Practical manual on

Plant Pathogenic Prokaryotes

APP 503 3(2+1)

For

M.Sc. (Ag.) Plant Pathology

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Department of Plant Pathology
College of Agriculture

Rani Lakshmi Bai Central Agricultural University
Jhansi, Uttar Pradesh-284003

Practical manual

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M.Sc. (Ag.) Plant Pathology

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General Safety Rules and Procedures

1. No food or drinks are permitted in the laboratory at any time.

2. Only closed-toe shoes are to be worn in the laboratory. Sandals are not permitted.

3. Keep hands and other objects away from your face, nose, eyes, ears, and mouth. The application of cosmetics in the laboratory is prohibited in the laboratory

4. Work areas/surfaces must be disinfected before and after use.

5. Laboratory coats must be worn and buttoned while in the laboratory.

6. Long hair should be secured behind your head.

7. Hands must be washed before leaving the laboratory.

8. All unnecessary books, purses, briefcases, etc., must be kept off the countertops.

9. Label all materials with your name, date, and any other applicable information (e.g., media,organism, etc.).

10. Dispose of wastes in their proper containers.

11. When handling chemicals, note the hazard code on the bottle and take the appropriate precautions indicated.

12. Do not pour chemicals down the sink.

13. Return all chemicals, reagents, cultures, and glassware to their appropriate places.

14. Flame (sterilize) transfer loops, wires, or needles before and immediately after use to transferbiological material.

15. Do not walk about the laboratory with transfer loops, wires, needles, or pipettes containinginfectious material.

16. Turn off incinerators before leaving the laboratory.

17. Report any broken equipment.

18. If you are injured in the laboratory, immediately contact your course instructor or TA.

19. Follow all instructors given by your course instructor or TA in the lab.

20. Always wipe and clean the lenses of your microscope before putting it away. Use the appropriate tissue paper and cleaning solution for this purpose. And make sure to carry the microscope carefully in the correct manner

Practical No. 1

Isolation and Purification of Bacteria

Collection of diseased material

The collections of the diseased material should be representative of all signs and symptoms of a disease (leaves, stems, roots, flowers or fruits). In case of root invading bacteria, part of the collection of stems and roots should be washed free from soil and excess of moisture should be removed before dispatch; a second portion of root material should be kept separate with adhering soil attached in case isolations are to be made from the soil. A polyethylene bag should be tied firmly around the base of the stem to prevent soiling of the rest of the plant. The earliest stages of the disease should always be included, where they are present in the crop because the pathogen is usually isolated more readily from such plant material. In the case of disease affecting the foliage this means lesions that are still water soaked in appearance and which appear translucent when held to the light, rather than lesions which are brown and necrotic. Material of canker and shoot die back should be always included the edge of the lesions and a few centimetre of the healthy tissue beyond it. Pressed and dried specimens of leaf spots and blights should always be retained for the use as herbarium material and also as a reference source.

Practical 1.1. Ooze test for the diagnosis of bacterial diseases

Requirements: Diseased leaves of cauliflower, microscopic glass slide, razor blade, microscope

Procedure

- Cut a piece of infected and healthy parts of leaf with the blade.
- Place the piece in a few drops of water on the slide and put a cover glass.
- Examine under low power objective (10X) of microscope after 1-2 min

Observations: If it is a bacterial infection, cloudy mass of bacterial cells will be visible through the cut ends of the tissue piece.

By using the test, it may also differentiate vascular or parenchymatous infection. In vascular infection, bacterial ooze comes out forcefully at distinct points corresponding to the vascular strand, whereas in diseases like crown gall, hairy root and leafy gall, oozing is difficult. It may be due to affected tissue may not contain the bacterial cells. Bacterial oozing is also difficult in tissues which contain large quantities of starch, dispersal of starch grains in water may mask the bacterial ooze.

Practical 1.2 Isolation of *Xanthomonas campestris* pv.*campestris* from the infected leaves of cauliflower

Requirements: Diseased leaves of cauliflower, microscopic glass slide, razor blade, inoculation needle, nutrient agar medium, Sx agar (selective isolation), sterilized distilled water, sterilized petriplates, spirit lamp, gas burner.

Nutrient Agar medium

Beef extract	3.0 g
Peptone	5.0 g
Agar*	15.0 g
Distilled water	1000 ml

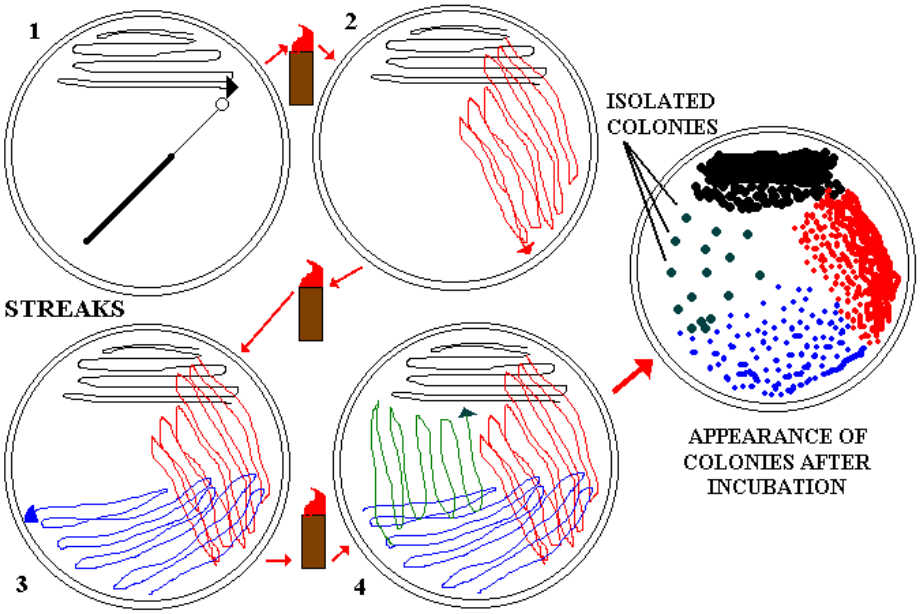
*Do not use agar if nutrient broth is desired

Procedure

- Select young developing lesions with healthy part of leaves for isolation.
- Cut the infected leaves into small pieces with the help of sterilized razor blade.
- Rinse the selected lesion with spirit and immediately dip in mercuric chloride solution (1:1000) for 15 seconds
- Rinse it with sterile distill water and pass for 3-4 changes
- Rinse a glass slide in spirit, flame and allow it to cool
- Place the surface sterilized lesion tissue on slide with the few drops of SDW
- Cut the infected leaves in 3-4 small pieces with sterile razor blade and keep for 2-3 mins to permit diffusion of bacteria into water drops
- Streak loop full of suspension over nutrient agar surface of 3-4 Petriplates by to and fro motion of inoculated needle
- Label the plates and incubate in an inverted position at 25º C and examine daily.

To get the single colony, pick a section of bacterial colony on loop and streak it on medium in single line (not back and forth). The loop is then sterilized in flame and streaked along another single line 90 degree from first streak overlapping few initial streaks. The loop sterilized again

and plate is turned another 90 degree. The second streak is used to make next line. This is done one last time. The bacteria in the plate should look like individual colony in the last streak.



Observations: The colony of *X. campestries*pv.*campestries*and other pathovars will be appeared on the medium as mucoid, convex and raised yellow colour within 4-5 days, but some of them will take as long as 10 days.

Practical 1.3 Isolation of *Xanthomonas oryzae*pv*oryzae* from the infected rice leaves

Requirements: Diseased leaves of rice, microscopic glass slide, razor blade, inoculation needle, nutrient agar medium/ Suwa's medium, sterilized distilled water, sterilized Petriplates

Suwa's medium

Sodium glutamate	2.0 g
$MgCl_2.6H_2O$	1.0 g
KH_2PO_4	5.0 g
Fe-EDTA stock solution*	1ml
Peptone	5.0 g

Sucrose	5.0 g
Agar	5.0 g
Distilled water	1000 ml

* Fe-EDTA 0.657 g dissolved in 100 ml distilled water and added it to medium at the time of plating after filter sterilization.

Procedure

- Collect bacterial blight infected leaves of rice and keep in ice-box for cooling.
- Wash the leaves with 70% ethanol for 10 sec and then rinse twice with sterilized distilled water. Cut each leaf into small pieces (3-5mm).
- Add 1 ml of sterilized 0.85% sodium chloride and grind the pieces of leaf by sterilized pastel-mortar. After grinding, add again 1 ml of sodium chloride.
- Dilute the grinded sample upto 10^{-7}
- Take 100 µl of supernatant and spread onto the Petriplates contain g medium.
- Incubate the plates in the BOD incubator at 28±1° C for 3-5 days

Observations: Light yellow, translucent, fluidal, raised convex colonies appear in the plates after 3-5 days. (Plate 1).

Practical 1.4 Isolation of *Ralstonia solanacearum* from infected tomato plant

Requirements: Bacterial wilt infected tomato plants, CPG, TTCand SMSA media, beaker razor blade, inoculation needle, sterilized distilled water, sterilized Petriplates, spirit lamp.

CPG and TTC agar media (Kelman, 1954)

Peptone	10.0 g
Agar	15.0 g
Casamino acid	1.0 g
Distilled water	1000 ml

To make TTC agar medium, cool the medium to 55ºC and add 1% stock solution of 2,3,5 triphenyltetrazolium chloride. The stock solution can be filter sterilized or autoclaved for 5 min at 121ºC and stored at 4 ºC or frozen.

SMSA medium (Elphinstone et al., 1996)

Peptone	10.0 g
Glycerol	5.0 ml
Casamino acid	1.0 g
Agar	15.0 g
Distilled water	1000 ml
Bacitracin	25 mg
Polymyxin β sulphate	100mg
Chloramphenicol	5mg
Penicillin G*	0.5 mg
Crystal violet	5 mg
TTC	50 mg

*Dissolve this antibiotic 5ml of 70% ethanol 30 minutes prior to use.

Procedure

- Collect wilt diseased tomato from the field.
- Wash the plant with tap water thoroughly to remove soil and dust particles

- Cut the stem and put it in a beaker containing sterilized distilled water and leave it for half an hour.
- The water would become milky in colour
- Streak bacterial suspension in Petriplates containing CPG, TTC and modified SMSA medium.
- Incubate the Petriplates at 28 ºC for 72 hr

Observations: The typical colony character of *R. solanacearum* is mucoid, whitish creamy and having pink colour in the centre of TTC medium(Fig 4 b).

Practical 1.5 Isolation of *Erwinia* species from soft rot infected potato tuber

Requirements: Soft rot infected potato/ carrot, medium (CVP, CPG, polypectate enrich medium), microscopic glass slide, inoculation needle

i) CVP medium (Kuppels and Kelman, 1974)

1N NaOH	4.5 ml
10% $CaCl_2.2H_2O$	3.0 ml
$NaNO_3$	1.0 g
Agar	1.5 g
Sodium polypectate	10.0 g
Sodium dimethylsulphide	0.5 ml
0.075 Crystal Violet	1.0 ml
Distilled water	500ml

Add NaOH, $CaCl_2.2H_2O$, $NaNO_3$ and Agar into 300 ml of boiling distilled water in a waring blender jar and blend it a higher speed for 15 sec. Add slowly sodium polypectate and blend it another 15 secwhile adding another 200 ml of boiling distilled water. Medium should be poured in a 2 l flask and add sodium dimethyl sulphide and crystal violet. Sterilize the medium at 120 ºC for 25 min and pour in Petriplates.

ii) CPG medium (Kuppels and Kelman, 1974)

Casamino acid	1.0 g
Peptone	10.0 g

Glucose	10.0 g
Agar	18.0 g
Distilled water	1000ml

iii) Polypectate enrichment medium

Sodium polypectate	1.5 g
Ammonium sulphate (10%)	10.0 ml
KH_2PO_4 (10%)	10.0 ml
$MgSO_4.7H_2O$ (5%)	5.0 ml

Procedure

- Select a small apiece of tissue from the periphery of decayed lesion of potato
- Add 1-2 drops of sterilized distilled water on flame sterilized microscopic glass slide and macerate it properly
- Streakloop full of suspension over CVP medium containgPetriplate by to and fro motion of the inoculated needle.
- Label the plates and incubate it in inverted position at 25 ±2 ºC in an incubator and observe daily

Observations: The soft rot *Erwinia* may appear on CVP medium as iridescent, cross hatched, translucent colonies in a cup like depression or pits. (Plate 1, Fig. 1c & 1d).

Note: The pure culture of Erwinia ssp. can be obtained by streaking directly from the deep pits in CVP on to CPG medium.

A selective- differential medium for the isolation of soft rot *Erwinia*

PT medium

Polygalacturonic acid	5.0 g
$K_2H.PO_4$	4.0g
$NaNO_3$	1.0 g
$MgSO_4.7H_2O$	0.2g

Sodium heptadecyl sulphate	0.05g
Agar	9.0g
NaOH(1M)	17 ml
Distilled water	1000ml

Procedure

- Put 24-48 h old bacterial culture at the centre of the plate containing PT medium with the help of the inoculation needle piece of tissue from the periphery of decayed lesion of potato
- Incubate 2-3 days at 25±2 ºC and then flood with 1% solution of cetrimide (Cetyl trimethyl ammonium bromide).

Observations: The soft rot *Erwinia* may appear on CVP medium as iridescent, cross hatched, translucent colonies in a cup like depression or pits. (fig 5 b).

A differentiation of *Erwinia carotovora* subsp.*carotovora* on Logan's differential medium

The medium was developed by Logan in 1966 to distinguish*E.carotovora*subsp. *atrospectica*from subsp.*cartovora.*

Medium

Nutrient agar	28.0g
Yeast extract	5.0 g
Glucose	5.0 g

After autoclaving, cool the medium to 60 ºC and add 10ml of filetred-steriliized 0.5% solution of 2, 3, 5 triphenyl tetrazolium chloride to it.

Procedure

- Put 24-48 Streak test culture of *Erwinia* on the dried plates.
- Incubate the plates for 24 h at 27 ºC

Observations: *Erwiniacarotovora*subsp.*carotovora*reduces the tetrazolium to insoluble red formazan and colonies (about 1.5mm diameter) develop a pink to red/ purple centre. Single colonies of subsp. *atrospectica*remain colourless and less than 0.5 mm in diameter. After 48 h the subsp. atrospectica colonies reduce the tetrazolium but remain smaller than subsp. carotovora. Those of *E. chrysanthemi* are larger than subsp.*carotovora* (2mm)and completely dark red/purple.

Practical 1.6 Isolation of *Bacillus* species from rhizospheric soil of plant by dilution plate techniques.

Requirements: Rhizospheric soil, culture tubes containing 9 ml of sterilized distilled water, sterilized 250 ml conical flask, medium (TSA, Mundt and Hinkle medium), pipette, Petriplates.

i) Mundt and Hinkle medium (Mundt and Hinkle, 1976)

Yeast extract	3.0g
Dextrose	5.0 g
Cyclohexamide	2.0 g
Agar	15.0 g
Distilled water	1000ml
pH	6.8

ii) Tryptic Soya Agar (TSA) medium

Tryptone	17.0g
Soytone	3.0 g
Dextrose	2.5 g
NaCl	5.0 g
$K_2H.PO_4$	2.5 g
Agar	15.0 g
Distilled water	1000ml
pH	7.3

Procedure

- Take 10.0 g of rhizospheric soil of plant and mix into 100 ml of sterilized distilled water in 250 ml flask
- Mix soilwell and heat the sample at 80ºC for 15 min
- Agitate it again and again
- Take 1 ml of soil suspension and pour into 9 ml of sterilized distilled water and further dilute upto 10^{-7} for each sample separately.
- Then take 100 µl of diluted suspension at different level as per population of the bacteria in the soil (10,$^{-3}10^{-4}$ &10^{-7}) and spread it with the help of a L-shaped glass rod onto solid TSA and Mundt and Hinkle media.
- Incubate the plates for 24 h at 28±1 ºC in the BOD incubator.

Practical 1.7 Isolation of *Pseudomonas* spp. from the rhizospheric soil of the plants by the dilution plate technique.

Requirements: Rhizospheric soil, culture tubes containing 9 ml of sterilized distilled water,100 ml of distilled water in 250 ml conical flask, medium, pipette, Petriplates.

Mundt and King et al.'s (1954) medium B agar (KB)

Proteose peptone	3.0g
$K_2H.PO_4$	5.0 g
$MgSO_4.7H_2O$	2.0 g
Glycerol	15.0 g
Agar	15.0 g
Distilled water	1000ml

Procedure

- Take 10.0 g of rhizospheric soil of plant and mix into 100 ml of sterilized distilled water in 250 ml flask
- Mix soil well and keep for 30 min
- Agitate it again and again
- Take 1 ml of soil suspension and pour into 9 ml of sterilized distilled water and further dilute upto 10^{-7} for each sample separately.
- Then take 100 µl of diluted suspension at different level as per population of the bacteria in the soil (10,$^{-3}10^{-4}$& 10^{-7}) and spread it with the help of a L-shaped glass rod onto solid TSA and Mundt and Hinkle media.
- Incubate the plates for 24 h at 28±1 ºC in the BOD incubator.

