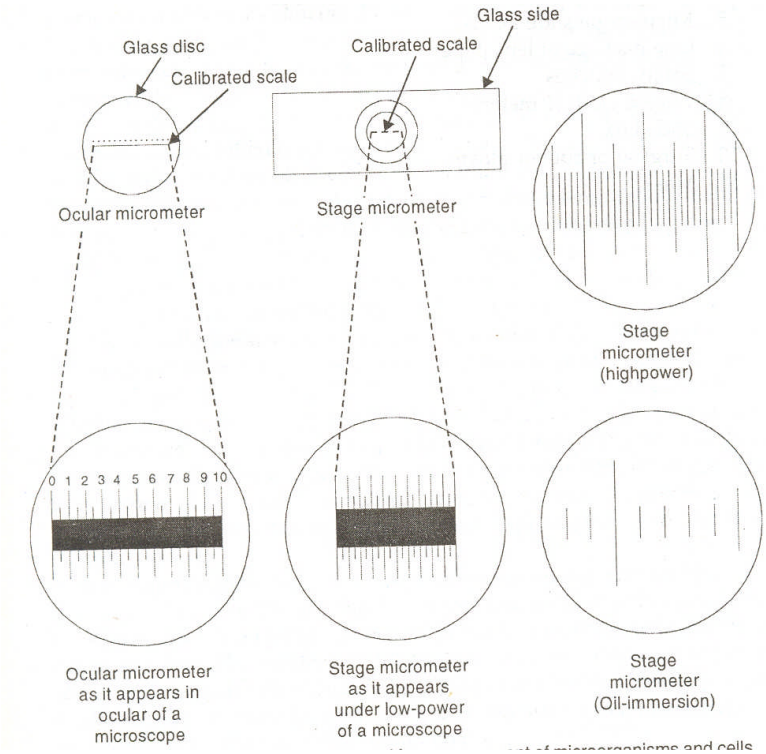
Procedure:

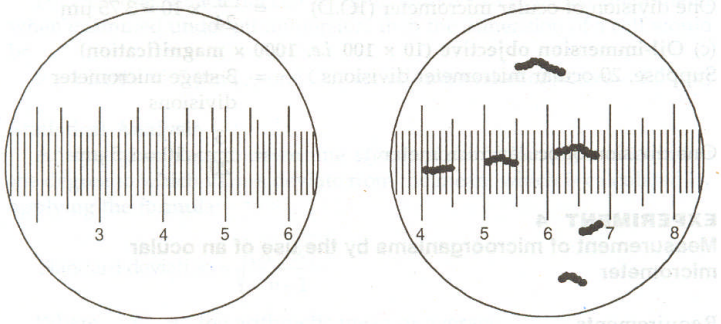
1. Calibrate the ocular micrometer for different objectives (low-power, high-power and oil-immersion) of a microscope as follows –
    (a) Remove the eye piece or ocular lens from the microscope, unscrew it and insert the ocular micrometer disc on the circular shelf. Screw the eye piece back and insert it in the microscope.
    (b) Place the stage micrometer on the microscope stage and bring its scale to the center of the microscopic field area under sharp focus.
    (c) Turn the ocular lens until the parallel lines of the ocular micrometer be parallel with those of the stage micrometer.
    (d) Make the lines coincide at the left end and find another line set which coincides.
    (e) Count the number of divisions in both ocular and stage micrometers, between the two coinciding lines.
    (f) Take at least five readings and average them.
    (g) Repeat the same with high-power and oil-immersion objectives.
    (h) From the data determine the calibration factor for different objectives by using the formula

$$\text{One division of ocular micrometer (in } \mu\text{m)} = \frac{\text{Number of stage micrometer divisions between the coincided sets}}{\text{Number of ocular micrometer divisions between the coincided sets}} \times 10$$

2. After calibration, replace the stage micrometer with the slide of bacterial cells or fungal spores.
3. While viewing with the high-dry or oil-immersion objective, move the stage so that the ocular micrometer is over number of cells or spores.
4. Now, count the number of ocular micrometer divisions that a single cell or spore occupies.
5. By rotating the ocular lens determine the division or divisions of several individual cells or spores. Take at least 10 readings, if the organism is round and take 10 readings each for length and width of cells or spores, if they are not round, and average them.
6. Basing on the readings determine the size of the cell or spore by multiplying the number of ocular micrometer divisions occupied with calibration factor for one ocular division for the objective used.

Result: The size of the cells or spores differ depending on the types of cells and spores, and will be in μm units.





A. Calibration of ocular micrometer. Ocular micrometer super imposed (below graduations which are numbered) over stage micrometer above (dark coloured graduations). Count the number of OM divisions coinciding with SM divisions; Six graduations on the OM coincide with two graduations on the SM.

B. Measurement of microorganisms with the calibrated OM. Count the number of divisions on the OM which the different cells occupy. Each filament occupies 5-7 divisions on the OM. Multiply OM divisions with the calibration factor.

Micrometry

Experiment No. 16

ANTIBIOTIC SENSITIVITY TESTING BY DISC DIFFUSION METHOD

Aim: To demonstrate the sensitivity or resistance of *Bacillus subtilis* against the selected antibiotic.

Requirements: 24 hr. old culture of *Bacillus subtilis*, Petri plates, Nutrient agar medium, Inoculation chamber, Forceps, selected antibiotic discs.

Composition of Nutrient agar medium:

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

Procedure:

1. Prepare the bacterial suspension of *Bacillus subtilis* by using sterile distilled water.
2. Prepare the nutrient agar medium as per the composition and autoclave at 121^{o}C temperature and 15 lbs pressure for 20 minutes.
3. Cool the sterilized medium to molten state and add the bacterial inoculum to it.
4. Disperse 20 ml aliquots of the inoculated medium into petri plates to prepare nutrient agar plates seeded with inoculum.
5. Allow the plates for the solidification of the medium.
6. After solidification, place the selected antibiotic disc at the center of the plate with the help of sterilized forceps and gently press it towards the medium surface.
7. Incubate the plates in inverted position for 24 hours at 37^{o}C temperature.
8. Observe the plates after incubation, for the formation of clear zone around the disc.
9. Calculate the area of clear zone by using the formula πr^2 for the comparison of antibiotic sensitivity against different antibiotics.

Result: Development of clear zone around the disc indicates the sensitivity of organism to that antibiotic and absence of any clear zone is an indicative of resistance of the organism.

