Practical Manual on

Plant Pathogenic Prokaryotes

PPA 503 3(2+1)

M.Sc. (Ag.) Plant Pathology

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2024

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Syllabus: Plant Pathogenic Prokaryotes PPA 503 3(2+1)

Practical: Study of symptoms produced by phytopathogenic prokaryotes. Isolation, enumeration, purification, identification and host inoculation of phytopathogenic bacteria. Stains and staining methods. Biochemical and serological characterization. Isolation of genomic DNA plasmid. Use of antibacterial chemicals/antibiotics. Isolation of fluorescent *Pseudomonas*. Preservation of bacterial cultures. Identification of prokaryotic organisms by using 16S rDNA, and other gene sequences. Diagnosis and management of important diseases caused by bacteria and mollicutes.

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Batch .....

Session .....

Semester .....

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Date: Course Teacher

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Practical No. 1

Objective: Isolation and purification of bacteria.

Activity:

1. Collection of diseased samples from the field.
2. Ooze test and isolation of bacteria.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Practical No. 2

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

Activity:

1. Collection of infected plant samples from the field.
2. Preparation of liquified suspension for endophyte isolation.

Materials Required: ……………………………………………………………

Procedure: ……………………………………………………………

Observation: ……………………………………………………………

Precautionary Measures: ……………………………………………………………

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1. Selection of suitable media for bacterial growth.
2. Purification of bacterial colony.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Practical No. 4

Objective: Isolation of bacteriophages from different sources.

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2. Purification, transfer and storage of isolated bacteriophage.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Practical No. 5

Objective: Morphological characterization of Bacteria (Shape/ Flagellation/ Sporulation)

1. Preparation of fresh bacterial culture.
2. Staining of a bacterial colony with different chemicals.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Practical No. 6

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1. Preparation of fresh bacterial culture.
2. Application of the stain on a bacterial colony.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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2. Dilution of the prepared fresh culture then observe under electron microscope.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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1. Preparation of fresh bacterial culture.
2. Preparation of the different chemicals and application to test bacterium.

Materials Required: ……………………………………………………………

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Procedure: ……………………………………………………………

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Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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1. Preparation of fresh bacterial culture.
2. To test bacterial activity on different reducing substances.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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1. Preparation of fresh bacterial culture.
2. To check the pigment production on different media.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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1. Isolation and purification of bacterial culture from infected plant sample.
2. Observation of symptoms and disease severity.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Practical No. 15

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1. Isolation and purification of test bacterium from infected plant sample.
2. Serological assay of test bacterium.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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2. DNA isolation and gel-electrophoresis.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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1. Extraction of the genomic DNA.
2. Primer designing and DNA amplification.

Materials Required: ……

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Procedure: ……

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Observation:……

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Precautionary Measures: ……

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Objective: Storage and Preservation of Bacteria.

1. Preparation of media.
2. Bacterial growth on a media and storage at low temperatures.

Materials Required:

Procedure:

Observation:

Precautionary Measures:

ISOLATION AND PURIFICATION OF BACTERIA

Collection of diseased material: The collections of the diseased material should be characteristic of all signs and symptoms of a disease (leaves, stems, roots, flowers or fruits). In the case of root-invading bacteria, part of the collection of stems and roots should be washed and free from soil and excess moisture 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 polyethene bag should be tied firmly around the base of the stem to prevent the rest of the plant from being dirty. 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 appear translucent when held to the light, rather than brown and necrotic lesions. The diseased portion of canker and shoot dieback should always include the edge of the lesions and a few centimetres of the healthy tissue beyond it. Pressed and dried specimens of leaf spots and blights should always be retained as herbarium material and as a reference source.

OOZE TEST

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

Procedure

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

Observations: If it is a bacterial infection, then the cloudy mass of bacterial cells will be visible through the cut ends of the infected piece of tissue. Using the test 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 the affected tissue may not contain the bacterial cells. Bacterial oozing is also difficult in tissues that contain large quantities of starch, dispersal of starch grains in water may mask the bacterial ooze.

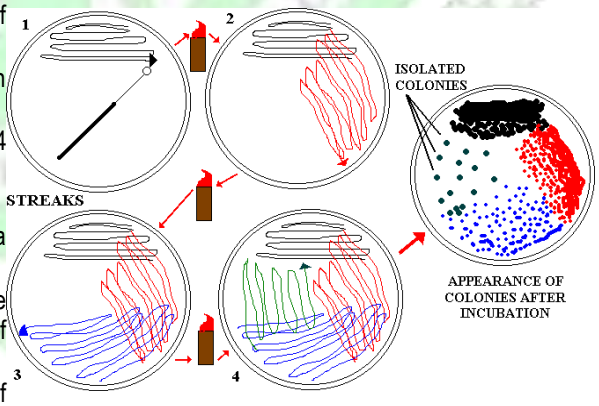
ISOLATION OF *XANTHOMONAS CAMPESTRIS* PV. *CAMPESTRIS*

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

Nutrient Agar medium: Beef extract – 3g; Peptone – 5g; Agar* - 15g; Distilled water – 1000ml (*Do not use agar if nutrient broth is desired)

Procedure:

- For isolation select young developing lesions with healthy parts of leaves for isolation,
- Cut the infected leaves into small pieces with the help of sterilized razor blade,
- Wash the selected lesion with spirit and immediately dip in mercuric chloride solution (1:1000) for 15 seconds,
- Wash it with sterile distilled water and pass for 3-4 changes,
- Wash a glass slide in spirit flame and allow it to cool,
- Place the surface sterilized lesion tissue on the slide with a few drops of SDW,
- Cut the infected leaves into 3-4 small pieces with a 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 Petri plates by to and from the motion of the inoculated needle,



- Label the plates and incubate them in an inverted position at 25° C and examine daily.
- To get the single colony, pick a section of a bacterial colony on a loop and streak it on medium in a single line (not back and forth). The loop is then sterilized in flame and streaked along another single line 90 degrees from the first streak overlapping a few initial streaks. The loop is sterilized again and the plate is turned another 90 degrees. The second streak is used to make the next line. This is done one last time. The bacteria in the plate should look like individual colonies in the last streak.