Observations: Whitish slightly raised, convex, florescent green /bluish colonies appear on the medium after 24 h (Fig 6).

Practical No. 2

Isolation of endophytic bacteria from plants (McInroy and Kloepper, 1995)

Requirements: Plants**,** culture tubes containing 9 ml of sterilized distilled water, 100 ml of distilled water in 250 ml conical flask, media (TSA, KB, NA), pipette, Petriplates.

Media: Composition of TSA, KB and NA media as described earlier.

Preparation of 0.02M (20mM) Phosphate buffer

$Na_2H.PO_4$	1.704g/liter
$KH_2.PO_4$	1.088 g/ litre

Make up the volume of the solution by adding 1000ml of distilled water and adjust the pH to 7.0

Procedure

- Take 10.0 Collect the plants from the field and bring them to the lab.
- Wash the collected plants with the tap water and keep for a while to remove the free moisture.
- Cut the root and stem sections (2-3cm long) by using a sterile scalpel. For younger plants, cut the root samples just below the soil line, it depends on the height of the plants.
- Mix Weigh the samples and then surface sterilize with 20% hydrogen peroxide for 10 in and rinse four times with 0.02M potassium phosphate buffer (pH 7.0).
- Disinfect the root samples with 1.05% sodium hypochlorite and wash in four changes of 0.02 M potassium phosphate buffer (pH 7.0).
- Take 1 ml aliquot from the final buffer wash and transfer in 9.9 ml Tryptic Soya broth to serve as a sterility check within 48 h.
- Take known quantity of sample (1.0g) and crush it in 9.9 ml of buffer in sterile pestle and mortar. Dilute serially triturated suspension upto 10^{-7} in potassium phosphate buffer solution.
- Pour 100 µl suspension of different dilution 10,$^{-3}$ to 10^{-7} on plates containing sterilized media. Transfer the representative colonies to fresh media plates to establish pure cultures.
- Incubate the plates for 4 days at 25 ºC

Observations: *Erwiniacarotovora*subsp.*carotovora*reduces the tetrazolium to insoluble red formazan and colonies (about 1.5mm diameter) develop a pink to red/ purple centre. Single colonies of subsp. *atrospectica*remain colourless and less than 0.5 mm in diameter. After 48 h the subsp. atrospectica colonies reduce the tetrazolium but remain smaller than subsp. carotovora. Those of *E. chrysanthemi*are larger than subsp.*carotovora*(~ 2mm)and completely dark red/purple.

Practical No. 3

Purification of phytopathogenic bacteria

Spreading dilution on solid agar

- Take Prepare nutrient agar plates as in the streak plate methods.
- Dilute the bacterial suspension prepared from the infected tissue serially 10^{-3} to 10^{-8} in 5 ml sterile water blanks.
- Inoculate 100µl on to the medium in petriplates from last 2 or 3 different dilutions
- In each plate spread the drop uniformly with a sterile angled L-shaped glass spreader
- Incubate the Petriplates in BOD incubator at 28±2 ºC for 2-6 days or more.
- Pick up a single colony of the desired bacteria.

Spreading dilution on solid agar

- Pour about 25 ml of molten suitable medium (nutrient agar) having 2.0% agar cooled about 45ºC
- Allow the medium to solidify for 1 h and then invert the plates. After 2-3 h, these plates may be used for the isolation purpose.
- Streak loop full of suspension prepared as in the preceding section, over the agar surface by the to and fro motion of the inoculated needle 3-4 times.
- Streak two or more plates without recharging the wire loop with bacterial suspension.
- Label the plates and incubate in an inverted position at 28±2 ºC and examine daily.
- Most bacterial plant pathogens develop colonies within 4-5 days, but some of them may take as long as 10 days.
- Single colonies are usually obtained in the second or third plate.

Pour plate methods

- Dispense 20 ml of nutrient agar medium containing 1.5% agar in test tubes before sterilization
- Autoclave the tubes at 121.6ºC for 20 min.
- Put three such tubes in boiling water to melt the medium and allow the cooling to 45 ºC
- Inoculate one tube with a loop full of suspension from the diseased tissue.
- Mix thoroughly by rotating the test tube within the palms.
- Transfer one loop full of this mixture into second tube and mix thoroughly
- Transfer one loop full of mixture into second tube and mix thoroughly

- ❖ Pour the inoculated medium of all the tubes into separate petriplates
- ❖ Label the plates and incubate at 28 ±2ºC for 2-6 days in inverted position.
- ❖ Pick up the single colony of desired bacteria.

Selection of pathogenic bacterial colony

With well-chosen diseased tissue, it is often possible that only the colonies of the pathogen develop. If more than one type of colonies is seen, it is preferable to select those, which are in more abundance and consistently found in several different suspensions from the affected tissue. Further the colonies which come up more slowly are likely to be that of a pathogen. During the isolation of undescribed pathogens, sometimes it may be desirable to select two or three types of colonies. After multiplication on the nutrient agar medium, these colonies are tested for the pathogenicity. The one which proves to be pathogenic is retained and the rest are to be discarded.

Purification of the selected colony

After selecting the right type of colonies, transfer them on to the nutrient agar slants. Touch wire loop of the inoculation needle on a well isolated colony and streak it on a agar slant in a tube. The cultures obtained from singly colony needs to be checked for purity. Make a dilute suspension of culture in water and streak on the nutrient agar plates. If the culture is pure only one type of colony with original characteristics should be seen.

Practical No.4

Isolation of bacteriophages from different sources

Bacteriophages are viruses infecting bacteria. The phages are found wherever the host bacteria exist. They can be categorized into two groups as per their specificity. Specific phages attack only one group of closely related bacteria, while the non-specific phages occur universally in the soil, water and often in healthy planting material in a very low concentration. Specific phages are often found in high concentrations where their host bacteria are present eg, infected plant tissue, rhizosphere of the infected plant, field water and soil.

(i) Isolation of specific bacteriophages from citrus canker infected leaves

Requirements: Canker infected leaf samples of citrus, scissors, 100 ml sterilized blank conical flask, sterilized distilled water, Petriplates containing nutrient agar medium, pipette, tips, 24h old culture of *Xanthomonas citri*

Nutrient sucrose agar medium

Beef extract	3.0g
Peptone	5.0g
Sucrose	10.0g
Agar	15.0 g
Distilled water	1000 ml

*Do not add agar if nutrient broth is desired.

Procedure

- Collect canker infected leaves of citrus from orchard
- Wash the leaves thoroughly with the tap water
- Cut the leaves in 2-3 mm pieces about 1.0 g quantity from the infected portions.
- Put the pieces in sterilized conical flask and add 50 ml of sterilized distilled water
- Keep the flask overnight for incubation at room temperature.
- Inoculate 10 ml broth culture of *Xanthomonas citri* and incubate for 24 h at room temperature.
- Mix 1 ml of phage culture into 9 ml of broth culture of *X. citri*
- Inoculate 100 µl of mix broth culture on to the NSA medium in Petriplates by spreading method.
- Incubate the Petriplates at 25 º C for 24-48 h.

Observations: Clear lytic areas are developed within 24 h which is called plaque.

Purification: After 48 h, the plaques differing in size are cut along with the medium and transfer separately into 5ml sterilized distilled water columns in the tubes. The phage from the single plaque is again purified twice by plaque test. The plaque containing purified phage is transferred into the 5ml of water column and stored at 5-7 º C.

(ii) Isolation of non-specific bacteriophages from soil and field water

Requirements: 250 ml sterilized blank conical flask, sterilized distilled water, Petriplates containing nutrient agar medium, pipette, tips, 24h old culture of *Xanthomonas oryzae*pv. *oryzae*

Procedure

(a) Soil sample:Take 10 g infected soil in 250 ml of conical flask and suspend it in 50 ml of water. Vortex well and allow standing for a few hours at room temperature.

(b) Field water sample:Collect the water from the field and allow settling for a few minutes

- ❖ Decant the supernatants in above source material (a&b). Centrifuge the decanted sample at 6000 rpm and filter the supernatant through bacteria proof filter (0.45µm pore size). The filtrate is tested for the presence of phages.
- ❖ In place of bacteria proof filters, chloroform treatment can be used. For this purpose, mix 0.5 ml of chloroform in 10 ml of supernatant in a test-tube. Shake vigorously for a while and allow the chloroform to settle down. Supernatant is tested for the presence of phages.
- ❖ Inoculate 1 ml broth culture on NSA medium in Petriplates by spreading method.
- ❖ Incubate the petriplates at 25 ºC for 24-48 h.

Observations: Clear lytic areas are developed within 24 h.

PFU (Phage forming unit) =Number of plaque X dilution factor

Note: Purification of phages may be done as mentioned earlier with the care of phage isolation from infected plant sample.

Practical No. 5

Morphological Characterization of Bacteria (Shape/ Flagellation/ Sporulation)

Practical 5.1 KOH test

It is a preliminary test of gram staining to differentiate the gram +ve and gram –ve bacteria. The destruction of the cell wall of the gram –ve bacteria and subsequent liberation of DNA which is very viscid in water and produces the string of slime. The wall of gram +ve is resistant to KOH it remains intact. DNA is not released.

Requirements: 24 hour old bacterial culture, potassium hydroxide (3% aq w/v), inoculation loop and a clean microscopic slide

Procedure

- Put 1-2 drops of 3% KOH onto a clean microscopic slide with a pipette
- Take a 24 h old single, well separated colony or culture bacteria from slant with the help of a cooled sterile loop.
- Mix loop full bacteria with KOH until an even suspension is obtained. Lift the loop from the slide

Observations: Gram negative bacteria will become gummy upon mixing with a loop and a string of slime is lifted while gram positive bacteria will not produce a watery suspension. If questionable results are obtained gram satin will be used.

Practical 5.2 Negative staining of bacteria

Bacteria are negatively charged and the dye (nigrosin) is an acidic dye. Hence, the acidic stain with the negative charge will not penetrate the bacterial cell due to the negative charge on the bacterial cell. Therefore the unstained cells are clearly discernible against coloured background.

Requirements: 24 hour old culture ofbacteria, clean microscopic glass slide, cotton, tap water, cleaning agent, microscopes, oil emulsion, blotting sheets, spirit lamp, nigrosin.

Procedure

- Put a drop of nigrosin on a slide.
- Put a loop full of bacterial culture in to the drop of stain and mix with the sterile inoculating loop.

- ❖ Put a slide vertically against the drop of suspension of organism and allow the drop to spread along the edge of the slide.
- ❖ Take a Make the smear of the culture on the slide uniformly.
- ❖ Observe under the microscope.

Observations: Bacteria will be seen clearly discernible against dark coloured background (Fig 8).

Practical 5.3Simple staining (Positive staining) of bacteria

For simple staining, basically charged dyes are used to stain negatively charged bacterial cell.

Requirements: 24 hour old culture ofbacteria, clean microscopic glass slide, cotton, tap water, cleaning agent, microscopes, oil emulsion, blotting sheets, crystal violet, spirit lamp.

Procedure

- ❖ Take a drop of distilled water and put on the cleaned microscopic glass slide.
- ❖ Take a drop of test bacterial culture and mix it in a drop of water on the slide.
- ❖ Spread uniformly on the slide to make smear and air dry.
- ❖ Gently heat to fix the smear.
- ❖ Stain with crystal violet.
- ❖ Put a slide under microscope and use a drop of mineral oil (Cedar wood oil) on the selected zone and see under 100x emulsion lens.

Observations: Violet colour of bacterial cell wall will be visibl**e** (fig 9).

Practical 5.4Gram staining of bacteria (Schaad et al., 2001)

For Gram reaction is essential for primary division of bacteria. The gram staining differentiates gram-ve and gram +ve bacteria on the basis of the chemical composition of the cell wall of bacteria. Gram staining involves the use of primary stain (crystal violet), the trapping reagent (iodine solution), decolouring agent (95% alcohol) and counter stain (Safranin). The gram +ve cells stain purple and the gram –ve stain red. Most plant pathogenic bacteria are gram –ve . Gram staining generally gives satisfactory results for the plant pathogens although older cultures of corny form bacteria may stain

gram –ve. The cultures tested should be less than 24 h old. Simple staining, basically charged dyes are used to stain negatively charged bacterial cell.

Requirements: 24 hour old culture ofbacteria,grease free microscope glass slide, glass rods, cotton, tap water, cleaning agent, microscopes, blotting sheets, Huckers crystal violet, safranin 95% ethyl alcohol, gram modification of lugol solution.

Preparation of the solution

1. Huckers ammonium oxalate crystal violet

Solution A	
Crystal violet	2.0 g/l
Ethyl alcohol (95%)	20 ml
Solution B	
Ammonium oxalate	0.8 g/l
Distill water	80.0 ml

Mix the solutions A and B. store it for 24 hr before use. Filter through muslin cloth. Keep it in a bottle.

2. Gram's modification of lugol solution

Solution A	
Iodine	1.0 g/l
Potassium Iodide	2.0 g/l
Distilled water	300.0 ml

Allow iodine solution to dissolve several hours or overnight in a room.

3. Decolorizes

i) Ethyl alcohol, 95% slowest agent (mostly used)

ii) Acetone : Fastest agent

iii) Acetone- alcohol: intermediate (95% ethyl alcohol, 100ml: 100ml).

With practice any of three colorising agents will yield good results.

4. Counter stain

Stock solution

Safranin	2.5 g/l
Ethyl alcohol, 95%	100.0 ml
Working solution	
Stock solution	10.0 ml
Distilled water	90.0 ml

Procedure

- ❖ On a grease free slide, dry a thiny spread bacterial film in air, without heat. Then lightly flame the underside of the slide twice to fix the bacteria to the slide.
- ❖ Flood the smear with crystal violet solution for 1 min.
- ❖ Wash in tap water for a few seconds. Drain off the excess water, and lightly blot dry on a paper towel.
- ❖ Flood the smear with iodine solution for 1 min
- ❖ Wash in tap water for a few seconds and do not blot dry.
- ❖ Decolorize with solvent, eg ethyl alcohol, until the solvent flows colourlessly from the slide (about 30 sec) blot dry. (If decolouriser is used longer, gram positive bacteria may loose colour.
- ❖ Take Rinse in the tap water for about 2 sec
- ❖ Counter stain with safranin solution for about 10 seconds
- ❖ Wash briefly in tap water. Blot dry and examine it under microscope (100x with oil emulsion)

Observations: Gram +ve bacteria stain purple to blue black, the gram-ve bacteria stain red or pink on the other hand.(Fig 10 a & b).

Practical 6.

Stainingof bacterial flagella

1) Silver impregnation methods

Requirements: 24-48 h bacterial culture (*Erwinia carotovora*, *Pseudomonas marginalis*), alcohol cleaned glass slide, tannic acid solution, ammoniated silver nitrate solution, distilled water, inoculation needle tap water.

Preparation of the solution

A. Tannic acid solution

Tannic acid	5.0g
Ferric chloride	1.5 g
Formalin (15%)	2.0 ml
Sodium hydroxide (1%)	0.8 g/l
Makeup the volume to with distilled water	1000ml

B. Ammoniated silver nitrate solution

Silver nitrate (2%)	100 ml

24-48 h Out of 100 ml solution, 10 ml of silver nitrate should be removed and kept separately then add ammonium hydroxide in remaining 90 ml until a heavy precipitate is formed. Add ammonium hydroxide until precipitate s dissolved. Then use from saved 10 ml silver nitrate, back titrate until a clouding appears and persist. The pH of solution should be adjusted to10 with ammonium hydroxide silver nitrate. The solution should be used within 4 hours of preparation.

Preparation of bacteria and staining procedure

- Grow the bacteria on YDC medium slant for 24-48 h
- Use Erwinia spp. having peritrichous flagella of pseudomonas marginalis having amphitrichous flagella as control and test bacteria
- Prepare a faintly cloudy suspension by carefully adding 1.0 ml of distilled water to bottom of the slant.
- Do not agitate the slant mechanically
- Place a loop full of distilled water on an alcohol cleaned slide
- Place a loop full of bacterial suspension just touching the drop

- ❖ Allow the slide to air dry
- ❖ Add reagent B (ammonised silver nitrate solution)for about 10 sec and immediately wash in distilled water.
- ❖ Air dry and examine under the microscope (100x with oil emulsion).

Practical 6.1Capsule staining of bacteria

Capsules are polysaccharides substances surrounding the bacterial cells. Since the capsule material is water soluble, use of water should be avoided as much as possible. Otherwise a capsular material may be washed away.

1. Prepare thin smears of bacterial culture on a microscope slide.
2. Allow the smear to only air-dry. Do not heat-fix as this will cause the capsule to shrink or be destroyed.
3. Apply 1% crystal violet and allow it to remain on the slide for 2 minutes.
4. With the slide over the proper waste container provided, gently wash off the crystal violet with 20% copper sulfate. Caution: Do not wash the copper sulfate and stain directly into the sink.
5. Blot the slide dry with bibulous paper.
6. Observe with the oil immersion lens.