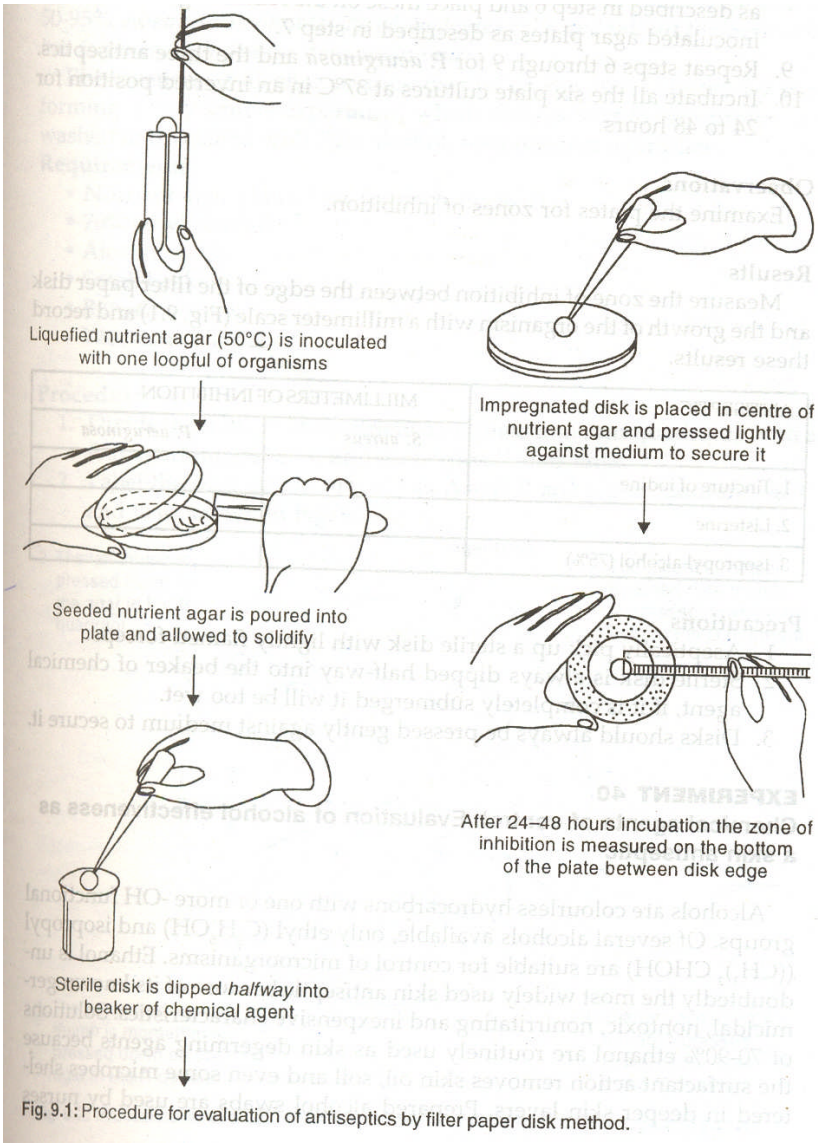


Fig. 9.1: Procedure for evaluation of antiseptics by filter paper disk method.

Antibiotic Sensitivity test by filter paper disk method

Experiment No. 17

OLIGODYNAMIC ACTION OF METALS ON BACTERIAL GROWTH

Aim: To demonstrate the toxicity of heavy metals to bacteria.

Requirements: Bacterial culture, Nutrient agar medium, Sterile petri plates, Sterile forceps, Inoculation chamber, Metal coin (25 paise coin).

Principle: Certain metals are toxic and inhibit growth of bacteria. A concentration gradient is established when a metal is placed on the medium resulting in the inhibition of growth of bacteria.

Composition of Nutrient agar medium:

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

Procedure:

1. Sterilize the petri plates in hot-air oven at 160^{o}C temperature for 2 hours.
2. Prepare the nutrient agar medium as per the composition and sterilize in autoclave at 121^{o}C temperature and 15 lbs pressure for 20 minutes.
3. Cool the sterilized medium to molten state and add the bacterial suspension to it and mix well.
4. Pour about 20 ml aliquots of inoculated molten nutrient agar medium into sterilized petri plates.
5. Place a piece of round metal coin (25 paise) aseptically on the surface of medium, just before it solidifies.
6. Maintain a control plate without a coin.
7. Incubate the plates at room temperature for 48 hours.
8. Observe the plates after incubation for the development of inhibition zone around the coin.

Result: A zone of inhibition of bacterial growth can be seen around the coin, if the bacteria used are sensitive to the toxicity of that metal coin.

Experiment No. 18

DETERMINATION OF THERMAL DEATH POINT (TDP) OF BACTERIA

Aim: To study the lethal effect of temperature by determining the thermal death point of the organism.

Requirements: 48 hr. old culture of *Bacillus subtilis*, Nutrient agar plates, Test tubes, Hot water bath, Pipettes, Marking pencil, Inoculating loop.

Composition of Nutrient agar medium:

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

Procedure:

1. Divide the bottom of the sterile nutrient agar plate into 6 sectors with glass marking pencil.
2. Label the six sectors of plate as control, $40^{o}C$, $50^{o}C$, $60^{o}C$, $70^{o}C$ and $80^{o}C$.
3. Label the plate with the name of the organism.
4. Inoculate the organism in the sector marked 'C' on the plate by means of a streak inoculation.
5. Label five sterile test tubes with organism's name and temperatures $40^{o}C$, $50^{o}C$, $60^{o}C$, $70^{o}C$ and $80^{o}C$.
6. Aseptically transfer 2 ml of bacterial suspension into each of five test tubes.
7. Suspend the tube labeled with $40^{o}C$ in the water bath and keep it for 10 minutes at a constant $40^{o}C$ temperature of the water bath.
8. Exactly after 10 minutes, remove the tube from water bath and immediately streak the inoculum from the removed tube in the sector labeled as $40^{o}C$ of the petri plate. Discard the $40^{o}C$ broth tube.
9. Repeat the steps 7 and 8 for the remaining temperatures viz., $50^{o}C$, $60^{o}C$, $70^{o}C$ and $80^{o}C$.

10. Incubate the bacterial inoculated plates at 32^{o}C temperature for 24 hrs. or until growth occurs in the control sector in an inverted position.
11. Observe the plates for the presence or absence of growth in all the labeled sectors of the petri plate.

Result: The labeled temperature of the sector in which growth is not seen will be the TDP of the organism.

Experiment No. 19

DETERMINATION OF THERMAL DEATH TIME OF THE BACTERIA

Aim: To study the lethal effect of temperature on bacteria in terms of Thermal death time.

Requirements: 48 hr. old culture of *Bacillus subtilis*, Nutrient agar medium, Petri plates, Inoculating loop, Marking pencil.

Composition of Nutrient agar medium:

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

Procedure:

1. Pour 20 ml aliquots of the sterilized molten agar medium into petri plates and allow for solidification.
2. Divide the bottom of petri plates into six sectors with glass marking pencil.
3. Label the 2 petri plates with time intervals as 0,3,6,9,12,15,18,21,24,27 and 30 minutes in continuation.
4. Inoculate '0' minute sector of nutrient agar plate with bacterium as streak inoculation.
5. Suspend the bacterial suspension containing tube into water bath set at a particular selected temperature for 4 minutes (1 minute for equalization of temperatures of broth and water and 3 minutes time of exposure).
6. Remove the tube and cool quickly under tap water.
7. Make streak inoculation on sector 3 on nutrient agar plate.
8. Replace the tube in water bath and allow 1 minute for the broth and water to become equalized before starting the next time interval i.e., 6 minutes.
9. Repeat the steps 6 and 7 quickly.
10. Repeat the steps 5 to 7 to the time intervals of 9,12,15,18,21,24,27 and 30 minutes.

11. Incubate the inoculated plates at $30^{o}C$ in inverted position for 24 hrs. or until growth occurs in the '0' time sector of the plate.
12. Observe the plates after incubation for the presence or absence of growth in different sectors of the plates and determine the time required to kill the organism at the given temperature.

Result: The labeled time interval of the sector in which no growth is seen will be the TDT of the organism at that selected temperature.

Experiment No. 20

ISOLATION OF BACTERIOPHAGES BY PLAQUE ASSAY METHOD

Aim: To isolate the bacteriophages (coliphages) from sewage water.

Requirements: Sewage sample, phage broth, *Escherichia coli* culture, Centrifuge, Incubator, Tryptone agar plates, Tryptone soft agar, Membrane filters, Pipettes.

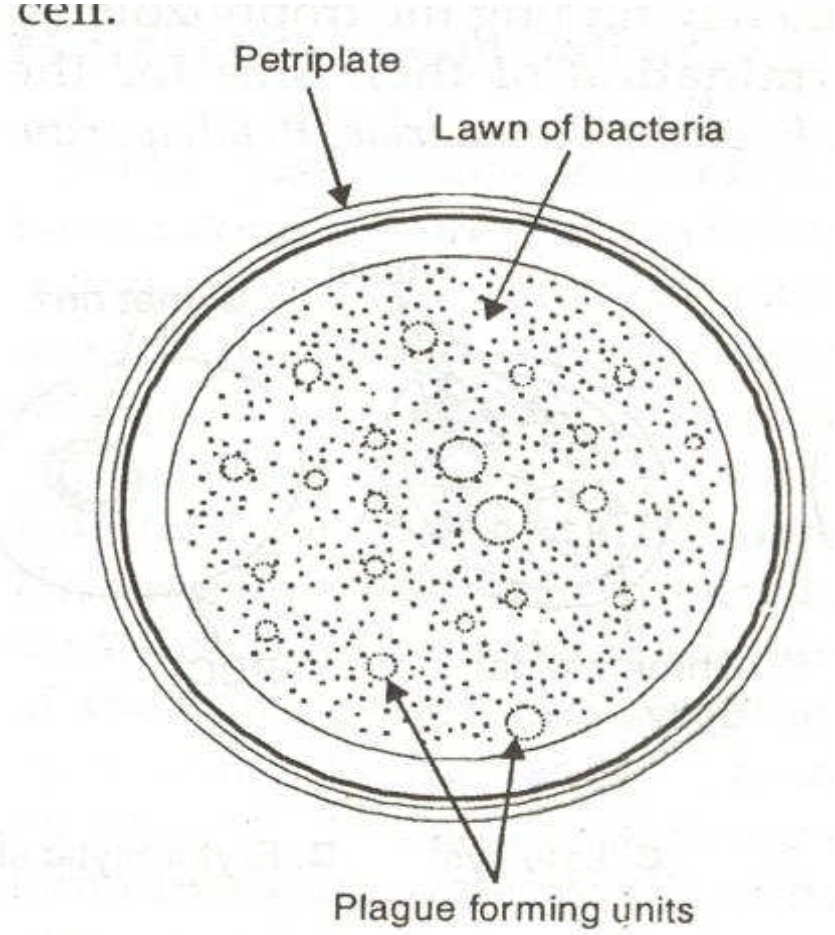
Principle: The phages of the sewage sample when added to molten soft agar medium containing the host bacterial culture and allowed for incubation result in the formation of plaques amidst the lawn of bacterial growth. Each plaque represents a large number of phages that are formed from a single phage particle on multiplication in their host and release to the outside.

Procedure:

1. Add 5 ml of bacteriophage broth, 5 ml of *E. coli* culture and 45 ml of raw sewage sample to 250 ml conical flask in aseptic conditions.
2. Incubate the culture for 24 hours at 37^{o}C and centrifuge the culture at 2,500 rpm for 20 minutes.
3. Decant the supernatant into a 125 ml flask.
4. Filter the supernatant through sterile membrane filter to collect the bacteria-free but phage containing filtrate.
5. Prepare the tryptone agar plates by pouring sterilized tryptone agar into the plates at molten state and allow the medium to solidify.
6. Prepare tryptone soft agar tubes and sterilize the medium.
7. To the molten tryptone soft agar tubes, add 1 ml of *E. coli* culture and few drops of filtrate, mix thoroughly and pour into the tryptone agar plates.
8. Allow the plates for the solidification of soft agar.
9. Incubate the plates in inverted position at 37^{o}C for 24 hours.
10. Observe the plates after incubation for plaques formation.

Result: Formation of plaques indicate the presence of coliphages in the raw sewage sample.

cell.



Plaque formation

Experiment No. 21

OBSERVATION OF SYMPTOMS OF VIRUS INFECTED PLANTS

Aim: To observe and note the important symptoms of plants infected with disease causing viruses.