Practical 6.2 Staining of endospore of bacteria

Spores of resting bodies produced by species of bacillus and clostridium bacteria with the cell i.e endospore. Most of the phytopathogenic bacteria do not produce the endospore. The staining is not necessary once the pathogenicity of the bacterial pathogen is established.

Requirements: 24 hold cultures of *Bacillus*/ *Clostridium* bacteria, clean glass slide, glass rods, cotton, tap water, Cleaning agent, microscope, blotting sheet, 5% malachite green, 0.5% safranin, spirit lamp.

Procedure

- ❖ Grow bacteria on the nutrient agar slant for 72 h
- ❖ Use the cultures of Bacillus/ clostridium bacteria as control and test bacteria
- ❖ Prepare a faintly cloudy suspension by carefully adding 1.0 ml of distilled water into the slant.
- ❖ Place a loop full of distilled water on alcohol cleaned slide.
- ❖ Place a loop full of bacterial suspension just touching the drop
- ❖ Dry and fix the smear by holding the slide high over low spirit lamp flame.

- Cover the smear with 5% aqueous solution of malachite green and allow to cool for 50-60 sec.
- Then heat the slide until it steams for 30 sec.
- Wash the slide in tap water
- Cover the smear with a 0.5% aqueous solution of safranin and allow to stain for 30 sec.
- Wash the slide tap water, drain the water blot and air dry.
- Examine under the microscope (100x with oil emulsion).

Observations: The spore stains green colour and remainder bacterial cell be red (Fig 12)

Practical No. 7 Electron microscopic study of bacterial flagella

Requirements: Electron microscope, 15-72 h old test cultures of bacteria, grids, culture tubes, double distilled water.

Preparation of bacterial cultures:

The test bacterium should grow on an optimal growth medium for 15-72 hand it is better to incubate the culture 2-3ºC below the optimum temperature for growth. The culture should be sampled when it is in logarithmic growth phase (15-72h).

Procedure

- Rinse the base of the agar slant about 5 times with double distilled water and discard the water. This is to remove the cells that accumulate at the base of the slant,
- Gently rinse the bacteria from the surface growth of agar slant with about 1 ml of double distill water.
- Prepare a dilution series using suspension of about 10^9 cells as the initial concentration. Mix the dilution very gently to avoid the loss of flagella.
- Dilute this suspension by ½, ¼, 1/16, 1/32. Then put a small drop of suspension on different grids. For optimal viewing, the final concentration of the suspension should be 5 cells/grid hole on 200 mesh grids.
- To shadow, freeze dry grids at -10 ºC in desiccators and shadow with carbon platinum at an angle of 8 º
- To stain, place a grid on the top of 1% uranyl acetate for 1 min or 2% phosphotungstate pH 6.5 for 30 min. Remove stain by touching the edge of grid to filter paper.
- Observe under electron microscope.

Observations: Flagella attachment to bacterial cell will be seen.

Practical No. 8

Biochemical and Physiological Test for the Characterization of Bacteria

In biochemical test, the ability of a bacterium utilize C or N sources or to produce enzymes/gas are determined. Recently some commercial kits are available to perform these test. For example, API systems strip test for carbon source utilization and other systems use oxidation-reduction reactions to determine C and N utilization in a miniaturized (ELISA- plate) format (BIOLOG system).

When conventional biochemical test have given a certain pattern, this pattern can be compared with those of plant pathogens described earlier. Furthermore, it may be necessary or fruitful to compare the isolated bacteria with a reference culture of the pathogen in the test performed. Some typical test used for the biochemical characterization of plant pathogenic bacteria are given below.

Practical 8.1 Utilization and decomposition of carbon compounds

i) Levan production

Levan or poly fructose is an extracellular capsular substance produced through the action of enzymes levan source. Most florescent *Pseudomonas* which utilises sucrose as a carbon source produce these enzymes. The colonies formed on 5% sucrose agar medium are translucent to opaque, shining, mucoid, with distinctive raised convex (doomed) appearance in young cultures (Plate-4, Fig 4d). Colonies are typical levan producing *Pseudomonas* may be observed on 2% sucrose peptone agar (SPA) or on nutrient agar on which 5% sucrose is added.

ii) Voges-Proskaur (VP) and methyl red test

Enterobacteriacea subgroups (*Erwinia*) are routinely differentiated by these tests. The test medium included

Glucose	5.0g
$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	5.0 g
Yeast extract	5.0 g
Distilled water	1000 ml

The V-P reaction depends on the ability of an organism to produce acid from glucose and subsequently convert acid end products to neutral end products, acetone (acetylmethylcarbinol) or 2,3 butanediol, which reacts with the medium on the addition of alkali to impart a pink colour (enhanced by the addition of creatine). Cultures are kept in the shaker incubated at 27 ºC for 5 days. One ml of culture is added in

a culture tube with 0.6 ml of naphthol (5% w/v in absolute ethanol) and 0.2 ml of 40% KOH and shaken vigorously for upto 2h. Optimal addition of a few grains of solid creatine may accelerate the reaction. (Fig 13)

Observations: Methyl red indicator (0.1 g methyl red is dissolved in 300 ml of 95% ethanol and made upto50ml with distilled water) is added to samples of culture, which will turn red if pH is at or below 4.2

iii) 3-ketolactose production test

This test can differentiate species of *AgrobacteriumA. tumefaciens* produces 3-ketolactose, while *A.rhizogens* and *A. vitis* shows negative result. 40-48 h old culture of bacteria is inoculated about 1.0 cm diameter on the medium containing a-lactose 1.0%, yeast extract 0.1% and agar 2.0%. The plates are incubated at 27ºC for 2 days. Four to six bacterial strains can be used in one plate. Then the agar surface in the plate is flooded with Benedicts reagent and leave at room temperature for one hour.

Observations: If 3-ketolactose is present, yellow ring of Cu2O becomes visible around the cell mass in about 1h

Note: Benedict's reagent: Dissolve 17.3 g of sodium citrate and 10 g of anhydrous sodium carbonate in 60 ml of distilled water with heating. Filter the resulting solution if a precipitate forms. Dissolve 1.73g of cupric sulphate in 15 ml distilled water. Slowly add the cupric sulphate solution into a large beaker containing the sodium citrate sodium bicarbonate solution, while stirring constantly. Dilute it in one litre.

iv) Acid production from erythritol and D (+) melezitose

Basal medium

$NH_4H_2PO_4$	1.0g
KCl	0.2 g
Yeast extract	1.0 g
Bromthymol blue 1% (w/v) in 50 % ethanol	3.0 ml
$MgSO_4.7H_2O$	0.2 g
Agar	1.5 g
Distilled water Adjust pH 7.1 before adding agar	1000 ml

Add 1 part of filter sterilized 10% (w/v) erythritol, melezitose or sucrose solution to 9 parts sterile and cooled basal medium after autoclaving. Then dispense about 4 ml of medium to sterile plugged tubes.

Observations: Development of a yellow colour in the medium indicates the production of acid from the oxidation of erythritol, melezitose or sucrose *Agrobacterium rhizogens* produces acid from the oxidation of erythritol, while *A. tumefaciens* and *A. vitis* shows negative results. *A. tumefaciens* produces acid from the oxidation ofmelezitose, whereas *A. rhizogens and A. vitis*shows negative results.

v) Acid production from carbohydrates

Medium C

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	5.0 g
Yeast extract	5.0g
Cysteine hydrochloride	0.1 g
Distilled water	1000 ml

vi) Acid production from carbohydrates

Medium C

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	5.0 g
Yeast extract	5.0g
Cysteine hydrochloride	0.1 g
Distilled water	1000 ml

- ❖ Gently disperse the medium into the culture tubes for preparing agar slants
- ❖ Autoclave the test tubes properly. After sterilization, add carbon source 0.5% (v/v) aseptically from the filter sterilized stock solution.
- ❖ Inoculate the test bacterial culture on to the slant and kept at 25±2 ºC for two days.

Observations: The culture should be observed for 28 days at an interval of 2 days. Production of the yellow colour indicates that acid is produced.

Practical 8.2 Decomposition of Nitrogenous compounds

i) Hydrogen sulphide production

Hydrogen sulphide production from organic sulphur compounds is of differential value for *Xanthomonas and Erwinia*

Medium

$NH_4H_2PO_4$	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	5.0 g
Yeast extract	5.0g
Cysteine hydrochloride	0.1 g
Distilled water	1000 ml

Dispense 3 ml medium in each tube and autoclave. A lead acetate strip is suspended over the medium after inoculation and held by a plug or screw cap. The lead acetate strip is prepared by immersing strip of Whattman filter paper in 5% lead acetate solution, air dried and autoclaved. The test tubes are examined for 14 day.

Observations: The paper strip becomes black in colour due to release of H_2S

ii) Indole production (Miller and Wright, 1982)

Indole is a putrefactive compound produced during anaerobic utilization of tryptophan by some of the bacteria. Indole is volatile and on reaction with oxalic acid, it forms indole oxalic acid which is in pink colour. The reaction is utilized in detecting the presence of indole.

Tryptophan broth medium

Tryptophan or casein digests	10.0g
NaCl	5.0 g
water	1000 g
pH	7.0

Dispense medium 5-10 ml in each tube and autoclave. To detect indole production, the Gnezda oxalic acid strips are prepared as follows. Soak Whattman no 1 strips (5x50mm) in warm saturated solution of oxalic acid. Cool down the solution and air dry at room temperature. Strips may be used without sterilization. Inoculate a test organism and oxalic acid strips into the solution. Incubate tubes at 25 ºC. (Fig 14).

Observations: Colouration of oxalic acid crystals at regular intervals for 14 days. If indole is produced, oxalic crystals on test strip become pink or red.

iii) Nitrate reduction

Pseudomonas cannot reduce nitrogen, but some non-pathogenic species such as *P. fluorescens, P. chlorophylusand P. aeruginosa* can use nitrate as a terminal electron acceptor and grow in the absence of oxygen.

Medium

Peptone	10.0g
Beef extract	5.0 g
KNO_3(nitrite free)	3.0 g
Distilled water	7.0

Dispense the medium about 10 ml into tubes, autoclave and cool. Then inoculate the test bacterium and plug each tube with 3% noble agar. Growth at 27 ºC after up to 5 days is recorded as positive test for denitrification. Add a few drops of sulphanic acid (0.8% in 5N acetic acid) and di methyl-alpha naphthylamine (0.5% in 5N acetic acid) to the nitrate broth culture.

Observations: Nitrite is present if the mixture become distinct pink or red. No colour would mean that nitrate id present as such or has been reduced to ammonium and free nitrogen. To confirm either of these two possibilities are there, add few zinc crystals to the above broth reagent mixture and shake for a few minutes. Nitrate are present without reduction if the broth becomes pink or red. No colour in either of the above two tests would mean that nitrate is reduced to ammonia or free nitrogen (fig 15)

iv) Arginine hydrolase test (Thornley, 1960)

This test is important in distinguishing Pseudomonads

Medium

Peptone	10.0g
NaCl	5.0 g
K_2HPO_3(nitrite free)	3.0 g
Agar	
Phenol red	
L-arginine	7.0
Distilled water	
pH	

Dispense 5 ml the medium in each tube and autoclave. Stab inoculates the medium with 48 h growth of the bacterium and cover the medium with sterile liquid paraffin to a depth of 1 cm. incubate the tubes for 7 days at 25-30 ºC and observe daily.

Observations:A change in colour of the medium to red indicates arginine- hydrolase activity (Fig 16)

Practical 8.3Decomposition of macromolecules

i) Gelatin hydrolysis (Liquefaction)(Dye, 1968)

Proteolytic bacteria decomposes gelatin, which produces an extracellular enzyme gelatinase. As a result of decompositions the gelatin losses its gel forming property.

Medium

Peptone	10.0g
Beef extract	5.0 g
Gelatin	4.0 g
Agar	20.0 g
Water	1000ml
pH	7.0

Autoclave the medium in flask and cool at 45 ºC. Then pour into the petridishesand allow for solidification. The medium is spot inoculated by 48 h growth of the test bacterium. Four cultures can be

tested on a plate. After incubation of 48h to 7 days at 25 ºC, flood the agar surface with mercuric chloride solution in dilute (20%) hydrochloric acid and allow acting for a few minutes.

Observations: The reagent forms a white precipitate with gelatin. If the bacterium has liquefied gelatin the growth is surrounded be a clear zone (Fig 17).

ii) Starch (Cowan, 1974)

The hydrolysis of starch occurs when the bacterium produces the extracellular enzyme amylase. The enzyme beta-amylase hydrolysis starch completely to maltose units, whereas alpha amylase cause partial hydrolysis of erythrodextrinand dextrin.

Medium

Peptone	10.0g
Beef extract	5.0 g
Starch (soluble)	2.0 g
Agar	20.0 g
Water	1000ml
pH	7.0

Sterilize the medium in flask by autoclaving and cool at 45 ºC.Then pour into the petridishes and allow for solidification. For one culture medium 4 bacterium should be maintained. The medium is spot inoculated by 48 h growth of the test bacterium. Test for starch hydrolysis one plate at a time after 2, 4, 7 and 14 days (Fig 18).

Observations: Flood the agar surface with Lugol's iodine and allow acting for few minutes. If the starch is hydrolysed, a colourless or reddish brown zone is observed around the bacterial growth in contrast to the blue background of the medium .

Practical 8.4 Other physiological and biochemical test

i) Kavacs' Oxidase test (Fahy and Persley, 1983)

The oxidases are enzymes catalysing the transfer of hydrogen directly to molecular oxygen, resulting in the formation of water.

$$2H_2 + O_2 = 2H_2O$$

For Oxidase test streak a 24-48 h slant growth of the test bacterium on a filter paper saturated with 1% tetramethyl-para-phenylene-diamino-dihydrochloride. The reaction is positive if a red or purple colour appear within 10 seconds.

Observations: The reaction is delayed positive if the colour appears in 10-60 seconds. Most non-pathogenic *Pseudomonas* are positive, whereas pathovars of *P.syringae*and *P. savastanoi*and *P. viridiflava* are negative (Fig 19)

ii) Catalase test

Catalase is an enzyme which converts hydrogen peroxide into water by removal of oxygen.

$$2H_2O_2 = 2H_2O + O_2$$

To detect the production of this enzyme, smear a loopful of 24-48 h slant growth of the test bacterium on a slide and cover it with a few drops of 20 volume hydrogen peroxide.

Observations: The reaction is positive if gas bubbles are produced. It is necessary to examine for gas bubbles under a microscope, if catalase activity is mild.

iii) Fluorescent Pigment production

The KB medium is used for the detection of fluorescein, a florescent green or blue water soluble chloroform insoluble petridinepigment (i.e. pyoverdine siderophores). After 24-48 h growth at 27ºC, colonies are examined for fluorescence with a long wave-length (366nm)ultraviolet lamp. Impurities such as iron will repress pigment formation and quench the florescence of formed pigments

King et al., B Medium (KB)

Proteose peptone	20.0g
K_2HPO_4	5.0 g
$MgSO_4.7H_2O$	2.0 g
Glycerol	

Agar	20.0 g
Distilled Water	1000ml

Observations: After 24-48 h growth at 27ºC, colonies are examined for florescence with a long wave-length (366nm) ultraviolet lamp. Impurities such as iron will repress pigment formation and quench the florescence of formed pigments

iv) Potato soft rot test

Cut 7-8 mm, peeled and alcohol-flamed potato tuber, and place in sterile Petriplates. Cover surface of slices immediately with sterile distilled water till slices are half immersed. Place a loopful of 48 h grown test bacterium in a nitch made in the centre of each thick slice. Keep inoculated slices under control. Note rotting of slices after 3-5 days.

v) Hypersensitive reaction of tobacco leaves (Klement, 1963)

Most of the phytopathogenic bacteria produce hypersensitive reaction on tobacco leaves. Prepare a dilute suspension (10^7 cfu/ml) of the test bacterium and inoculate the culture described by Klement (1963) injection infiltration method.

Observations: Quick necrosis of tissue within 12-24h on infiltrated point on leaves.

vi) Ferric ammonium citrate broth test

This test can be used to differentiate the species of *Agrobacterium*. *A. tumefaciens*produces reddish brown pellicle at the surface of the medium, whereas *A. rhizogens and A. vitis*show negative result.

Medium

Ferric ammonium citrate	10.0g
$MgSO_4.7H_2O$	0.5g
K_2HPO_4	0.5 g
$CaCl_2$	0.2 g
Distilled Water	1000ml

Adjust pH 7 before autoclaving. 0.1% L glutamic acid or 0.001% yeast extract can be added for auxotroph to grow. 24-48 h old culture of test bacteria is inoculated to the culture tubes containing above mentioned broth medium and incubated the culture tubes in stationary position.

Observations: In positive reaction, reddish brown pellicles is formed at the surface of medium i.e. *A. tumefaciens*

vii) Citrate utilization

Sodium citrate agar medium

Sodium citrate (anhydrous)	2.0g
$MgSO_4.7H_2O$	0.2g
$NH_4H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
NaCl	5.0 g
Agar	20.0g
Bromomethyl blue, 1%(w/v) in 50% ethanol	15 ml
Distilled Water	1000ml

For the preparation of medium dissolve the salts in distilled water and then add bromothymol blue and adjust the medium pH 6.8. Add the agar and heat the suspension to melt the agar. Dispense the medium in the test tubes to cool. Inoculate 24-48h old culture of test bacteria on to the slant the tubes to cool and incubate 24-48 h old culture of test bacteria on to the slant and incubate for 24-48h (Fig 20).

Observations: The inoculated medium turns green to a deep Prussian blue if citrate has been utilized.

Practical No. 9

Protein digestion (liquid or agar) and litmus milk test

i)Liquid test

Add bromcresol purple (w/v) 0.004% in powdered skim milk. Adjust 7.0 with 1.0 NaOH. Sterilize the medium steaming for 30 min on three successive days. Inoculate milk solution in test tubes with loopful of test bacterial cells and incubate it at 27ºC.

Observations: Clearing reaction on the medium indicates positive digestion of casein. No change in colour or red colour (acid) or blue colour (alkaline) will be observed.

ii)Agar plate test

It is constituted powdered skim milk, sterilize as above and mixed at 48ºC with sterile melted YNA to obtain a 10% v/v concentration and pour over the surface of a thin layer of nutrient agar in Petriplates. The plates should be dried and spot inoculated on the medium.

Observations: A clear zone around the colonies after 3,5 and 7 days will be observed**.**

To detect the production of this enzyme, smear a loopful of 24-48 h slant growth of the test bacterium on a slide and cover it with a few drops of 20 volume hydrogen peroxide.

Observations: The reaction is positive if gas bubbles are produced. It is necessary to examine for gas bubbles under a microscope, if catalase activity is mild.

Practical 9.1 Production of alkali in the medium

i)Alkali from malonic acid

Basal medium

$(NH_4)_2SO_4$	2.0g
KH_2PO_4	0.4g
K_2HPO_4	0.6 g
NaCl	2.0 g
Yeast extract	0.1g
Malonic Acid, sodium salt	3.0g
Bromothymol blue, 1.0% (w/v) in 50% ethanol	2.5 ml
Distilled Water	1000ml
pH	7.0

Dispense 3-4 ml basal medium to each test tube before sterilization. Inoculate test bacterium in each test-tube and incubate at 27 ºC for about two weeks.

Observations: The medium will turn blue when alkali is produced.

ii)Alkali from mucic acid and L-tartaric acid

Medium

$NaNH_4PO_4$	2.0g
NaH_2PO_4	0.4g
K_2HPO_4	0.6 g
KCl	2.0 g
Bromothymol blue, 1.0% (w/v) in 50% ethanol	2.5 ml
Distilled Water	1000ml
pH	7.0

Dispense 4.5 ml basal medium to each test tube before sterilization. After sterilization, add 0.5ml of filter sterilized 1% solution of either L-tartaric acid or mucic acid previously neutralised with NaOH. Inoculate test bacterium in each test-tube and incubate at 27 ºC for about two weeks.

Observations: The medium will turn blue when alkali is produced

Practical 9.2Pectate degradation test

i)Hildebrand's medium

Sodium polypectate	22.0g
$CaCl_2.H_2O$10%(Freshly prepared)	6.0 ml
Bromothymol blue, 1.0% (w/v) in 50% ethanol	1.0 ml
Distilled Water	1000ml

The basal medium should be prepared in the following order as 1000 ml distilled water should be heated to near boiling, and then add 1 ml bromthymol blue, 6 ml of $CaCl_2.H_2O$ and 22g of sodium polypectate. The mixture is stirred with a mechanical stirrer on hot plate. After dissolving sodium polypectate. The mixture is stirred with a mechanical on hot plate. After dissolving sodium polypectate, add 100 ml of sterile 4% agar solution to each medium and adjust three pH level given below-

Medium 1: adjust the pH 4.5-4.7 with 1N HCL and autoclaved

Medium 2: adjust the pH 6.9-7.1 with 1N HCL and autoclaved

Medium 3: autoclaved and then adjust pH 8.3 with sterile 1N NaOH

Pour the medium in to Petri plates, when the temperature of medium not below 70ºC. Store the plates at room temperature for a while to remove free moisture from the surface of medium. Spot 24-48h old culture of test bacterium on the medium by using a sterile toothpick or transfer loop. Incubate the plates at 26±2 ºC for 6 days.

Observations: The inoculated plates should be examined daily upto 6 days before the occurrence on the medium, which indicate positive test.

Practical No. 10

Reducing substances from sucrose

i)Medium

Peptone	10.0g
Agar	17.0g
Distilled Water	800ml

Sterilize the medium and then add filter sterilized sucrose (40g dissolved in 200 ml water) pour into Petri plates. Inoculate dense aqueous suspension of test bacterium in grid pattern onto the medium. Incubate the plates at 25±2 ºC for 2 days. Flood the inoculated plates with 10 ml of Benedict's solution and incubate at 30-45 min at 60 ºC.

Benedict's solution: Sodium citrate 35.0 and $Na_2CO_3.H_2O$ 20.0 g is dissolved in 160 ml of distilled water and heat to dissolve. Dissolve 3.5g of $CuSO_4.5H_2O$ in 40 ml distilled water. Mix both the solutions. This mixture can be kept for several months in room temperature.

Observations: An orange zone around the colony of bacteria against a blue background will be visible in positive test.

Practical 10.1 Phosphate activity (Holt et al., 1994)

To test the phosphatase activity, prepare nutrient agar medium and when it is ready to be poured into Petriplates, add filter sterilized solution of phenolphthalein diphosphate sodium salt to give final concentration of 0.05%(w/v) and thoroughly mix in the medium and pour into petriplates. Inoculate the dense aqueous suspension of test bacterium in grid pattern on to the medium. Incubate the plates at 25±2 ºC for 2 days. Then place a drop of (ca. 100 µl) concentrated ammonia in lid of Petriplates and invert the medium over it.

Observations: The colonies of bacteria that have phosphatase activity will turn pink in colour immediately.

Note: Use only glass Petriplates for this assay because ammonia softens Plastic Petriplates.

Practical 10.2 Utilization of keto-methyl glucoside (methyl a-d-glucoside)

Medium

KH_2PO_4	2.0g
NH_4CL	10.0g
$MgSO_4.7H_2O$ (10% conc)	2.0 ml
K_2HPO_4	7.0 g
Casamino acid	1.0g
Agar	15 g
Distilled water	998 ml

Autoclave, cool it to around 50 ºC and add 20% keto-methyl-glucoside and 2ml of Trizolium chloride. Then pour the medium into Petriplates. Stab inoculate dense aqueous suspension of test bacterium in a grid pattern on to the medium. Incubate the plates at 25±2 ºC for 2 days.

Observations:*Erwinia carotovora* subsp. *atroseptica* grows well and produces colonies with red centres. *E. carotovora* subsp. carotovora grows poorly and colonies are white, although a little red colour may develop at the point, where the inoculum is stabbed into the medium.

Practical 10.3 Glucose dehydrogenase activity assay

Medium

Mannitol	10.0g
L-glutamate	2.0g
$MgSO_4.7H_2O$	0.2g
K_2HPO_4	0.2g
Yeast extract	0.25g
Glucose	20 g
Agar	15.0g
Distilled water	1000 ml
pH	7.0

Streak the test bacteria on the medium. Colonies are overlaid with a 4 ml of 0.5% agar containing mixture of methylene blue (65 µg/ml) and eosin yellow (400g/ml). Incubate the plate for 1-5 min at 30ºC.

Observations: A purple halo around individual colonies indicate glucose dehydrogenase activity due to the production of 2-gluconate from glucose.

Practical 10.4 Arbutin hydrolysis(α –glucosidase activity) (Crosse et al., 1963)

Medium

Arbutin	5.0g
Peptone	10.0g
Yeast extract	3.0g
Glucose	1.0 g
Ferric citrate	0.5g
Agar	12.0g
Distilled water	1000 ml
pH	7.0

Autoclave the medium and pour into petriplates. Spot two strains of bacteria in each plate containing medium. The plates of medium well separately and incubate them in a BOD incubator at 25±2ºC for 10 days.

Observations: The medium will become brownish in colour due to the growth bacteria in positive case. Most of pathovars of Pseudomonas syringae are positive but other splain of *Pseudomonas* like *morspronorum, helanthi*, *Cannabina, glycinia and phaseolicola* are negative as in *P. savastoni*.

Practical 10.5 Poly-β-hydroxybutyrate PHB accumulation

Media: Two media are used to promote PHB accumulation by *Ralstonia solanacearum*

Media

1) Nutrient agar with 5% sucrose
2) Mineral medium as given below

$(NH_4)_2SO_4$	0.2 g
L-glutamate	0.2 g
$MgSO_4.7H_2O$	0.2g
DL- Poly K_2HPO_4	0.2g
β-hydroxybutyrate	0.25g
Distilled water	1000 ml
pH	7.2

Grow bacteria for 24-48 h in above mentioned media before testing of PHB inclusion as a procedure given below.

Procedure: Technique A

- Sudan black B solution (0.3g in 100 ml of 70% ethanol) is prepared by shaking the solution at intervals to dissolve the dye and allow it to stand overnight.
- Make bacterial smear on a glass slide and air dry it well as well as fix the bacteria by heating.
- Flood stain Black B on the entire slide and leave undisturbed for 10-15 min
- Drain excess solution, blot dry and clear the slide with xylol (xylene) in a copling jar or by adding it with a dropper.
- Blot the cleared slide to dryness and counter satin with safranin (0.5% aqueous solution) for 5-10 sec. Wash slide in water, blot and dry the slide.
- Examine the slide under oil immersion with light microscope.

Observations: The PHB grannules are dark blue-black in colour. PHB granules also show up well like egg shaped under electron microscope medium will become brownish in colour due to the growth bacteria in positive case.

Technique B: Detection ofβ-hydroxybutyrate in bacteria through UV light using NB medium

Medium

$NH_4H_2PO_4$	1.0 g
KCL	0.2 g
$MgSO_4.7H_2O$	0.2g
DL-Poly β-hydroxybutyrate sodium salt	5.0 g
Proteose peptone number 3	20.0g
Nile blue solution (1%)	1.0 ml
NaOH	4.5 ml
Distilled water	900 ml
Agar	17.5 g
pH	7.2

Add 100 ml of filter sterilized solution of 20% glucose in the medium after autoclaving. Inoculate the test bacteria onto the medium and incubate at 26± 2ºC for 24-48 h. days.

Observations: The PHB bacterial colonies will be seen as florescence bright orange under long wave (366 nm) ultraviolet radiation. Colonies of fluorescent pseudomonads will not florescent in this medium, and the florescent granules are not visible microscopically.

Practical 10.6 Detection of pectin enzyme produced by *Xanthomonas* spp.

Requirements: Viscometer, pipette, stopwatch, 1% pectin solution in phosphate buffer, pH 5.0, 0.5% solution of pectinol (100 OD) in water, culture filtrate of 35 h old culture of Xanthomonas sp. Grown on nutrient broth + 0.5% pectin or pectic acid.

Procedure: Technique A

- Mix 1 ml of water to 10 ml of pectic solution.
- Add 6 ml of this mixture to viscometer
- Determine also the flow time for a comparable water sample.
- Boil a small sample of pectinol 100 OD (not previously heated) with 10 ml of pectin solution and add 6 ml of this mixture to viscometer.

- ❖ Record the time for the sample to run through viscometer at 0,5,10 and 20 min after enzyme sample is added to pectin solution. .

Observations: Record the time for the sample to run through viscometer at 00,5,10 and 20 min after enzyme sample is added to pectin solution. .

Practical No.11

Study of pigment production by phytopathogenic bacteria

The bacteria which produces pigments are called chromogenic bacteria. Bacteria produce two types of pigments **(i) Water insoluble**, which do not diffuse into the medium eg carotenoids-yellow colour (*Xanthomonas* spp.) and **(ii) Water soluble**, which diffuse into the medium eg.Fervenulin, toxoflavin-florescent (*Burkholderiaglumae*), pyrocverdins-green / blue fluorescent (Fluorescent *Pseudomonas* spp.), melanin- brown colour (*Streptomyces scabiei* and *Ralstonia solanacearum*)

i) Test of water insoluble pigment produced by *Xathomonascampestries*pv. *campestries*

Yeast Glucose Chalk Agar

Yeast extract	10.0 g
$CaCO_3$	20.0 g
Glucose	10.0g
Agar	20.0 g
Distilled water	1000 ml

The Dissolve yeast extract and glucose in 200 ml of water and melt agar in remaining 800 ml of water. Mix the two volumes and then add chalk. Dispense the medium into the tubes. Sterilize and prepare slants.

Observations: *Xanthomonas campestries*pv.*campestries*produces yellow pigment after 48h

ii) Test of water soluble pigment produced by *Pseudomonas fluorescens*

The water soluble florescent pigment is detected on Kings B medium

Medium King's B medium

Peptone	20.0g
Glycerine	10 ml
$K_2H.PO_4$	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	20.0 g
Distilled water	1000ml
pH	7.2

Autoclave the medium in flask and pour the sterilized medium into the petriplates. Inoculate the 48 h old culture of Pseudomonas fluorescens onto the Petriplates with inoculation needle. Incubate the petriplates for 24-48 h at 28ºC

Observations:*Pseudomonas fluorescens* produces diffusible fluorescent green/ blue pigment, a pigment zone is produced around the bacterial growth, which is easily detected especially when viewed under ultra violet light.

Practical No. 12

Characterization of biovars of *Ralstonia solanacearum*

For the differentiation of the biovars of *Ralstonia solanacearum* basedon various test including utilization of single alcohols and carbohydrates such as dextrose, mannitol, sorbitol, dulcitol, trehalose and oxidation of lactose, maltose, D(+) cellobiose and nitrite from nitrate and gas from nitrate.

Basal medium

$NH_4H_2PO_4$	1.0g
KCL	0.2 g
$MgSO_4.7H_2O$ Yeast extract	0.2 g
Peptone	1.0g
Agar	3.0 g
Bromthymol blue	80 mg
Distilled water	1000 ml
pH	7.0-7.1 ml

Autoclave the medium at 121ºC for 20-30 min and cool it to 50-60 ºC. Prepare 10% aqueous solution of different carbon sources such as dextrose, mannitol, sorbitol, dulcitol trehalsoe and oxidation of lactose, maltose, D (+) cellobiose. Sterilize all carbon sources with 0.22µm membrane filter except dulcitol which is sterilized by separate autoclaving. Add 1% of carbon sources into the basal medium, after through mixing, dispense 3 ml of molten medium into the sterilized culture tubes and allow to solidify. Add 100 µl of 48 h old culture of *R. solanacearum* into the basal medium and incubate at 28-30 ºC. Observe the tubes at 2, 4, 7 and 10 days.

Observations: The change in colour of the medium from the olivaceous green to yellow (acid pH, < 6) indicates oxidation of carbohydrate. Those biovars capable of oxidising the disaccharides will be take a few days longer to give a clear positive result.

Practical No. 13

Identification of bacteria by using Biolog system

There are approximately 4,000 named bacterial species and this is just a fraction of the total number of species in the environment. The Micro Log (Biolog) system provides the unique feature of user defined custom databases involving 91 carbon source utilization assays*23 chemical sensitivity assays. If an organism is outside the Micro Log (Biolog) data base, the user can save the pattern to a custom database for future refrence. If the organism is isolated again, the laboratory will have the pattern saved instead of simply getting a “no ID”. Some other methods provide supplemental off-line test for use alongside the identification panel. This approach is inconvenient and does not produce an expanded pattern library. Identification from the Microplate is superior to less precise methods, because: 1. The Micro Log system bases its identification on a larger number of test. There are over $4x10^{28}$ possible patterns from a single Microplate 2. This covers far more species. The larger number and more diverse range of test in the microplate provide greater accuracy and precision. Various methods have different numbers and types of organisms within their database. The MicroPlate has a much larger number of test, which provides greater fingerprint discrimination and larger database.

Procedure:

The test procedure is fast and simple, involving 5 steps, and requiring only 2 to 3 minutes hands on time per sample.

- A pure sample of a bacterium is grown on a Biolog Universal Growth w/5% Sheep blood agar plate for a 500 g jar of dehydrated powder.
- The bacteria are swabbed from the surface of the agar plate and suspended to a specific density in GN/GP inoculating fluid
- Add l50 il of bacterial suspension is pipetted into each well of microplate
- The Microplate is incubated at 30ºC or 35ºC (depending upon the nature of the organism) for 4-24 h.
- The Microplate are read either visually or with the Biolog Micro station or Omni Log System, compared to the GN database and a result is determined.