Tomato leaf curl:

- Characterized by severe stunting of the plants with downward rolling and crinkling of the leaves together with mottling.
- Newly formed leaves show chlorosis.
- Older, curled leaves become leathery and brittle.
- Stunting of plant is due to shortening of the internodes.
- The diseased plants look pale and produce more later branches resulting in bushy growth.
- Causes complete sterility of plant.
- Virus is transmitted by whitefly and by grafting.

Spotted wilt of tomato:

- Characterized by bronze markings on the upper surface of young leaves, which extend from leaf blade to the petiole and stem.
- The upward rolling of leaves occurs.
- Yellow spotting of fruits and finally wilting of stem occurs.
- Disease is transmitted by vectors.

Leaf curl of papaya:

- Prevalent in several parts of India.
- Characterized by severe curling, crinkling and deformation of leaves. Mostly young leaves are affected.
- Other symptoms like vein-clearing, reduced size, inward rolling of leaves and thickening of veins are also common.

- Twisting of petioles may occur.
- Diseased leaves become leathery and brittle, plants become stunted, fruit yield reduced, defoliation often results.
- Caused by virus which is transmitted readily by grafting and by means of whitefly.

Papaya mosaic:

- Most devastating viral disease prevalent in the central belt of India.
- Most serious in young plants.
- The top young leaves of diseased plant are much reduced in size and show blister-like patches of dark green tissue, alternating with yellowish-green lamina.
- The leaf petiole is reduced in length and the top leaves assume an upright position.
- The infected plants show degeneration and marked reduction in growth.
- Fruits develop circular water-soaked lesions with a central solid spot.
- Fruits are elongated and reduced in size.
- Virus is mechanically transmitted.

Yellow vein mosaic of Bhendi:

- Characterized by yellowing of entire network of veins in leaf blade.
- In severe cases young leaves turn yellow, reduced in size, plant is stunted .
- Infection may start at any stage of plant growth.
- Vein clearing is followed by veinal chlorosis of leaves.
- Veins and veinlets may appear thickened.
- Due to infection, flowering of the plant is restricted, fruits if formed are small, rough and harder.
- Caused by bhendi vein-clearing virus transmitted by vectors.

Potato mosaic:

- Caused by potato virus Y.
- Chief symptom is a blotching mottle, which becomes apparent about 3 weeks after planting.
- The mottled mosaic symptoms are more pronounced on young and newly formed leaves.
- Later necrosis appears on the veins on the lower surface of leaf followed by similar lesions on the upper surface.
- Necrotic lesions spread along the veins to the petiole and stem.
- The affected leaves droop and wither.
- The entire plant becomes stunted and is easily identified even from a distance.

Potato streak:

- Caused by virus namely Solanum virus 4.
- Causes chlorophyll mottling, which appears as streaks on the leaf.
- Often necrotic spots are produced on the young shoots, and the tender shoots and buds may die.

Rosette disease of Ground nut:

- Virus is transmitted through grafting and by *Aphis craccivora*.
- Characterized by a clumping together of the foliage.
- Reduction in the size of leaf blade occurs.
- Occurrence of severe mottling.
- Bunching and erect appearance of the plant occur.

Little leaf of apple:

- Characterized by reduction in the size of leaves.
- Reduction of internode length.
- Mottling and deformation of the leaf blade occur.
- The leaf buds either open late or not open at all and eventually die.

Sugarcane mosaic disease:

- The causative virus of sugarcane mosaic disease belongs to the Potato Virus Y group.
- More commonly, elongated yellowish stripes alternate with the normal green portions of the leaf to give a mosaic appearance.
- In severe infections, the chlorotic area may be greater than the healthy.
- The mosaic symptoms are more clearly apparent on the younger basal portions than on older leaves.
- Similar symptoms are seen on the leaf sheaths and stalks, and in highly susceptible varieties yellow stripes are seen even on the canes.
- Sometimes, elongated necrotic lesions are produced on stalks, in which case stem splitting is common.
- The entire plant is stunted and becomes chlorotic, and easily identified from a distance.

Tobacco mosaic disease:

- Disease is caused by Nicotiana Virus I.
- It is the first recognized viral disease of plants.
- First symptoms are light discoloration along the veins of the youngest leaves.
- Soon after the infection, leaves develop a characteristic light and dark green pattern, the dark green areas usually associated with the veins.
- Sometimes, the disease is accompanied by a blistered appearance of the leaf due to more rapid growth of the dark green tissues.
- Plants that are infected early in the season are very much stunted while those infected late in the season show little reduction in size.

Leaf curl of Tobacco:

- Disease appear in fields usually about 4-6 weeks after transplanting.
- Characterized by downward curling of young leaves i.e., leaf margin turn downwards and come together at bottom, exposing the middle upper surface of the leaf blade.

- Usually thickened leaf blade exhibits vein clearing symptoms.
- As disease advances, plant becomes dwarf and most of the leaves are curled.
- The inflorescence is greatly condensed and the veins of the calyx thickened and green enation and leaf-like outgrowths along the veins are also common.

Tungro disease of Rice:

- Disease is characterized by stunting of plant and discoloration of leaves, ranging from various shades of yellow to orange and rusty blotches spreading downwards from the leaf tip.
- Young leaves how a mottled appearance and slightly twisted, whereas older leaves appear rusty colored.
- In less susceptible varieties infection delays flowering.
- In highly susceptible varieties, if infection takes place at early stages the plants may die before flowering.
- The virus is transmitted by green leaf hoppers.

Orange leaf disease of tobacco:

- The disease is characterized by an intense orange color which may later change to a golden or brownish yellow.
- Reduced tillering occurs in diseased plants.
- As the disease advances, there may be longitudinal rolling of leaves and rapid death of plant.
- Transmission occurs by leaf hopper.

Experiment No. 22

ESTIMATION OF CHLOROPHYLL IN VIRUS INFECTED LEAVES

Aim: To estimate and compare the total chlorophyll content in healthy and virus infected leaves.

Requirements: Healthy and virus infected leaf samples, Pestle and mortar, Centrifuge, Centrifuge tubes, Test tubes, Measuring cylinder, Spectrophotometer, Acetone.

Principle: Chlorophyll is extracted in 80% acetone and the absorption at 663 nm and 645 nm are read in spectrophotometer. Using the absorption coefficients, the amount of chlorophyll is calculated.