Practical no. 14

Pathogenicity test of phytopathogenic Bacteria

Practical 14.1 Pathogenicity test of *Xanthomonas campestris* pv. *Campestris* (Xcc) on cauliflower

Requirements: 48 hour old bacterial culture of Xcc, 40-45 days old plants of cauliflower, scissor, and levels tags, saline solution.

Procedure

- Grow Xcc isolates in the Petri plates containing nutrient glucose agar medium for 48 h at 28ºC
- Scrap bacterial growth from the plates and suspend it in 10 ml of sterile distilled water or saline solution (0.85%NaCl) to produce turbid suspension (10^8 to 10^9 cfu/ml)
- Inoculate the culture at the margin of the leaf by clipping secondary veins with mouse tooth forceps
- Wrap the teeth of the forceps in cotton wool to hold inoculums and dip into the bacterial suspension.
- Inoculate approximately 10-12 points per leaf, and three youngest leaves of ach plant
- The number of infected points per leaf and the severity of the sysmpotms are accessed 2 and 3 weeks after inoculation.

Scoring of resistance:

The total number of inoculated points, number of points showing symptoms are recorded and then percentage of infected points is calculated. The severity of symptoms (Plate 7, Fig. 7a and 7b) are accessed on a six point scale of 0-9 based on relative lesion size as given below

0= no symptom

1= Small necrosis or chlorosis surrounding the infected area

3= typical small V-shaped lesion with black veins

5=typical lesion half way to middle vein

7=typical lesion progressing to middle vein

9=lesion reaching the middle vein

Segregation analysis:

Plants are grouped into eight categories based on resistance scores and percentage of inoculated points showing symptoms (Vicente et al., 2002)

Resistant = a score of 0, 1or 3 with less than 25% of points showing symptoms

Partial resistance = a score of 3 with more than 25 % of points showing symptoms and with a score of 5 with less than 50 % of points showing symptoms

Susceptible = a score of 5 with more than 50% of points showing symptoms and with a score of 7 with less than 75% of points showing symptoms

Very susceptible =a score of 7 with more than 75%of points showing symptoms and with a score less than 75% of points showing symptoms

Practical 14.2 Pathogenicity test of *Xanthomonas oryzae*pv. *oryzae* (Xoo) on paddy

Requirements: 48 hour old bacterial culture of Xoo, 30-40 days old palnts of rice, scissor, and levels tags, saline solution or distilled water

Procedure (Kauffman et al., 1973)

- Select 35-40 days old rice seedlings
- Scrap bacterial growth from the plates and suspend it in 10 ml of sterile distilled water or saline solution (0.85%NaCl) adjust the OD (10^8 cells /ml)
- Cut 1-2 cm of leaf tip with scissors previously dipped in bacterial suspension, In each treatment 10-15 leaves were inoculated. To ensure better inoculation the cut leaves are also dipped in bacterial suspension
- Record data 21 days after inoculation.

Observation: Disease severity is assessed based on the estimation of percent diseased leaf area (Plate 8, Fig. 8a and 8b)

4th edition of Standard evaluation system for rice (1996)

BB Score (Green house test)	Percent lesion area
1	0-3
2	4-6
3	7-12

4	13-25
5	26-50
6	51-75
7	76-87
8	88-94
9	95-100
BB Score (Field test)	Percent lesion area
1	1-5
3	6-12
5	13-25
7	26-50
9	51-100
The percent lesion area =	Mean lesion length (cm)/ Mean lesion length (cm) x 100

Practical 14.3 Pathogenicity test of *Ralstonia solanacearum* in tomato plant

Requirements: 48 hour old bacterial culture of *R. solanacearum*,35-40 days old palnts of tomato, scissor, and levels tags, 0.85% saline solution

Procedure (Kauffman et al., 1973)

- Grow tomato plants at 25-30ºC
- Scrap 48 h old bacterial growth from the plates and suspend it in 10 ml of sterile distilled water or saline solution (0.85%NaCl) to produce turbid suspension of (10^8 to 10^9 cfu/ml)
- Make slight injury with the help of a scalpel on the root and pour bacterial suspension at root zone in soil
- Water the plants from the bottom to prevent washing the bacteria out of soil after inoculation.

Observation: The inoculated plants should be observed daily basis uoto 21 day. The plants will show wilting symptoms on the leaves initially and later whole plant. Then calculation will be done for the

average percentage wilt on each day for each treatment. Disease rating is recorded by using following scale: 1= no symptoms; 2= one leaf wilted; 3= 2-3 leaves wilted; 4= 4 or more leaves wilted; 5= whole plant wilted (dead). Calculate the wilt intensity 21 days after the inoculation, using the following formula.

$$\text{Wilt intensity (\%) (I)} = \{(n_1 x v_1) \div (V \times N)\} 100$$

The where n_1 = number of plants with respective disease rating; v_1 = disease rating (1, 2,3, 4 or 5); V= the highest disease rating and N= the number of plants observed

Practical 14.4 Pathogenicity test of soft rot causing bacteria *Erwinia carotovora* subsp. *carotovora*

Requirements: 48 hour old bacterial culture of *E.carotovora* subsp. *carotovora, healthy potato tubers / plant, needle,*scissor, levels tags, 0.85% saline solution, micropipette tip or syringe

Procedure: Two methods are used to test pathogenicity of soft rot causing bacteria as given below

i) Tissue maceration

- Take healthy potato tubers and dip the tuber in to 5.25% sodium hypochlorite solution for 10 min
- Dry the treated tuber in the air to remove free moisture. If require repeat the same procedure.
- Cut tissue into convenient size pieces, place the Petriplates on moist sterile filter paper
- Inoculate 100-1000µl of bacterial suspension containing 10^6cfu/ml from 24 h old culture
- Incubate the plates 20-27ºC for 48 h

Observation: The tissue surrounding the inoculation site will be decay and tissue maceration will be visible. It may be examined by spatula surrounding the inoculation point.

ii) Pathogenicity test

- Select healthy potato plants of 30-35 days old
- Prepare suspension of 24 h old bacteria containing 10^2 to 10^9 cfu/ml
- Inoculate the plants by using a micropipette tip/ syringe at least 8-10 plants each concentration of bacterial culture.
- Injection point should be sealed after inoculation by application of small amount of petroleum jelly over the puncture
- Keep the plant at a high relative humidity for better result.

Observation: Symptoms of soft rot will appear within 1 week of inoculation

Practical 14.5 Differentiation of virulent and avirulent colonies of bacteria.

Requirements: TTC medium, culture of phytopathogenic cultures of *Ralstoniasolanacearum, Erwinia carotovora, Pseudomonas syringae*pv. *Phaseolicola*, *Clavibactermichigenesis* subsp. *insidious*, Petriplates

Medium

Peptone	10.0g
Casamino acid	1.0 g
Agar	20.0 g
Distilled water	1000 ml

Autoclaving the medium in flask. Cool the medium at 45ºC. Add 1 ml of sterile 1% solution of 2, 3,5 trio phenyl tetrazolium chloride (TTC) into the medium and maintain 0.005% concentration of TTC in agar medium. The TTC solution should be sterilized by filtration or by autoclaving for 8 min and store in dark. Pour medium into petriplates**.**

Procedure

- Inoculate 100 µl of 48 h old culture of test bacterial culture by dilution plate technique. Spread the bacterial suspension by L-shaped glass rod uniformly.
- . Incubate the plates for 48 hat 28 ±2ºC.

Observation: Virulent and avirulent colony types may be differentiated on the basis of characterstics given below

Bacterium	Virulent strain	Avirulent strain
Ralstonia solanacearum	Irregularly round, fluidal white with pink centre	Round butyrous deep red with a narrow bluish border
Pseudomonas syringae*pv. *Phaseolicola	Intense red	Reddish white
Erwinia carotovora	Larger colonies, reduce dye more	Small colonies reduced dye slightly intensely

In case of*R. solanacearum,* within the virulent colonies more virulent can be disytingusihed from less virulent on the amount of reddening present in the centre. The more virulent has less reddening and less virulent has more reddening.

Practical No. 15

Sero-diagnostic and Fatty acid profiling of phytopathogenic bacteria

Serodiagnostic reactions between antigen and antibody are usefully exploited. The monoclonal antibody technology was developed by Kohler and Milstein (1975) allows production of unlimited quantities of antibodies against virtually any molecule. The monoclonal anti-bodies have unique epitope specificity and the reaction is reproducible. Although the monoclonal anti-body have obvious advantages over conventional polyclonal antibodies, however, it involves higher cost and great deal of labour. In most cases, the generation of polyclonal antibodies requires nothing more than the antigen, a rabbit and a syringe. The resulting polyclonal anti-serums are adequate for most needs in bacteriology at an fraction of cost of the monoclonal antibody production. Antibodies have been used extensively and in many test formats to detect and identify bacteria. The most popular and successful formats include agglutination, the enzyme linked immunosorbent assay (ELISA), Immunofluorescence (IF), lateral flow strips test and flow through assays.

Practical 15.1 Antigen preparation from *Xanthomonas campestries*pv. *campestries*

*X. campestries*pv.c*ampestries*(Xcc) is isolated from the infected cauliflower leaf on the nutrient agar (NA) plates by using standard procedure. Individual colonies appearing yellow, raised and mucoid are isolated and repeatedly subcultured for pure colonies and subjected to confirmatory test such as pathogenicity, hypersensitivity and biochemical tests. The Xcc is cultures on the nutrient broth under static conditions for 48 h at 27ºC and harvested by centrifugation at 3000 rpm for 10 min and cells suspended in 20 mmol/l phosphate-buffered saline (PBS; pH 7.4). These intact whole cells are washed three times in the same buffer and used as antigen for immunization and ELISA.

Sheep erythrocytes (SRBC) as carriers

Requirements: Both carbohydrates and protein antigens can easily be adsorbed on to ship erythrocytes to render them agglutinable with antiserum specific for the “add on” antigens. SRBC can easily used as a carrier for the purified carbohydrate antigens, which by themselves are normally not very immunogenic in rabbits. By treating SRBC with 0.005% tannic acid, low molecular weight protein antigens can also be adsorbed**.**

Attachment of crude Lipopolysaccharide (LPS) onto SRBC

- Boil an overnight culture of gram –ve bacteria for 1 h; centrifuge it at 2,000 rpm for 10 min to sediment cell debris. The supernatant extract contains crude LPS antigen extract

- Mix 4.5 ml of bacterial supernatant extract to 6 ml of 2.5% SRBC at a bacterial extract to SRBC ratio of 3:4 (v/v). Isotonic solutions such as phosphate buffered saline (PBS) or Alserver's solution should be used for all manipulations of SRBA suspension. Incubate for 30 min with occasional shaking.
- Sediment SRBC by centrifugation at 200x for 10 min. and wash the cell twice with 10 ml saline or isotonic buffers.
- Resuspend the pellet with 6 ml of PBS. The suspension is now ready for injection.

Requirements:

(i) Alsever's solution (citrate saline solution)

Alsevers solution is an isotonic, anti-coagglutant blood preservative that permits the storage of whole blood at refrigerated temperature for 10 weeks or more.

Dextrose	20.5g
Sodium citrate (anhydrous)	8.0 g
Citric acid (anhydrous)	0.55 g
Sodium chloride	4.2 g
Distilled water	1000 ml

(ii)Tanning of SRBC for coating with protein antigens

- Add 3 ml of 0.005% tannic acid into the centrifuge tube containing 3 ml of 2.5% SRBC. Incubate at 37ºC for 10 min.
- Centrifuge the cells at 2000 rpm for 10 min and wash once in 5 ml of PBS. Centrifuge as before, and Resuspend the pellet in 3 ml of PBS
- To each centrifuge tube containing 3 ml of tanned SRBC and 3 ml of 0.3 mg/ml soluble protein, mix gently and incubate at 37ºC for 15 min.
- Centrifuge, and wash twice as above and Resuspend each pellet in 3 ml of PBS diluent. These cells are now ready for injection.

iii) Bacterial cells as carriers

Bacterial smooth LPS containing O-antigen sugars are extremely immunogenic, while it is often difficult to raise antibodies to the LPS core oligosaccharide epitope of gram negative organism. To

elicit a response to epitope of the core region, antigens such as pure oligosaccharide, lipid A, or roughLPS can be attached to bacterial cells of a rough strain.

- Prepare $5x10^9$cfu/ ml heat killed cells of the rough mutant per ml of 1% (v/v) acetic acid
- Heat the cell suspension to 100ºC for 1 h
- Wash three times in distilled water
- Lyophilise
- Dissolve lipid A or rough LPS in 0.5% (v/v) diethylamide at a concentration of 1mg/ml then add the lyophilized acid-treated bacteria to final concentration of 1mg/ml
- Stir slowly for 30 min at room temperature.
- Dehydrate the mixture in vacuo with speed-vac centrifuge.

Antiserum production

Circulating anti-bodies against a specific antigen do not appear in significant amounts until at least 7 days after an immunization. Most of the antibodies of an early (or primary) response are of the immunoglobulin M (IgM) class, whereas antibodies from latter (secondary) response are often will be mainly IgG. However, if the antigen used is pure carbohydrate, the secondary response will mainly be IgM. The amount of antibody formed after a second or booster injection of any given antigen is usually much greater than formed after first injection. Therefore, for the production of high titre antibodies the following schedule may be used for the rabbit.

- Raise the anti-body in a three month old female albino rabbit.
- Collect pre immune serum (before the first injection) for use as control to detect cross reactive antibodies.
- Mix bacterial cells (10^5/ml) in a proportion of 1:1 with Freunds complete adjuvant.
- Inject 0.1 ml of cells (1x 10^4 /ml) containing approximately 100 mg protein at four sites subcutaneously
- At the 4^{th} day, repeat 1 day injection steps with Freund's incomplete adjuvant. After this step, allow animal to rest for 14 days so that primary response subside to a base line level otherwise a secondary response may not be achieved.
- At the 18^{th} day, inject 1.0 ml (1:1 mixture of protein antigens with Freunds' incomplete adjuvant) intramuscularly into the thigh muscles of one leg of the animal.

- At 22 nd day, bleed and collect serum.
- At 25th day, repeat the above step of intramuscularly injection on another leg of the animal.
- At 29th day, bleed and collect serum and pool the serum with previous sample.

The intramuscularly injections may be kept up once a month or once every 2 weeks, and serum may be collected 3 to 4 days later. To avoid batch-to batch variability, the immune sera collected at different times should be pooled. Variations may be made at some of the steps; for example, intravenous injections without adjuvant, may be given as booster (on day 18 and 25) to elicit a more rapid response before bleeding to collect the antibodies.

Serum collection

Blood should be collected in a dry, sterile container without any coagulant. Routinely blood is withdrawn from the marginal vein of animal. A small cut (superficial venesection) to a dilated vein with the tip of a fresh scalpel blade will open it up. The droplets of blood that emerges are collected in a suitable container, usually a screw-cap glass bottle. With sufficient practice 20-30 ml of blood can easily be obtained. Blood withdrawn from animals should be allowed to clot at room temperature for 30 min. Serum can then be separated and centrifuged to get rid of all blood cells. Alternatively, the blood can be kept to clot at 4 ºC overnight for complete shrinkage of clot before removing serum. The latter procedure will yield more serum per litre of blood and will preserve anti-bodies from hydrolysis by the action of naturally occurring IgG proteases.

Practical 15.2 Sero based diagnostic assay of phytopathogenic bacteria Antigen

ELISA Protocol

ELISA is carried out on a 96 well microtiter plate (Nunc, Denmark) (Hobbs et al., 1987; Rajeshwari et al., 1998). Results are quantified by measuring the absorbance at A 410nm using the microtiter plate reader (MR 5000; Dynatech).

Reagents for ELISA:

(i) Coating buffer: 0.05M carbonate-bicarbonate buffer (pH 9.6)

Solution A Sodium carbonate	21.2g/l
Solution B Sodium bicarbonate	16.8g/l

To prepare the working buffer, mix 20 ml of solution A and 42.5 ml of solution B and adjust volume to 250 ml with distilled water. The pH should be 9.6 add 0.05g of NaN3. Store at

4 ºC in a dark bottle for upto 4 weeks.

(ii) PBS : (pH 7.4)

Solution A: $NaH_2PO_4.2H_2O$	31.2g/l
SolutionB:Na_2HPO_4	16.8g/l
or	
$Na_2HPO_4.2H_2O$	71.7g/l

The working buffer is prepared as follows: Mix 47.50 ml of solution A and 202.50 ml of solution B; adjust the volume with 800ml distilled water. Add 8.75g of NaCl and make up the volume upto 1000ml. The pH should be 7.4

(iii) Wash buffer: PBS containing 0.05% Tween 20

(iv) Blocking reagent: PBS containing 5% (w/v) skim milk. This should be made fresh

(v) Reagents for alkaline phosphatase conjugates

Substrate buffer: Sodium carbonate (0.05M;pH 9.8) containing 10 mM $MgCl_2$.To prepare the substrate buffer, us the buffer stocks made for the coating buffer (Step 1). Mix 27.5 ml of solution A with 35 ml of solution B and make upto 250 ml with distilled water. Add 0.05 g of $MgCl_2.2H_2O$. check the pH before use. Store in dark at 4 ºC.

Alternatively use 1M ethanolamine buffer pH 9.8 containing 0.5mM $MgCl_2$.

AP substrate: Dissolve in the buffer 1mg of p-nitro phenyl phosphate per ml.

the substrate can be obtained from Sigma in the form of 5mg tablets. Store the substrate at -20 ºC. Read the yellow colour at 414nm. Colour development can be stopped by the addition of 10 ml of 3M NaOH to the wells.

vi. Reagents for alkaline phosphatase conjugates

Substrate buffer: Make citric Sodium carbonate (pH 4.0) by dissolving 0.2 g of citric acid in 90 ml of distilled water. Adjust the pH 4.0 with 1M NaOH. Store at room temperature.

HRPO substrate: Immediately before use add to 10 ml of citric acid buffer, 5 ml of 30% H_2O_2 and 75 ml of 10 mg/ ml {2, 2-azino-bis(ethylbenthiazoline)-6 sulphonic acid}.

Alternatively use 1M ethanolamine buffer pH 9.8 containing 0.5mM $MgCl_2$.

Read the green at 414 n. Colour development can be stopped by addition of 0.08M NaF to be to wells.

Immunoblot assay

As Xcc positive and specific in ELISA reactivity, an immunoblot assay is performed to identify the antigenic determinant molecule in a pathogen. An equal number of bacterial cells should be loaded

in duplicate into 12% SDS-PAGE in a mini gel apparatus (Genie Pvt, Bangalore, India). After electrophoresis, the first half with the marker proteins is silver
stained for visualizationof the protein bands and the second half of the gel transfer to nitrocellulose membrane. The nitrocellulose paper should be probed with 1:500 PAb-Xcc
followed by 1:1000 alkaline phosphatase-conjugated swine ant-rabbit IgG. 5-bromo4-chloro-3-indoolyl phosphate is used as the chromogenic substrate, which on the alkaline phosphatase activity, yielded a reduced, stable purple-coloured complex with a band, which reacted specifically with PAb-Xcc (Kotani and Mc Garrity, 1985).

Purification of PAb-Xcc by Sephacryl-200-HR column chromatography

The anti-serum is subjected to 50% (w/v) ammonium sulphate precipitation and loaded onto the degassed column packed with Sephacryl-200-HR (65x1.0cm); 1-ml fractions are collected at a flow rate of 18 ml/h and fractions monitored at 280 nm spectrophotometrically (U-2000; Shimadzu, Japan). Subsequent peak fractions are pooled at peak1, P1Ab-Xcc, and a peak 2, P2AB-Xcc, and examined for antibody reactivity by ELISA. After lyophilisation; they are evaluated for antibody titre, sensitivity and detection of cells as well as culture filtrates. Crude antibody is also included in the experiment. Appropriate controls, such as detection of cells as well as culture filtrates. Crude antibody is also included in the experiment. Appropriate controls, such as detection with pre-immune serum and irrelevant xanthomonads, are prepared. The put on pare or more budlets two peaks showed differential reactivity with the virulent and avirulent isolates in ELISA.

Characterization of Polyclonal antibodies- Xcc

To determine sensitivity and antibody titre. Antigen (10-50 ml) at $10^2$and 10^5 cells/ml is loaded onto the ELISA plate in different rows and tested with 100ml developed antibody at various dilutions, 1:500, 1:1000, 1:2000 and 1:4000/ well. The ELISA reactivity was examined and the maximum dilutions showing significant reactivity is considered as the antibody titre.

Evaluation of specificity

Various xanthomonads such as *X. campestries*pv. *campestries* (Xcv), *X. axonopodis* and *X.oryzae*pv. *oryzae*(Xoo) are tested for cross reactivity at 10^4 and 10^2 cells/ ml with different doses of antigen (10-50ml/ well). Culture filtrates (100ml) of all these organisms at equal cell concentration of 10^8 cells /ml are also examined by ELISA.

Practical 15.3Fatty acid methyl ester (FAME) analysis for bacterial identification

A fatty acid methyl ester (FAME) is the product of reaction between fats or fatty acids and methanol. Every microorganism has its specific FAME profile and FAME is used for microbial identification.

Using FAME analysis one can know the types and proportions of fatty acids present in cytoplasm membrane and outer membrane (gram negative) lipids of bacterial cell. More than 300 fatty acids are now in bacteria. From FAME analysis one can know lengths, bonds, Ning's, and branches of the fatty acid. The simplified steps of FAME analysis are discussed below

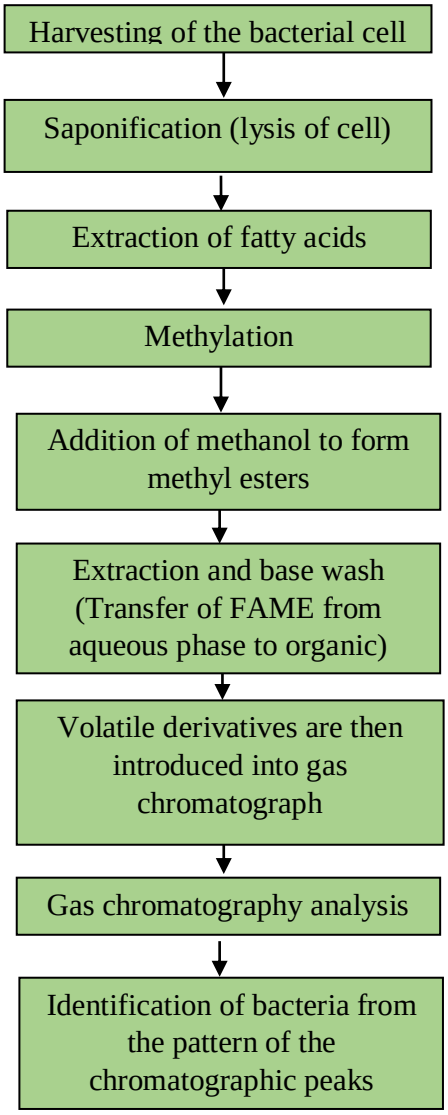


Fig8: Flow chart for the analysis for fatty acid methyl ester for bacteria

Practical No. 16

Molecular Techniques for the Characterization and Identification of Bacteria

For the detection and identification of plant pathogenic bacteria, DNA based molecular technique is an indispensable tool. The main advantage of DNA based method is that reliability, accuracy, sensitivity and fastness. The identification assay is not dependent on the environmental conditions, age and physiological stage of the target pathogen. However, some techniques are dependent upon the quality of extracted DNA.

Practical 16.1 Extraction and quantification of total genomic DNA OF bacteria

i) **Characterization Rapid extraction of bacterial genomic DNA with guanidium thiocyanate (GES methods) and quantification** (Pitcher et al., 1989)

This procedure yields high quality DNA which is useful for most applications in bacteriology for DNA homology studies, RFLP analysis and PCR

Materials and preparation of solutions

1. 10 ml disposable inoculation loop
2. 0.5 M EDTA pH 8

   Prepare 1000 ml using:

Sodium EDTA	186.1g
NaOH	20.1g
Deionised water	800 ml

Adjust pH 8.0 with 5N NaOH. Adjust final volume 1000 ml with deionised water

3 100 ml x1.0 ml Tris 0.1 M EDTA

Prepare 1000 ml using:

Tris-HCl	186.1g
Distilled water	20.1g
Adjust pH 8.0 by adding 42 ml concentrated HCl	800 ml

0.5 M EDTA pH 8.0	200 ml

Adjust final volume 1000 ml with deionised water

4 1X TE buffer, 10 mM Tris, 1Mm EDTA, pH 8.0

Prepare 1000 ml using

100X TE Tris, pH 8.0	10 ml
Distilled water	990 ml

5 GES solutions- Guanidine thiocyanate-EDTA sarkosyl

Prepare 100 ml GES solution:

Guanidine thiocyanate	186.1g
0.5M EDTA pH 8.0	20.0 ml
Add 20 ml water	800 ml
Dissolve all ingredients at 65ºC, cool down	
Add 1.0g N- Lauryl sarkosine	

Adjust volume to 100 ml with distilled water. Filter sterilize using a 0.45mm filter, store at room temperature.

(Guanidine thiocyanate is harmful; use protective wear.)

6 Resuspension buffer -0.15M NaCl, 0.01 M EDTA, pH 8.0

Prepare 1000 ml using:

NaCl	8.77g
0.5M EDTA, pH 8.0	20 ml

Adjust volume to 1000 ml with distilled water

7 Ammonium acetate 7.5 M

Prepare 1000 ml using

Ammonium acetate	578.1g

8 Chloroform/ iso-amyl alcohol 21:1 ratio (v/v)

Prepare 1000 ml using

Isoamyl alcohol	40 ml
Chloroform	960 ml

Note: Chloroform is highly toxic; wear suitable protective clothing and work under a fume hood.

9 Chloroform/ RNAse solution (2.5mg/ml)

RNAse	50mg
Sterile distill water	20 ml

Incubate 10 minutes at 100 ºC, aliquot in working solutions and store at -20ºC

10 RNAse solution (250mg/ml); prepare 1 ml using

RNAse solution (2.5mg/ml)	100 ml
Sterile distill water	900 ml

Note: All materials need to be sterilized by autoclaving except solution number 5,8,9 and 10

Procedure (Kauffman et al., 1973)

- Take a well grown 24-48 h old culture on agar plate/broth in 1.5 ml tube
- Harvest cells with loop, homogenise/ vortex if grown in agar plates
- Centrifuge 3 min at 13000 rpm and then remove supernatants carefully and tubes having bacterial pellet should be put on the ice
- Add 100 µl 1X TE (Tris-EDTA) pH 8 and homogenise (vortex or use a pipette)
- Add 500 µl GES (the cells are lysed by sarcosyl and guanidium thiocyanate, the latter is strong denaturing agent and inactivates endonucleases. Keep on ice always.
- Gently shake by hands until the solution becomes viscous because of cell lysis
- Add 250µl ice cold chloroform : isoamyl alcohol (24:1)

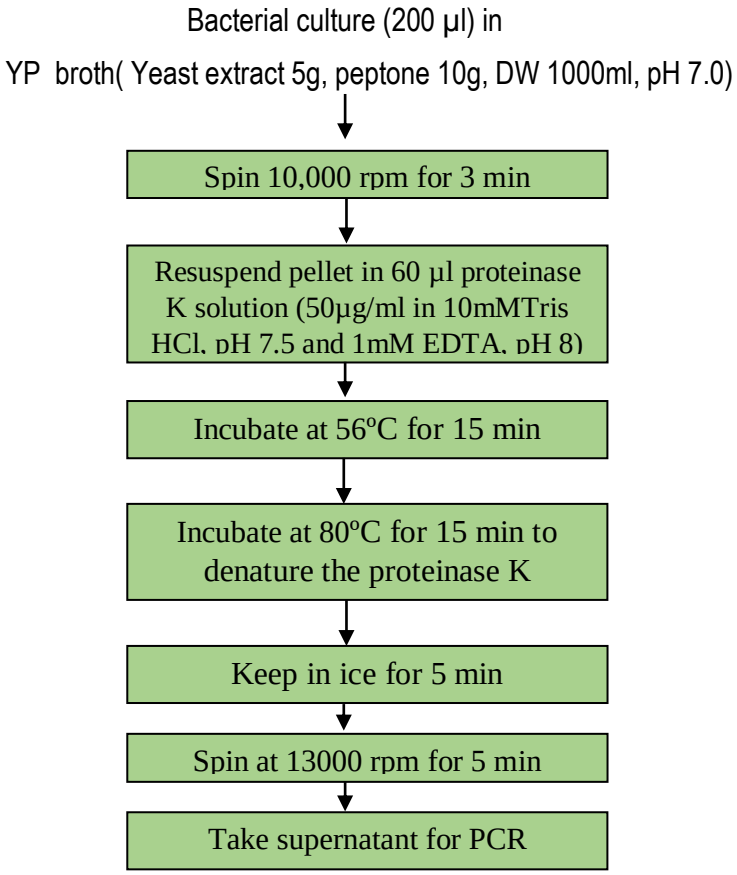
- Shake well till homogenous milky solution is obtained.
- Centrifuge approximately 10 min at 13000 rpm until upper phase is clear
- Prepared numbered Eppendorf tubes with 400 µl isopropanol ready in deep freezer at -20ºC
- Take off 700 µl DNA solution (Clear upper phase) with 1000 µl tips carefully, whixch the top is cut off (to make the opening wider to avoid DNA from shearing) and trabnsfer to Eppendorf with ice cold
- Shake gently till white cloud of precipitated DNA becomes visible
- Centrifuge for 10 min at 13000 rpm
- Remove the supernatant carefully and the tubes having pellet
- Add 180 µl absolute ethanol (70%) for washing and centrifuge for 1 min at 13000 rpm
- Remove ethanol with 200 µl pipette carefully and repeat washing three times
- Dry DNA pellet in desiccators or until pellet becomes clear (do not dehydrate DNA)
- Add 100µl 1X TE pH 8 and flick to uniform mixing
- Keep in fridge until DNA is dissolved (or at room temperature if it does not dissolve easily)
- Store DNA solution at -20ºC

ii) Bacterial DNA isolation by CTAB method (Murray and Thompson, 1980)

- For each isolate, inoculate a single colony (from) in pre-autoclaved 1.5 ml Eppendorf tubes containing 1.0 ml NSB broth and keep for inoculation at 28ºC for 48 h with vigorous shaking. Take atleast two tubes per isolate
- Harvest the bacterial cell by centrifugation for 5 min at 15000 rpm in centrifuge to pellet
- Remove the supernatant and Resuspend the pellet in 576 µl TE (1X) buffer, 30 µl of 10% SDS and 3 µl proteinase K (20 mg/ml) to give a final concentration of 100 µg/ml. Mix thoroughly and incubate for 1 h at 37 ºC.
- Add 100 µl of 5M NaCl and mix thoroughly.
- Add 80 µl of CTAB/NaCl, mix thoroughly.
- Add equal volume of phenol:chlroform: isoamyl alcohol (25:24:1) shake vigorously and centrifuge for 10 min at 13000 rpm

- Transfer the aqueous viscous supernatant to a fresh tube and add 0.6 vol isopropanol to precipitate the nucleic acids. Shake the tubes, white DNA precipitates clearly visible.
- Wash resulting pellet with 70% ethanol, dry and dissolve in 50 µl TE buffer.
- Digest the RNA contaminant by adding 5 µl of RNAse (100 µl/ml)

iii) Rapid DNA extraction protocol (50 min)



Quantification of DNA by Fluorometry

- To ensure that stock DNA is adequately dissolved heat for 1 min at 65 ºC.
- Take 10 µl of DNA and add 390 µl of sterilized distilled water in 1.5 ml Eppendorf tubes
- Measure optical density (OD) at 260 nm (Warm up the UV spectrophotometer for at least 30 min)
- Wipe out the ouster surface of cuvette using lint free tissue paper before placing the cuvette in the spectrophotometer
- Pipette 1 ml distilled water as blank standards and adjust zero

- Rinse the cuvette with water. Do not change the scale settings
- Fill 1 ml of sample in cuvette and select read sample programme on screen monitor
- If the reading is greater than the standard concentration, use a higher standard concentration, if the reading is low, use a lower standard concentration. If fluctuation ifs frequent or variable pre heat the instrument for longer
- If the standards has not been changed repeat it
- It is best to recalibrate the instrument after every 10 sample reading
- Calculate concentration of purified DNA (assume ODI= 50µg/ml DNA; use the following formula: ODx50 µg x DF = original DNA concentration in µg/ml). Dilute

Quantification of DNA by Nano drop spectrophotometer

- If Put 1µl of DNA into nanodrop and record the quantity of DNA in nanogram/ µl
- Purity of DNA samples, OD values are recorded at 260 nm and 280 n. The A260/A280 nm ratio around 1.9 (1.85-1.9) indicate best quality of DNA
- It is best to recalibrate the instrument after every 10 sample reading

Practical 16.2 Isolation of plasmid from *Pseudomonas fluorescens.*

Preparation of stock solution

1. Tris-HCl (1M)-pH 8.0 157.6g/l
2. EDTA(0.5M) 146.12g/l
3. Sodium acetate (3M) 100.0g/l
4. NaOH (12N) 480g/l
5. Glucose (1M) 180.16g/l
6. SDS (10%) 100ml/ 900 ml SDW

Preparation of working solution

Working solution Chemical	**Stock solution**	**Working solution**	**Amount/ 100 ml**
A: Solution I			
Tris-Cl (pH 8.0)	1M	25mM	2.5 ml

EDTA (pH 8.0)	0.5M	10 mM	2.0 ml
Glucose	1M	50 mM	5.0 ml
Lysozyme	-	-	200 mg
SDW	-	-	90.5 ml
B. Solution II			
NaOH	12N	0.2N	1.66
SDS	10%	1.0%	10 ml
SDW			88.44 l
B. Solution II			
Sodium acetate	3M	3M	100 ml