Procedure:

1. Weigh 1.0 g of finely cut and well mixed representative sample of healthy leaves into a clean mortar.
2. Grind the leaf material to a fine pulp with addition of 20 ml of 80% acetone.
3. Centrifuge the ground contents for 5 minutes at 5,000 rpm and transfer the supernatant to a 100 ml volumetric flask.
4. Grind the residue with 20 ml of 80% acetone, centrifuge and transfer the supernatant to the same volumetric flask.
5. Repeat the same procedure until the residue become colorless. Wash the mortar and pestle thoroughly with 80% acetone and collect the clear washings in the volumetric flask.
6. Make up the volume to 100 ml with 80% acetone.
7. Read the absorbance of the solution at 645 and 663 nm wavelength in spectrophotometer against the solvent blank (80% acetone).
8. Repeat the steps from 1-7 with virus infected leaf sample.
9. Calculate the total chlorophyll amount present in healthy leaves and virus infected leaves per gram tissue using the following equation

$$\text{mg total chlorophyll / gram tissue} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \quad X \quad \frac{V}{1000xW}$$

where A = absorbance at specific wavelengths

V = Final volume of chlorophyll extract in 80% acetone

W = Fresh weight of tissue taken

Result: Relatively less amount of total chlorophyll is seen in virus infected leaves compared to that of healthy leaves.

Experiment No. 23

SEPARATION OF PIGMENTS BY CHROMATOGRAPHIC METHOD

Aim: To isolate and separate the bacteriochlorophyll and or carotenoid pigments by column chromatographic method.

Requirements: Cultures of *Pseudomonas* or *Xanthomonas* or *Serratia*, 6% KOH in methanol, diethyl ether, benzene, sodium sulphate, petroleum ether.

Procedure:

- Inoculate 100 ml of minimal medium with 1 ml of log phase culture of bacteria known to produce pigments. Incubate for 48 hrs at 30^{o} C in a shaker.
- Harvest the cells by centrifugation at 10,000 rpm for 15 min and resuspend the cells in 100 ml of distilled water and recentrifuge.
- Resuspend the cells in 50 ml of methanol and transfer the suspension to Erlenmayer flask. Keep the flask in a boiling water for 5-10 min. and intermittently swirl the flask to facilitate the release of bacterial pigments.
- Cool the suspension, centrifuge to remove the cells and transfer the supernatant to a flask and add equal volume of 6% KOH in methanol. Warm the content at 40^{o}C for 10 min and swirl the content intermittently.
- Transfer the content to a separating flask and add two volumes of diethyl ether and enough water to separate the layer. Gently shake the flask and the bacterial pigments get into ether phase.
- Allow to stand for few minutes for separation of two phases. Remove the ether phase and wash with water and dehydrate by adding anhydrous solid sodium sulfate.
- Transfer the ether to flask evaporator and evaporate to dryness at 30^{o}C and redissolve the material in 10 ml of petroleum ether and filter through Whatman No.1 filter paper.
- Fill a glass chromatographic tube with a fritted glass disc. Pack the tube with magnesium oxide up to within 5 cm of the top. Mount the tube on a heavy-walled filter flask and a gentle suction is applied to the side arm.

- Using a glass-stirring rod, pour the petroleum ether solution of pigments down the side of tube. Enough of the solution should be poured to nearly fill the tube.
- When nearly whole of the pigment solution has passed into the magnesium oxide, add the benzene to develop and spread out the bands.

Result: Each pigment forms a distinct colour band as the sample solution is drawn through the column.

Experiment No. 24

SEPARATION OF PROTEINS BY ELECTROPHORETIC METHOD

Aim: To separate the proteins from the sample by using SDS PAGE method.

Requirements: Vertical slab gel electrophoresis apparatus, power pack, Acrylamide-bisacrylamide stock solution, Stacking gel buffer stock, Resolving gel buffer stock, Ammonium persulfate (1.5%), TEMED, Reservoir buffer or electrode buffer, Staining solution, Destaining solution.

Principle: SDS is an anionic detergent which binds strongly to, and denatures, proteins. The number of SDS molecules bound to a polypeptide chain is approximately half the number of amino acid residues in that chain. The protein-SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the protein.

Procedure:

Preparation of reagents –

- Stock Acrylamide solution : Dissolve 30 g of Acrylamide and 0.8 g of Bisacrylamide in 100 ml of distilled water and filter the solution through Whatman No. 1 filter paper.
- Stacking gel buffer : Dissolve 6.0 g of Tris in 48 ml of 1M HCl and adjust the pH to 6.8 and make it to a final volume of 100ml with deionized water. Filter the solution through Whatman No. 1 filter paper.
- Resolving gel buffer : Dissolve 36.3 g of Tris in 48 ml of 1M HCl and adjust its pH to 8.8 and make it to a final volume of 100ml with deionized water. Filter the solution through Whatman No.1 filter paper.
- Reservoir buffer: Dissolve 3.0g of Tris, 14.4 g of Glycine and 1.0 g of SDS in 1000 ml and adjust the pH to 8.3.
- SDS : Dissolve 1.0 g of SDS in 10 ml of distilled water.
- Ammonium persulfate (APS) solution: Dissolve 0.15 g of APS in 10 ml of water.
- Sample buffer: Prepare by adding 12.5 ml of 1M Tris-HCl (pH 6.8), 4.0 g of SDS, 10.0 ml of β-Mercaptoethanol, 20.0 ml of Glycerol and 4.0 ml of 1%

Bromophenol blue solution and make up to a final volume of 100 ml by adding water.

- Staining solution: Dissolve 1.25 g of Coomassie brilliant blue R-250 in 200 ml Methanol and 35 ml of Glacial acetic acid and make the final volume to 500 ml with distilled water. Filter the solution through Whatman No. 1 filter paper.
- Destaining solution: Mix 75 ml of Glacial acetic acid and 50 ml of Methanol and make up to 1000 ml with water.

Sample preparation: Mix the protein sample with equal volume of sample buffer. Boil the mixture for 3 min in a boiling water bath and cool to room temperature. If the protein sample is too dilute, precipitate the proteins with 10% Trichloroacetic acid (TCA), centrifuge at 10,000 x g for 5 min. Wash the precipitate with 1:1 ethanol-ether to remove TCA. Dissolve the precipitate in the sample buffer diluted with water in 1:1 ratio, boil for 3 min and cool.