Preparation of working solution

- Inoculate the culture in nutrient broth at 37 ºC and 200 rpm
- Transfer about 1.5 ml culture in micro centrifuge and centrifuge it at 10,000 rpm for 5 min
- Resuspend the pellet in 100 µl of solution (1) and incubate it at room temperature for 5 min.
- Then add 200 µl solution (2) gently mixed and incubate in ice 5 min
- Then add 150 µl of solution (3) and mix gently by inverting the tube 5-6 times and incubate it on ice for 1h
- Centrifuge the mixture at 4 ºC, 12000 rpm for 20 min
- Decanted the clear supernatant to a fresh centrifuge tube and again centrifuge after incubating on ice for 10 min
- Mix the supernatant with equal vol. of isopropanol and incubate at room temperature for 30 min
- Precipitate the plasmid DNA by centrifugation at 4 ºC, 12000 rpm for 20 min
- Air dry the plasmid DNA for 30 min
- Dissolve the pellet in 30µl 1XTE buffer and directly use for gel electrophoresis

Practical 16.3 Fingerprinting of *Xanthomonasoryzae*pv. *oryzae* by Rep-PCR

(Taken from the laboratory handbook on PCR-based DNA fingerprinting technique (1995) prepared by M.L.C George for the Asian Rice Biotechnology Network, IRRI**)**

PCR reaction cocktail

Components	Final Concentration	Vol. needed (25µl/sample)
Sterilized dist. water		7.75
10X PCR buffer	1X	2.50
1M Tris, pH. 9.5		0.75
DMSO	10%	2.50
625µM dNTP	185 µM each dATP, dCTP, dTTP,dGTP	7.50
Primer J1***	0.5 µM	**1.25**
Primer J1***	0.5 µM	**1.25**
Taq polymerase[3]	2.5 units/ µl	**0.50**
Cocktail volume*Total		**24.0**
DNA	50 ng/ µl	1.0 µl separately for each sample
Total vol. for PCR		25.0 µl

10X PCR Buffer**:** 100 mM Tris HCl (pH 8.3), 500mMKCl, 15mM $MgCl_2$and 0.1gelatin;

DMSO-Dimethyl sulfoxide, Taq polymerase 1.0 µl- approximately 2.5 units

*Adjust the volume if the amounts of other components are changed

**Primer 1 (JEL1) Forward 5'-CTCAGGTCAGGTCCGCC- 3'

Primer 2 (JEL2) Reverse 5' GCTCTACAATCGTCCGC3'

Perform PCR reaction using following profile

Step	Temperature(ºC)	Time (Min.)	Number of cycles
1	94	1 min	1
2	94	10 sec	
3	62	1 min	
4	65	10 min	30
5	65	15 min	1
6	4	store	

Visualization of PCR product: Load PCR amplified products on to a products onto 0.75% agarose gel (1.5 g agarose+2.25 g synergel). Load on a 0.75% synergel gel/0.5% agarose gel (equivalent of 2% agarose) in 0.5X TBE buffer and run for 6 h at 125 volts (eg. to make 300 ml gel mix 1.5g agarose and 2.25g synergel^{Tm}). In preparing 0.75% Synergel^{Tm}/0.5% agarose gel make sure to mix the two components in a dry flask before adding the buffer. Then swirl to homogenize before microwaving. Synergel is from diversified Biotech, Newton, MA.

3.Loading buffers

The loading buffer gives colour and density to the sample to make it easy to load into wells. Also, the dyes are negatively charged in neutral buffers and thus move in same direction as the DNA during electrophoresis. This allows to monitor the progress of the gel. The most common dyes are bromophenol blue (Sigma B8026) and xylene cyanol. Density is provided by glycerol or sucrose.

Typical recipe of loading dye

- Bromophenol blue or Xylene cyanol 25mg
- Sucrose 4g
- H_2O to 10 ml

After electrophoresis, transfer the gel to an ethidium bromide bath (60 ml/10 mg/ litre stock solution for 30 min and per litre of TAE buffer or water for 30 min and distain in 1X TAE buffer or water 30 min. Photograph the gel on an ultraviolet transilluminator, using an orange UV filter and positive film eg. Digital images are more suited to computer assisted pattern analysis.

Practical 16.4 Detection of *Xanthomonas oryzaepv. oryzae*from leaf samples of paddy by PCR

Preparation of bacterial ooze

- Cut out 10 very thin (about 1 mm) cross sections of leaf containing the advancing tip of the lesions
- Soak the leaf sections in 200 µl of sterile distilled water for atleast 1 hr.
- Spin down at maximum speed for 5 min at 12000 rpm
- Decanted the water leaving behind 25 µl in the tube
- Pipette up and down to resuspend the cells and use 5 µl of ooze as template

PCR reaction cocktail

25 µl Reactions	For one reaction
10 mM dNTP	0.4 µl
10X Buffer	2.5 µl

Taq polymerase	0.4 µl
10 µM Primer F	0.8 µl
10 µM Primer R	0.8 µl
25mM MgCl2	0.6 µl
PCR water	13.5 µl
20 ng DNA sample	5 µl

Thermocycler Program

Step	Temperature(ºC)	Time (Min.)
1	94	2 min
2	94	1 min
3	62	1 min
4	72	2 min
5	Repeat 2-4 for 40x	
6	72	5 min
7	4	Forever
8	End	

Primer product size: 964 bp

TXT: 5'- GTCAAGCCAACTGTGTA3'

TXT4R' CGTTCGCGCGCCACAGTTG3'

Gel electrophoresis Agarose 2.0%, Voltage 100v; Period of running gel;2h

Visualization: Alfaimagar soft is used to visualize the gel by UV light

Practical 16.5 Detection of *Xanthomonas campestriespv. campestries*from leaf samples of paddy by PCR

Preparation of sample

- Collect black rot infected leaves of cauliflower
- Cut the infected leaves into 10 very thin (about 10 mm) cross sections and soak in 200 200 µl of sterile distilled water for atleast 1 h.
- Streak bacterial ooze in the nutrient sucrose medium and incubate for 72 h in BOD incubator at 28ºC.
- Pick up the bacterial colonies that appear on the Petriplates i.e. light yellow mucoid raised colonies directly used as DNA template as discussed in above methods

Primer product size:619 bp

Primer pair DLH 120 Forward 5'- CCGTAGCACTTAGTGCAATG3'

DLH 125 reverse: 5' GCATTTCCATCGGTCACGATTG-3'

PCR reaction cocktail

25 µl Reactions	For a reaction
10 mM dNTP	0.5 µl
10X Buffer	2.5 µl
Taq polymerase	0.5 µl
10 µM Primer F	0.5 µl
10 µM Primer R	0.5 µl
25mM MgCl2	1.5 µl
PCR water	19.0 µl
20 ng DNA sample used as the bacterial colony	

Thermocycler Program

Step	Temperature(ºC)	Time (Min.)
1	94	3 min
2	95	40 sec
3	63	40 sec
4	72	40 sec
5	Repeat 2-4 for 40x	
6	72	5 min
7	4	Forever
8	End	

Gel electrophoresis Agarose 2.0%, Voltage 100v; Period of running gel;2h

Visualization: Alfaimagar soft is used to visualize the gel by UV light

Practical 16.6 16S rRNA gene sequence based bacterial identification

It is a standard technique for the identification of unknown bacteria. The results of 16S rRNA sequence analysis are not influenced by environmental conditions such as the nutrition, temperature and age. Hence, the information is less variable and can be interpreted more precisely than the physiological or the biochemical test.In bacterial 16S rRNA gene is highly conserved with few variations. The combination of similar and variable sequences is useful for the identification of genera and species of bacteria.

Sequence analysis of 16S rRNA gene has excellent powers of discrimination from the level of domain (Starting at 50% homology) to moderately related species (97.5%) similarity. These are often species specific and present in multiple copies in bacterial genome. Thus they make excellent targets for identification of the bacteria at the species level. Ribosomal RNA is naturally amplified. When the bacterial cells are grown, usually more than 103 ribosomes and many copies of 5S, 16S, 23S rRNA genes are found.

The sequencing of 16 S rRNA gene is amplified by PCR using universal primer primers (Normand et al., 1992)

Primer pair FGPS6-63: 5' GGAGAGTTAGATCTTGGCTCA

FGPL132-38: CCGGGTTCCCCATTCGG-3'

PCR reaction cocktail

10X Buffer with 15mM $MgCl_2$	5.0 µl
dNTP 25 mM	0.4 µl
Primer FGPS6-63 10 µM	1.0 µl
FGPL132-3810 µM Primer	1.0 µl
Taq polymerase (5 unit)	1.5 µl
PCR water	39.6 µl
DNA sample	1.0 µl

Thermocycler Program

Step	**Temperature(ºC)**	**Time (Min.)**
1	94	3 min
2	94	1 min
3	55	30 sec
4	72	2 min
5	72	5 min
6	4	Forever
7	End	
8	Step 2-4 repeat 35 cycles	

Gel electrophoresis Agarose 2.0%, Voltage 100v; Period of running gel;2h

Visualization: Alfaimagar soft is used to visualize the gel by UV light

Practical 17.

Multilocus sequence typing (MLST) of phylogenetic bacteria

Multilocus sequence typing is a valuable molecular technique widely used for the epidemiological studies. It is one of the most powerful aspect of MLST is its ability to detect and measure the recombination. This is a PCR amplification based method, wherein housekeeping genes along with their conserved genes are analyzed according to nucleotide variation for the characterization of the bacterial pathogens. MLST directly measures the variations in the DNA sequence of the set of housekeeping genes and characterise strains by their unique allelic profile. The principle of MLST is simple technique involving PCR amplification followed by DNA sequencing. Nucleotide differences between strains can be checked at a variable number of genes µm depending on the degree of discrimination desired. The work flow of MLST involves 1) data collection, 2) data analysis and 3) Multilocus sequence analysis. In the first section, definitive identification of variation is obtained by nucleotide sequence determination of gene fragments. In the data analysis all sequences are assigned a sequence type (ST). If any new alleles or STS are found, they are stored in the database post verification. In the final sections of MLST, the relatedness of the isolates is made by comparing the allelic profiles. A huge set of data is produced during identification process so, bioinformatics issued to manage, align, arrange and merge all biological data.

Procedure

- Extract the total genomic DNA from *Xanthomonas campestries* strains according to the DNA isolation protocol
- 5µl of stock DNA is diluted 10 times by adding 45 µl of water and measure the quantity by using bio spectrophotometer and quality is accessed by gel electrophoresis
- Slight eight genes, seven housekeeping genes,*atpD* (ATP synthetase beta chain), *dnaK* (heat shock protein 70, molecular chaperone DnaK), *efp* (elongation factor P), *gln A* (glutamine synthetase I), *gryB*(DNA gyrase subunit B, *rpo D* (RNA polymerase sigma 70 factor and *tpiA(*triosephosphate isomerase) and one gene coding for a transmembrane protein *fyu A* (Ton B-dependent receptor for PCR and subsequent
- Prepare a 20 µl reaction mixture containing 20 mM of each dNTP, 400 nM of each primer, 1.5 mM $MgCl_2$ I unit of Taq polymerase and 50 ng of genomic DNA
- Run the PCR cycler each consisting of 35 cycles each of 50 sec at 94ºC., 50 sec at appropriate annealing temperature. And 1 minute at 72ºC with initial denaturation of 3 min at 94ºC and final extension for 7 min at 72 ºC.

- Purify the PCR amplicons and sequence on both strands by using primer defined in table 1
- Sequence the internal regions of the gens (400-500 bp) using another set of specific primers
- Do the sequence analysis by using the sequence analyser (vector Nti , CLC sequencer viewer, Bio edit etc)
- Do the blast analysis for the internal sequences in MLST data base.
- Assign each sequence of gene allele number and combine allele number for ach isolate defined the sequence type. Group sequence type into clonal complexes using BRUST v3 (http://ebrust,mlst.net). The majority of MLST database are hosted at 2 web servers currently located at imperial college London (mlst.net) and Oxford university (pubmlst.org).

Observations

A clonal complex constraint strains that have closer allelic profile including single locus, double locus variants (DLVs) i.e. strains that differ at two loci and satellite i.e strains that differ at there or more loci. The program identified the putative ancestral genotype which is ST with most single locus variants. The sequences of alleles that differ between the ancestral ST with the associated SLVs arte compared and were assigned as resulting from either recombination replacement or a point mutation. If an allele arises by a point mutation then it differs from the ancestral allele at a single nucleotide site; *denovo* point mutation will result in an allele that is likely to be unique within the data set. Those that differ at multiple nucleotide sequence but which occur several times in a data set are assigned by recombination. The role of recombination relative to the role of point mutations is creating diversity will be measured by the determining ration of recombination to point mutation (r/m ratio) per allele per nucleotide.

Table 1.Primers used for PCR amplification and sequencing of *Xanthomonas compestries*

Target gene	PCR Primer Name	Ta (°C)	Sequence (5'- 3')	Sequencing Primer Name	Sequence (5'- 3')
atpD	P-X-ATPD-Fb	60	GGGCAAGATCGTTCAGAT	emiATPD1F	TTCAGATCATCGGCGCGGT
	P-X-ATPD-Rb		GCTCTTGGTCGAGGTGAT	emiATPD2R	TTGGTCGAGGTGATGCGCT
dnaK	P-X-DNAK-Fb	62	GGTATTGACCTCGGCACCAC	emidnaK1F	ACCAAGGACGCCGAAGTGCT
	P-X-DNAK-Rb		ACCTTCGGCATACGGGTCT	emidnaK2R	CGATCGACTTCTTGACCAGG
efP	P-X-EFP-Fb	62	TCATCACCGAGACCGAATA	emiefp1F	TCACCGAGACCGAATACG
	P-X-EFP-Rb		TCCTGGTTGACGAACAGC	P-X-EFP-Rb	TCCTGGTTGACGAACAGC
glnA	P-X-GLNA-Fb	62	ATCAAGGACAACAAGGTCG	emiglnA1F	GCTGATCAAGGACAACAAGG
	P-X-GLNA-Rb		GCGGTGAAGGTCAGGTAG	emiglnA3R	ACTTCATCGTCAGCAGTTCG
gyrB	emigyrB1F	60	TGCGCGGCAAGATCCTCAAC	emigyrB2F	CGCTACCACCGCATCATCCT
	emigyrB4R		GCGTTGTCCTCGATGAAGTC	emigyrB3R	AGGTGCTGAAGATCTGGTCG
rpoD	emirpo11F	62	ATGGCCAACGAACGTCCTGC	emirpo27F	GAAATCGCCATCGCCAAGC
	mirpo13Re		AACTTGTAACCGCGAC GGTATTCG	emirpo12R	CGGTTGGTGTACTTCT TGG
fyuA	emifyuA3F	62	ACCATCGACATGGACTGG ACC	emifyuA5F	ACGGCACGCCGTTCTGGGG
	emifyuA4R		GTCGCCGAACAGGTTCACC	emifyuA6R	GATCAGGTTCACGCCGAACT
tpiA	emitpiA1F	57	GGAAATTGGAAGCTGCATGG	emitpiA2F	CTTCGCCACCGAACTGG
	emitpiA6R		GAARTCTTCGGCRACCAGT	emitpiA5R	TCTTCGGCRACCAGTGA

(Source: Fargier *et al.*, 2011 and Boudon *et al.*, 2005)

Practical No. 18

Storage and Preservation of Bacteria

Persons working with bacteria are interested to keep the cultures for longer duration alive with genetically stable. There are various techniques/methods available, which depends on the nature of bacteria and on the preservation objectives. The preservation methods have a common objective of reducing the organism's metabolic rate as low as possible while still maintaining viability. A high recovery or survival rate with a minimum of damage or change to the surviving organisms is also highly desirable. From long term preservation, lyophilization, (Freeze drying) and cryogenic storage are suitable techniques to preserve bacteria without losing genetic stability and viability. However these facilities are not available in all laboratories.

Practical No. 18 Storage of bacteria in mineral oil (Fahy and Persley, 1983)

This is a simple method of preservation of many bacteria. This technique is simple but inconvenient for the purpose of transport as freeze drying. Another disadvantage of this method is that there is a slow diffusion of oxygen through the oil, which allows the growth to continue at a slow rate and hence genetic stability might be poor.

Procedure

- Prepare a short slope of the medium in the culture tube to be preserved and the top of the agar is 5.0 cm below the cap of the tube.
- Sterilize white medicinal grade paraffin oil by autoclaving at 121ºC for 60 min. Then drive off any entrapped moisture by heating in a drying oven at 110 ºC for 60 min
- Inoculate the bacterial culture on the short slope and maintain the purity of the culture
- Pour 10 ml of sterile paraffin oil on the slope and do not disturb the bacterial growth on the slope. The oil must cover the top of the agar slope to prevent the drying of cultures.
- Store the oil layered slope at the same temperature as the agar slope is normally kept
- Establish programme of a viability check and routine stock maintenance.
- To recover cells from a culture which has been covered with oil, tilt the culture tube with the slope upper most and then harvest some growth and transfer it to the liquid medium. The little oil so transfered with the inoculums will float on the top and the culture subsequently recover from under the oil layer with a pipette.