Preparation of slab gels:

- Clean the glass slabs, spacers and comb with alcohol. Assemble the glass plates and seal the glass plates with agarose or agar after keeping the spacers and clamps.
- Mix 12.5 ml of Acrylamide stock solution with 3.75 ml of resolving gel buffer stock solution and 11.95 ml of water and degas the solution for 1 min. using a water pump and then add 0.30 ml of SDS, 1.5 ml of APS and 0.015 ml of TEMED.
- Mix the gel solution gently and pour it into the mould in between the clamped glass plates taking care to avoid entrapment of any air bubbles. Overlay distilled water on the top as gently as possible and leave for 30 min for setting of the gel.
- After the gel polymerization, remove the water layer and rinse the gel surface with stacking gel buffer.
- Prepare the stacking gel by mixing 2.5 ml of Acrylamide stock solution, 5.0 ml stacking gel buffer stock solution, 0.2 ml of SDS, 1.0 ml of APS, 11.3 ml of water and 0.015 TEMED. Pour this stacking gel on the polymerized gel surface and immediately insert the supplied plastic comb in the stacking gel. Allow the gel to polymerize for about 20 min.

- After the stacking gel has polymerized, remove the comb without distorting or damaging the shapes of the wells. Clean the wells by flushing with electrode buffer using a syringe.
- Install the gel plate assembly into the electrophoretic apparatus. Pour the reservoir buffer in the lower and upper chambers. Remove any trapped bubbles at the bottom of the gel.

Electrophoresis of sample:

- Load 10-20 µl sample in the sample wells. Also load molecular weight marker proteins in one or two of the wells.
- Switch 'ON' the current maintaining it at 10-15mA for initial 10-15 min until the samples have traveled through the stacking gel. Then increase the current to 30 mA until the bromophenol blue dye reaches near the bottom of the gel slab which may require 3-4 h.
- After the electrophoresis is complete, turn 'OFF' and disconnect the power supply and carefully remove the gel slab from in between the glass plates.
- Place the gel in a trough containing staining solution for 3-4 h or it can be kept for staining overnight. Destain the gel with destaining solution till a clear background of the gel is obtained.
- Measure the distance traveled by the tracking dye and also by the different protein bands and calculate relative mobility (R_m) as given below

$$R_m = \frac{\text{Distance travelled by the protein band}}{\text{Distance travelled by the tracking dye}}$$

Result: The separated proteins of the sample band at different locations on the slab gel basing on their molecular weights.

Experiment No. 25

OBSERVATION OF FUNGAL COLONIES AND SPORES

Aim: To know the important features of some common fungi.

Requirements: Fungal colonies or prepared slides or photographs.

Description of some common fungi:

Mucor –

- Taxonomic position:- Division -- Zygomycotina
  Class -- Zygomycetes
  Order -- Mucorales
  Family -- Mucoraceae
- Colonies are cottony and spreading type which covers petridish within 2-3 days of incubation.
- Hyphae are cottony, hyaline, grey or brownish, coarse, coenocytic and richly branched.
- Rhizoids and stolons are absent.
- Sporangia are developed terminally on sporangiophores
- Sporangia possess a basal columella and numerous minute non-motile sporangiospores.
- Sporangiospores are globose to sub-globose.
- Mucor is a saprophyte.

Rhizopus –

- Taxonomic position:- Division -- Zygomycotina
  Class -- Zygomycetes
  Order -- Mucorales
  Family -- Mucoraceae
- Colonies are cottony and spreading.
- Hyphae are hyaline, branched and coenocytic.

- The mycelium has 3 types of hyphae — the rhizoids, the stolons and the sporangiophores.
- The rhizoids are branched, penetrate the substratum and provide anchorage.
- The erect sporangiophores arise in groups from stolon opposite to rhizoids.
- The sporangia possess a basal columella and numerous minute non-motile sporangiospores.
- The sporangiospores are globose to sub-globose in shape.
- It is a saprophyte.

Aspergillus –

- Taxonomic position:- Division -- Ascomycotina
  Class -- Plectomycetes
  Order -- Eurotiales
  Family -- Eurotiaceae

- Hyphae are branched, hyaline and septate.
- Conidiophores are upright, simple, terminating in a globose or clavate swelling.
- Foot cells differentiate from basal hyphae, enlarge and develop a thick wall. From each foot cell an upright conidiophore arises.
- Conidiophores bear sterigmata at the apex or radiating from the entire surface of vesicle.
- Conidia are one-celled, globose, variously colored and are produced in basipetalous chains.
- It is either saprophyte or parasite.

Penicillium –

- Taxonomic position:- Division -- Ascomycotina
  Class -- Plectomycetes
  Order -- Eurotiales
  Family -- Eurotiaceae

- Hyphae are branched, hyaline and septate.
- Conidiophores arise from the mycelium in single or less often in synnemata.

- Conidiophores are branched, penicillate.
- It has a cluster of sterigmata on which conidia are produced in basipetalous chains.
- Conidia are one-celled, hyaline or brightly colored in masses.
- It is a saprophyte or parasite.

Alternaria –

- Taxonomic position:- Division -- Deuteromycotina
  Class -- Hyphomycetes
  Order -- Moniliales
  Family -- Dematiaceae

- Hyphae are branched, septate and light brown in color.
- Conidiophores are branched, simple, short or elongated, bearing a simple or branched chain of conidia.
- Conidia are borne in acropetalous succession in long chains.
- Conidia are dark, pyriform with a long beak and possess transverse and longitudinal septa and are called as muriform conidia or dictyospores.
- It is a parasite or saprophyte.

Curvularia –

- Taxonomic position:- Division -- Deuteromycotina
  Class -- Hyphomycetes
  Order -- Moniliales
  Family -- Dematiaceae

- Colonies are brown to black and spreading type.
- Hyphae are branched, septate and brown.
- Conidiophores are macronematous, mononematous, erect, brown, simple and sometimes branched.
- Conidia are dark, 3-5 celled, curved or fusiform, end cells are pale.
- It is either parasite or saprophyte.

Cladosporium –

- Taxonomic position:- Division -- Deuteromycotina
  Class -- Hyphomycetes
  Order -- Moniliales
  Family -- Dematiaceae
- Colonies are olivaceous and brown or dark green in color.
- Hyphae are dark brown, septate and branched.
- Conidiophores are dark brown, branched and verrucose near the apex or middle portion due to production of conidia.
- Conidia are generally ovoid to cylindrical in shape but sometimes irregular and lemon shaped.