Practical 18.1 Preservation of bacteria in sterile soil (Fahy and Persley, 1983)

Many bacteria survive well in dried soil for longer period (more than 20 years) at room temperature. This method is for the *Streptomyces* and spore forming bacteria such as species of *Bacillus* and *Clostridium*.

Procedure

- Select suitable loamy soil, pulverise it and remove plant debris and larger particles by screening
- Dispense soil samples to a depth of about 1 cm in a cotton wool plugged test-tube in a screw capped 25 ml bottles.
- Add distilled water to soil to bring it to about 60% of its maximum water holding capacity.
- Autoclave the soil at 121ºC for 60 min for three successive days.
- Test the soil for sterility before use
- Heat the soil to dryness in an oven at 105ºCand then store the soil samples in desiccators until required.
- Prepare a suspension of cells in 2% sterile peptone water from a slope culture.
- Place a 0.1 ml of cell suspension on each soil sample by using pipette and allow the moisture to absorb into the soil
- Check the viability of the culture routinely from the stock.
- To recover the culture aseptically transfer a s mall sample of soil to the suitable broth medium or make a suspension of soil in broth medium and use this to inoculate in an agar plate.

Practical 18.3 Preservation of bacteria in sterile distilled water (Fahy and Persley, 1983)

This method is the best used as a source of working stock cultures in conjugation with stocks preserved by other stable methods where contamination is less likely. This method is applicable to narrow range of phytopathogenic bacteria such as *Ralstonia, Pseudomonas, Agrobacterium* and *Corynebacterium.* The bacteria grow slowly continuously and genetic stability may not be expected for longer period. Due to liquid storage, chance of contamination is more when working with a similar range.

Procedure

- Dispense distilled water in a screw cap bottle 25 ml and sterilize by autoclaving at 121ºC for 15 min.
- Transfer an aliquot of sterile distilled water with a pipette to the slope culture of bacteria and prepare dense suspension of cells.

- Transfer the cell suspension to remaining distilled water. Use a few drops to perform purity check and tightly cap the bottle.
- Store the bottles at room temperature or preferably 10-15ºC
- Check the viability of culture routinely from the stock.
- To recover the culture aseptically transfer a few drops to a fresh agar plate or on broth medium and incubate.

Practical18.4 Preservation of bacteria by freeze drying method (Fahy and Persley, 1983)

This method is used for long term preservation (20-30 years) of bacteria. This technique preservation is highly suitable to cultures which are continuously in high demand. In lyophilization, two methods of preservation freezing and dying are combined. The overall process involves removal of water by vacuum sublimation from the frozen state. The method thereby overcomes the problems associated with drying from the liquid state and the dried ampules may be stored at room temperature in dark. Although the long term preservation is improved by storage in freeze.

Procedure

The lyophilization can be divided into 7 stages discussed below

1)Preparation of ampoules

- Type the accessions number of the culture on the Whatman No1 filter paper and date of freeze drying on the reverse side
- Cut the levels to dimensions of approximately 4 mm x 25 mm
- Insert the label into each 0.5 ml freeze-drying ampoule
- Allow sufficient space to avoid visual obstruction of the number by freeze dried pellet
- Using a swab tick, plug the neck of each ampoule with cotton wool to a depth of 12mm with a cotton wool projecting at the same distance.
- Pack the ampoule in a brown pare bag and sterilize in a hot air oven at 160 ºC for 1 h

2)Culture purity check and growth of culture

- Subculture the organism into 2ml of the liquid medium optimal for its development and incubate this until growth is visible or prepare a suspension from the stock culture.
- Using this suspension inoculate a sterile agar slope and a sterile plate of the growth medium and incubate both for the desired time until the early stationary phase of the population growth is reached at optimum growth temperature

- Examine the plate culture with a microscope for purity of the culture form and prepare a smear from an isolated colony. Stain by Gram's staining method and examine to establish that the culture has correct morphology and stain reaction.

3)Suspension of cells in the preservation medium

- Open the brown paper bag containing the sterile ampoule
- Using 1 ml pipette transfer 1 ml of mist. Desiccant to the slope culture and discard the pipette into the disinfectant cylinder
- Taking a sterile Pasteur pipette and bulb prepare a dense suspension (about 10^8cfu/ ml) of the culture in the mist. Desiccans by repeatedly drawing it into the pipette and washing down the growth of the slope
- When the growth is suspended dispense two drops (0.1ml) of suspension into bottom of each ampoule replace the cotton wool plug and place a complete ampoule into the rack. Take care not to contaminate the inner sides of ampoules as the remaining medium will be charred during ampoule sealing.

4)Operation of freeze dryer

- Prepare the slurry of crushed dry ice and 96% ethanol in the Dewar flask surrounding the vapour trap. Continue adding dry ice until mixture becomes viscous.
- Prepare slurry of crushed dry ice and 96% ethanol to a depth of 25 mm in plastic container (about 16 cm diameters) continue adding dry ice until the mixture becomes viscous
- Plunge the aluminium block into the dry ice ethanol mixture in the plastic container and allow it to cool down. Set the block upto an angle of 45º and slip the ampoules into the position. Allow 1 min for the contents of ampoules to freeze and then return the block into the upright position in the dry ice-ethanol bath for 5 min.
- Transfer the aluminium block containing the frozen ampoule into the evacuated chamber
- After lightly smearing the seals with high vacuum grease, fit the lid of the evacuation chamber and firmly seal it
- Close all stop cocks.
- Switch on the vaccum pump
- After 5-10 min check the vacuum indicated on the vacustat and ensure that the vaccum is increasing satisfactorily and that no leaks are indicated.
- After 6-8 h when the vaccum has reached 0.01mm of Hg turn off the vacuum pump and immediately fill the evacuation chamber and the ampoule with dry high purity nitrogen

- Open the evacuation chamber remove the aluminium freezing blocks containing the ampoules and place it in a holding chamber such as an anaerobic jar and flush with dry high purity nitrogen.

5)Sealing of ampoule under vaccum

- Use a pair of scissor to trim the cotton wool plugs off at the rim of the ampoules.
- Use a sterile swab stick and push the trimmed cotton wool plug down the ampoule until the bottom of the lug is approximately 5 mm above the top of the level.
- Constrict the neck of the ampoules using an ampoule constrictor or manually in a Bunsen flame
- Re-evacuate the ampoules on an ampoule manifolds
- Commencing the top, collapse the constriction of each ampoule by the means of small opposed gas flames using a micro cross fire burner.
- Ampoules are sealed under vaccum must be checked for leaks with a high frequency tester.

6)Sealing of ampoule under nitrogen

An alternative method of sealing the ampoules under vaccum is to seal it with an inert gas such as high purity nitrogen before sealing. It is technically easier than sealing under vaccum and there is a less chance of releasing fine particles containing microbes when they are opened. On the other hand it is easier to detect the faulty seals in vaccum ampoules.

7)Ampoule testing and Culture recovery

- With a glass ampoule cutting knife make a score mark on a glass near the centre of the cotton wool plug
- Swab the area of the ampoule around the score mark with the cotton wool dampened with 70% ethanol
- Apply a red-hot glass rod to the score mark upto break the ampoule leaving the cotton wool plug in the place
- With a sterile Pasteur pipette aseptically transfer 6-8 drops of liquid medium to the freeze dried material in the ampoule
- Resuspend the freeze dried culture and transfer a few drops of liquid medium to a suitable agar plate and remaining to the liquid medium.
- Incubate the inoculated broth and agar plate cultures at the suitable temperature
- The subsequent plate growth provides the convenient estimate of the survival of the organism by observing if the growth is confluent, individual colonies or no growth at all. This method may be easily quantified to measure the survival.
- Plate out the subsequent growth in broth and on the plates and incubate
- Check the subsequent colony morphology for purity

- ❖ Gram stain a smear prepared from the single colony and check the cellular morphology or the purity
- ❖ Test the culture for selected properties that characterize it and its specific properties such as pathogenicity
- ❖ Record the ampoules and their preservation date and store in dark at 4ºC

Practical 18.5 Cryogenic storage of bacteria (Fahy and Persley, 1983)

Bacteria can survive long term storage in the frozen state by markedly reducing their metabolic rate. The bacteria have been stored in freezers at a temperature around -20 ºC and -70 ºC. The lower the temperature the less is the loss of viability of most microorganisms and temperature higher than -70 ºC. The lower the temperature the less is the loss of viability of most of the microorganisms and a temperature higher than -70 ºC should not be used for long term storage but may be satisfactorily used for a period of 1 year. The use of ultra-low temperature obtained by freezing in the liquid nitrogen at -196 ºC has provided bacteriologist with a simple standardised technique which has been successfully used to preserve a wider range of bacteria with a much reduced viability loss and a high degree of genetic stability. Although many bacteria survive freezing in their growth medium, the addition of cry protectants such s 5-10% glycerol or dimethyl sulphoxide affords some protection from the stresses of freezing. Other cryoprotectants such as methanol, sugars, starch and polyvinylpyrrolidone have been used by various co-workers.

Procedure

The following method describes the procedure for the cryogenic storage in the liquid nitrogen. It is also applicable in principle to storage at other temperatures.

1)Preparation of ampoules

- ❖ Type Prepare filter paper labels as outlines in the section (1) of the freeze drying schedule above
- ❖ As these labels are not visible clearly when the culture is frozen, the ampoules (0.7 ml) are also labelled on the external glass surface with a permanent ink marker pen
- ❖ Cap each ampoule with a piece of aluminium foil and place the ampoules in a rack for sterilization. Sterile the ampoules ta 160 ºC for 1h. During the process the external painted label becomes etched into the glass. Alternatively pre-sterilized heat sealable polypropylene ampoules or polypropylene vials with silicon gaskets may be used, Mark the ampoules as suggested above.

2)Growth of the cultures

- ❖ Cultures are prepared and checked as outlined in freeze drying schedule.

3)Suspension of the bacterial cells in the preservation medium cultures

- ❖ Use a 5 ml graduated pipette transfer 5 ml of preservation medium (eg. suitable liquid growth medium consisting of (5-10% glycerol or dimethyl sulphoxide) to the slope culture and discard the pipette.
- ❖ Take a sterile Pasteur Pipette and bulb and suspend the culture in the preservation medium by drawing it into the pipette and wash down the growth of the slope to make a dense suspension.
- ❖ Suspend the bacterial growth aseptically, remove the cap of each ampoule and dispense 0.5 ml of the suspension into ampoule. Replace the cap of each ampoule.

4)Sealing of the ampoules

- ❖ Cultures Use the micro Bunsen burner with a gas air flame seal the glass ampoule and check under a stereo microscope for any hairline cracks. Polypropylene ampoules may be heated sealed or closed with its screw cap

5)Freezing of ampoules

- ❖ Pack completed ampoules in the holders in use
- ❖ Placed the sealed ampoules in a -30 ºC deep freezer or controlled rate freezer if available
- ❖ Leave the ampoule in the freezer until they reach-30 ºC. Allow approximately 1 h. The rate of cooling will approximate 1ºCper min. The rate of cooling may be controlled simply by wrapping ampoules in cotton wool or by placing in the boxes of cardboard or other suitable material. The rate of cooling should be predetermined
- ❖ Remove the pre-frozen ampoules from the -30ºCfreezers and quickly transfer them to their predetermined position in the liquid nitrogen flask. Glass ampoules should be stored in vapour phase.
- ❖ Catalogue the ampoules and make the provision for testing an ampoule from each culture for viability, survival rate and purity.

6)Ampoules testing and culture recovery

- ❖ After putting on protective clothing and a face shield, remove the test ampoule from liquid nitrogen. Any ampoule which has a hairline aperture through defective sealing may explode if liquid nitrogen has penetrated
- ❖ Thaw the ampoule by immersing it in 37 ºC water bath. Thaw the cell suspension until a small piece of ice remains, so that the remaining ice melts till the ampoule is transfered to the laboratory bench at room temperature
- ❖ Swab the area of the ampoule around the gold line with cotton wool dampened with 70% ethanol. Hold the cotton wool around the ampoule and apply the pressure with fingers and thumbs to break the ampoule at the gold line. Heat sealed polypropylene ampoules may be cut with sterile scissors

- With a sterile Pasteur pipette aseptically transfer a few drops of suspended culture to the surface of the suitable agar plate and the remaining to the broth medium of the culture is inhibited by the cryoprotectants. The cell suspension should be centrifuged and resuspended before being cultured.
- Incubate the media under suitable conditions
- Plate out the subsequent growth and carry out colonial and cellular purity check.

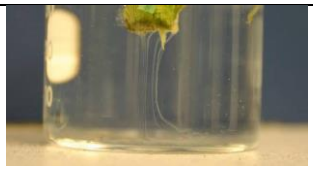


Fig.1: Cloudy mass of bacterial ooze

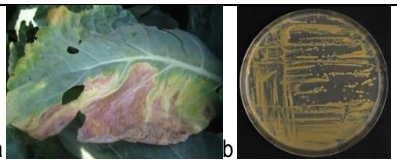


Fig.2: Black rot of cabbage *X. campestries* pv. *campestries*

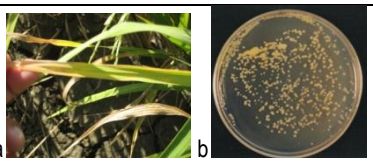


Fig 3 a. Symptoms of bacterial leaf blight of rice b.*Xanthomonas oryzae* pv. *oryzae*

Fig.4.:a.Bacterial wilt of tomato b. *Ralstonia solancearum*

Fig.5.a Soft rot of potato *Erwinia carotovora* pv. *carotovora* on CVP medium

Fig 6.: *Pseudomonas fluorescens* culture

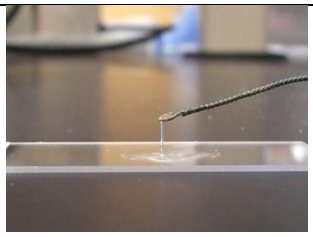


Fig. 7. String of slime showing Gram –ve bacteria

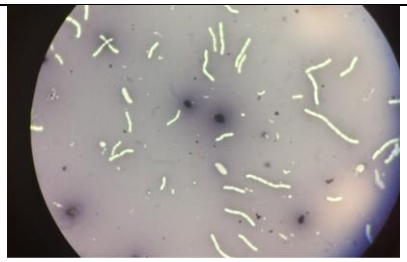


Fig 8. Negative staining of bacteria

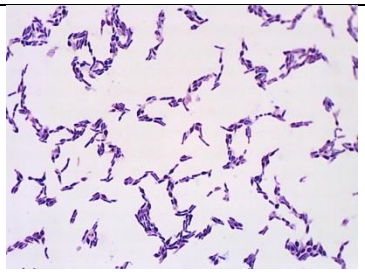


Fig 9. Simple staining of bacteria

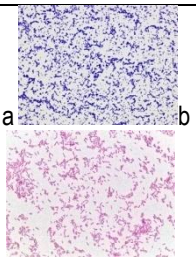


Fig. 10 a. Gram Positive Bacteria b.Gram negative bacteria

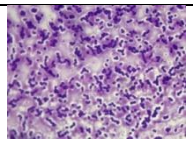


Fig 11. Gram positive cocci showing capsules

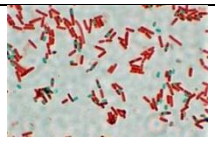


Fig.12. Endospores stained green

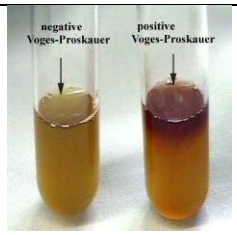


Fig 13.Voges-Proskauer test.

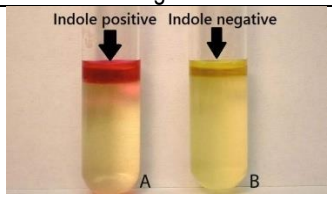


Fig.14 Indole production test

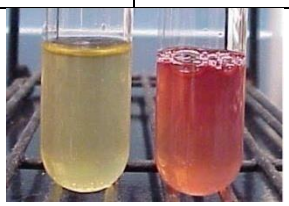


Fig 15. Nitrate reduction test

Fig 16: Arginine test

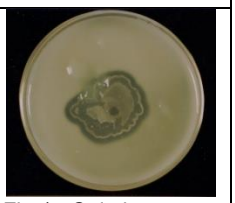


Fig.17 Gelatin liquefaction test

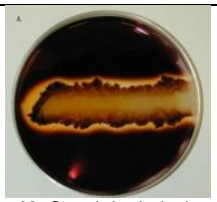


Fig. 18. Starch hydrolysis test

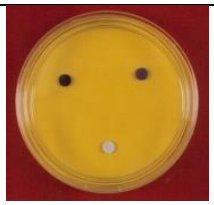


Fig. 19. Oxidase test

Fig 20. Citrate utilization test