Fusarium –

- Taxonomic position:- Division -- Deuteromycotina
  Class -- Hyphomycetes
  Order -- Moniliales
  Family -- Moniliaceae
- Colonies are white and spreading type.
- Mycelium is cottony in culture with a tinge of pink color, septate and branched.
- Conidiophores are variable, slender and simple or stout and may be single or organized into a sporodochium.
- Conidiophores are short, branched with a whorl of phialides.
- Conidia are hyaline, variable and are of two kinds – macroconidia and microconidia.
- Macroconidia are several celled, slightly curved or bent at the pointed ends.
- Microconidia are one-celled, ovoid or oblong and simple or in chains. Some are 2-3 celled, oblong or slightly curved.
- Chlamydospores which may terminally or intercalary may be produced in old cultures.
- It is a parasite or saprophyte.

Colletotrichum –

- Taxonomic position:- Division -- Deuteromycotina
  Class -- Coelomycetes
  Order -- Melanconiales
  Family -- Melanconiaceae

- Colonies are white, spreading type, turning black due to production of acervuli.
- Mycelium is branched, hyaline and septate.
- Acervulus is discoid, bearing conidiophores at the bottom bearing dark brown setae around the fruit body.
- Conidiophores are simple, unbranched, short, bearing single celled conidia, ellipsoid or sickle shaped.
- Gibberella is the teleomorph of the fungus.

Experiment No. 26

OBSERVATION OF IMPORTANT PROTOZOA

Aim: To observe and acquaint with the identification characters of protozoa.

Requirements: Prepared slides or photographs of protozoa.

Important features of some protozoa:

***Entamoeba histolytica*:**

- It is a member of class Sarcodina.
- It causes amoebic dysentery.
- Trophozoite is the adult stage of *E. histolytica*.
- Nucleus consists of a nuclear membrane with central karyosome and peripheral chromatin granules.
- Cytoplasm consists of red blood cells of host.
- Trophozoite stage can be found in diarrhetic stools.
- The cyst of *E. histolytica* may be round to oval with a wall.
- Early cyst consists of single nucleus and sausage shaped chromatin bodies, while the late cyst is quadri-nucleated.
- Cysts are found in diarrhetic stools.

Giardia lamblia:

- This protozoan belongs to class Mastigophora
- It is an intestinal parasite and causes severe diarrhea and abdominal discomfort.
- Cysts and trophozoites are seen in diarrhetic stools.
- Trophozoite is the adult and vegetative stage of the parasite.
- It is usually pear shaped with concave sucking disc.
- Two nuclei are bilaterally located with central karyosome and peripheral chromatin is absent.
- Cytoplasm is uniform and clear.
- Trophozoite consists of four pairs of flagella.

- Cysts of *G. lamblia* are oval to ellipsoidal in shape.
- Four pairs of flagella are found within the cyst.
- Cysts are 2-4 nucleated.
- Protoplasm is retracted from the cyst wall.

Balantidium coli:

- *Balantidium coli* belongs to class Ciliophora.
- This organism resides in the human sub-mucosa of the large intestine.
- It causes intestinal ulceration and alternating constipation and diarrhea.
- Trophozoite is the adult and vegetative stage of the parasite.
- It is oval shaped with peritrichous cilia.
- Cytoplasm is vacuolated.
- Trophozoite contains of a kidney-shaped macronucleus and a micronucleus.

Trypanosoma gambiense:

- *T. gambiense* belongs to the class Mastigophora.
- It is a homoflagellate and causes African sleeping sickness disease.
- It has a complex life cycle which is completed in two hosts viz., vertebrate hosts (humans) and invertebrate host (tse tse fly).
- Trophozoite is crescent shaped with a single flagellum along the undulating membrane.
- Cytoplasm is granular with a large, central and polymorphic nucleus.
- Trophozoites can be isolated from peripheral blood.

Plasmodium vivax:

- *P. vivax* belongs to class Sporozoa.
- Life cycle of *P. vivax* is very complex and completed in two hosts viz., man and mosquito.
- *P. vivax* causes malaria disease in human beings.

- It possess many stages in its complex life cycle, but erythrocytic stages in blood are important from diagnosis point of view.
- Erythrocytic stages are designated as signet ring, trophozoite, schizong, segmenters, merozoites and gametocytes.
- The blood smear may show one or more stages of erythrocytic cycle.

Experiment No. 27

OBSERVATION OF IMPORTANT MICROALGAE

Aim: To observe and acquaint with the identification characters of microalgae.

Requirements: Prepared slides or photographs of microalgae.

Important features of some microalgae:

Chlamydomonas:

- Chlamydomonas is mostly fresh water, but also grow in ditches, tanks, ponds, lakes marine water and moist terrestrial habitats.
- It is a motile, unicellular alga.
- It is a green algal member.
- Generally oval in shape and possess cellulosic cell wall.
- Possess two anterior whiplash type flagella of equal length.
- Contains a single haploid nucleus, large chloroplast, pyrenoid, and stigma or eyespot for phototactic response.
- At the base of the flagella, two small contractile vacuoles are present for osmoregulation.
- Reproduce asexually by zoospores formed through cell division.
- Sexual reproduction through gametic fusion.

Chlorella:

- Unicellular green alga found in freshwater, brakish water and terrestrial habitats.
- Non-motile, spherical, sub-spherical or ellipsoidal bound by a true cellulosic wall.
- Characterized by bell shaped or cup shaped parietal chloroplast with or without pyrenoid.
- Single small nucleus, mitochondria and golgi bodies are present.
- Flagella, eyespots and contractile vacuoles are absent.
- Only asexual reproduction is present and produce asexual autospores.

- Motile cells, zoospores or gametes are not produced.
- Few members may live as symbionts.

Eudorina:

- Members are common inhabitants of freshwater ditches, pools, ponds and lakes.
- This alga forms motile coenobia which are ellipsoidal or spherical.
- Each coenobium has 32 or 64 globose cells embedded in mucilaginous matrix and arranged in definite tiers of 4,8,8,8,4 cells each.
- In cell structure it very much resembles with chlamydomonas.
- Vegetative reproduction is by fragmentation of coenobia.
- Asexual reproduction through the formation of gonidia, which produce daughter coenobia.
- Sexual reproduction is anisogamous or oogamous. Zygote formation is by gametic fusion. Zygote loose flagella, germinate and meiotically produce one or more zoospores that give rise to new individuals.

Euglena:

- Unicellular and belongs to Euglenophyta.
- It is the representative genus.
- Typical ***Euglena*** cell is elongated and bounded by a plasma membrane.
- Inside the plasma membrane, an elastic pellicle which is composed of articulated proteinaceous strips lying side by side is present.
- Possess several chloroplasts that contain chlorophylls ‘a’ and ‘b’ together with carotenoids.
- Nucleus is large and contains a prominent nucleolus.
- Large contractile vacuole is present to regulate the osmotic pressure within the organism.
- Two flagella arise from the base of the reservoir.
- Near the reservoir, pigment spot called stigma is present.
- The primary storage product is paramylon.

Pinnularia:

- Unicellular alga belonging to Chrysophyta.
- Possess a distinctive two-piece wall of silica called a frustule.
- The upper, older, overlapping bigger part or lid is called the epitheca.
- The lower, smaller, overlapped half is called the hypotheca.
- Each half consists of the main top and bottom surfaces called the valves.
- The incurved lateral sides at right angles containing the overlapping connecting bands are called the girdles.
- Contains smaller plastids.
- The single large nucleus of the vegetative cell is diploid.
- The cells lack the flagella.

Volvox:

- Volvox is a freshwater planktonic form occurring as green balls of pin head size.
- The colonies, usually called as coenobia, are generally spherical, oval or ellipsoidal in their shape.
- A definite number of cells form a globular outer hull which is held together by a highly viscous, gelatinous, glycoprotein sheath.
- The cell number per coenobium range from 500 to 50,000.
- The cells in the posterior region of the coenobium are usually larger than those in the anterior region.
- Coenobia are motile by the joint action of the flagella of individual cells.
- Each cell of V*olvox* resembles the Chlamydomonas cell with a pair of anteriorly inserted flagella.
- Chloroplast is cup shaped. Two or more contractile vacuoles and an eyespot are found in the anterior region.
- Both asexual and sexual reproduction are present.
- Pyrenoids are one or more in number.

Spirogyra:

- Spirogyra is a freshwater, submerged or free-floating alga.
- It is unbranched and filamentous with uniseriate row of cylindrical cells.
- The basal cell frequently develops into a branched or highly lobed anchoring organ.
- The filaments are slimy to touch because of an outer mucilaginous wall layer.
- The cell wall consists of two layers, an inner cellulosic and an outer pectic layer.
- The septa between the cells may be plane or semireplicate or replicate.
- Contains one or a few spiral, ribbon-shaped chloroplasts, each with many pyrenoids.
- The cytoplasm forms a thin layer lining the cell wall.
- A single nucleus is situated in the central part of the cell and is connected with the peripheral cytoplasm through a number of radiating cytoplasmic strands.
- Vegetative reproduction is the common method and involves the fragmentation of filaments.
- The fusing gametes produced during sexual reproduction are morphologically isogamous and physiologically anisogamous.

Zygnema:

- Zygnema is a widely distributed free-floating freshwater alga.
- It is filamentous and unbranched .
- The cells of zygnema contain a pair of axile stellate chloroplasts.
- Each chloroplast harbours a single central pyrenoid with radiating starch grains.
- The single nucleus of the cell embedded in the middle of the cytoplasm that separates the two chloroplasts.
- Vegetative reproduction is by the fragmentation of the filament.
- Sexual reproduction mode is of isogamous type.

Vaucheria:

- Occurs widely in stagnant and flowing freshwaters, in shaded terrestrial habitats and on walls.
- The thallus is generally a sparingly branched, cylindrical tube lacking cross walls or septa.
- Rhizoid-like branches anchor the alga to the substratum, in terrestrial species.
- Thallus contains an outer cellulosic cell wall and a central vacuole that runs continuously from one end to the other end of the thallus.
- The protoplast is continuous and peripherally contains many discoid chromatophores devoid of pyrenoids.
- Numerous nuclei lie internal to the chromatophores.
- Asexual reproduction is through zoospore formation.
- Sexual reproduction is of oogamous type.

Oedogonium:

- *Oedogonium* is a widespread freshwater alga that grows epiphytically in ponds, pools, and shallow tanks.
- It is unbranched and filamentous with cylindrical cells except the basal holdfast cell.
- The cell wall is thick and differentiated into an inner cellulosic, middle pectic and outer chitinous layers.
- The cells are uninucleate and have a central vacuole containing cell sap.
- The chloroplast is elaborate, reticulate with many pyrenoids.
- Fragmentation is the usual method of vegetative propagation.
- Asexual reproduction is by the formation of multiflagellate zoospores.
- Sexual reproduction is of oogamous type.

Experiment No. 28

OBSERVATION OF IMPORTANT CYANOBACTERIA

Aim: To observe and acquaint with the identification characters of some cyanobacteria.

Requirements: Prepared slides or photographs of cyanobacteria.

Important features of some cyanobacteria.:

Spirulina:

- It is a member of cyanobacterial order, Oscillatoriales.
- It occurs more commonly in brackish and salt water.
- It forms phytoplankton covering the surface of water.
- It is a protein-rich fresh water cyanobacterial form.
- The filaments are spirally twisted.
- Filament is an unbranched trichome with only vegetative cells.
- The mode of reproduction is binary fission in single plane.
- It is usually motile and does not have the heterocysts.

Oscillatoria:

- It is a common filamentous form widely distributed in freshwater environments and damp soil and rocks.
- It is a member of cyanobacterial order, Oscillatoriales
- The filaments are unbranched containing a single row of cells.
- The individual cells are much shorter than length.
- It exhibits oscillatorian movement.
- Exhibits binary fission of reproduction in single plane and also fragmentation.
- Usually motile and does not have the heterocysts.

Nostoc:

- Found in soil and freshwater habitats.
- Member of the cyanobacterial order, Nostocales
- Occur in the form of minute balls of mucilage. Some occur in the intracellular spaces of bryophytes.
- The filaments are embedded in the mucilaginous mass and each individual filament is referred as trichome.
- Possess intercalary as well as terminal heterocysts in a trichome.
- Form hormogonia through fragmentation process.
- Reproduction is through single plane binary fission.
- Often motile and may produce akinetes.
- The heterocysts are prominently present.

Anabaena:

- It occurs as free-floating forms in ponds, lakes, paddy fields and other freshwater habitats.
- Some species of *Anabaena* inhabits the coralloid roots of cycads.
- The gelatinous sheath is not as viscous as in *Nostoc.*
- *Anabaena* do not form large colonies like *Nostoc.*
- Often motile and may produce akinetes.
- The heterocysts are prominently present.
- Form hormogonia through fragmentation process.
- Reproduction is through single plane binary fission.

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