Observations: The colony of *X. campestris* pv. *campestris* and other pathovars will appear 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.

ISOLATION OF *XANTHOMONAS ORYZAE* PV. *ORYZAE*

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

Suwa's medium: Sodium glutamate – 2g; $MgCl_2.6H_2O$ – 1g; $KH_2.PO_4$ – 5g; Fe-EDTA stock solution* - 1ml; Peptone - 5g; Sucrose - 5g; Agar - 5g; Distilled water – 1000ml (*Fe-EDTA 0.657g dissolved in 100 ml distilled water and added to medium at the time of plating after filter sterilization).

Procedure

- Collect bacterial blight-infected leaves of rice and keep them in an 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 a leaf with sterilized pastel- mortar. After grinding, add again 1 ml of sodium chloride,
- Dilute the grinded sample up to 10-7,
- Take 100 µl of supernatant and spread it onto the Petri plates containing 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).

ISOLATION OF *RALSTONIA SOLANACEARUM*

Requirements: Bacterial wilt-infected tomato plants, CPG, TTC and SMSA media, beaker razor blade, inoculation needle, sterilized distilled water, sterilized Petri plates, and spirit lamp.

CPG and TTC agar media (Kelman, 1954): Peptone - 10g; Agar - 15g; Casamino acid - 1g; Distilled water - 1000 ml

To make TTC agar medium, cool the medium to 55ºC and add 1% stock solution of 2,3,5 triphenyl tetrazolium 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 - 10g; Glycerol - 5ml; Casamino acid - 1g; Agar - 15g; Distilled water - 1000ml; Bacitracin - 25 mg; Polymyxin β sulphate - 100mg; Chloramphenicol - 5mg; Penicillin G* - 0.5mg; Crystal violet - 5mg; TTC - 50mg (*Dissolve this antibiotic 5ml of 70% ethanol 30 minutes before use).

Procedure

- Collect wilt diseased tomatoes 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 Petri plates containing CPG, TTC and modified SMSA medium,
- Incubate the Petri plates at 28 ºC for 72 hr.

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

ISOLATION OF ERWINIA SPECIES

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

i) **CVP medium** (Kuppels and Kelman, 1974): 1N NaOH - 4.5ml; 10% $CaCl_2.2H2O$ - 3ml; NaNO3 - 1g; Agar - 1.5g; Sodium polypectate - 10g; Sodium dimethylsulphide - 0.5ml; 0.075 Crystal Violet - 1ml; Distilled water - 500ml

   Add NaOH, $CaCl_2.2H2O$, NaNO3 and Agar into 300 ml of boiling distilled water in a waring blender jar and blend it at a higher speed for 15 sec. Add slowly sodium polypectate and blend it another 15 sec while adding another 200 ml of boiling distilled water. Medium should be poured in a 2 litter flask and add sodium dimethyl sulphide and crystal violet. Sterilize the medium at 120ºC for 25 min and pour in Petri plates.

ii) **CPG medium** (Kuppels and Kelman, 1974): Casamino acid - 1g; Peptone - 10g; Glucose - 10g; Agar - 18g; Distilled water - 1000ml

iii) **Polypectate enrichment medium:** Sodium poly pectate - 1.5g; Ammonium sulphate (10%) - 10ml; KH2.PO4 (10%) - 10ml; MgSO4.7H2O (5%) - 5ml

Procedure

- Select a small piece of tissue from the periphery of the decayed lesion of the potato,
- Add 1-2 drops of sterilized distilled water on flame sterilized microscopic glass slide and macerate it properly,

- Streak loop full of suspension over CVP medium containing Petri plate by to and from the motion of the inoculated needle,
- Label the plates and incubate them in an 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 onto to CPG medium.

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

PT medium: Polygalacturonic acid - 5.0g; K2H.PO4 - 4g; NaNO3 - 1g; MgSO4.7H2O - 0.2g; Sodium heptadecyl sulphate - 0.05g; Agar - 9g; NaOH(1M) - 17ml; 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 the decayed lesion of potato**,**
- Incubate for 2-3 days at 25±2ºC 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 - 5g; Glucose - 5g

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

Procedure

- Put a streak from 24-48 old test culture of *Erwinia* on the dried plates,
- Incubate the plates for 24 h at 27ºC.

Observations: *Erwinia carotovora* 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* remains 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* (2 mm) and completely dark red/purple.

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, Petri plates.

(i) Mundt and Hinkle medium (Mundt and Hinkle, 1976): Yeast extract - 3g; Dextrose - 5g; Cyclohexamide - 2g; Agar - 15g; Distilled water - 1000ml; pH - 6.8

(ii) Tryptic Soya Agar (TSA) medium: Tryptone - 17g; Soytone - 3g; Dextrose - 2.5g; NaCl - 5g; $K_2H.PO_4$ - 2.5g; Agar - 15g; Distilled water - 1000ml; pH - 7.3

Procedure

- Take 10.0 g of rhizospheric soil of a plant and mix it into 100 ml of sterilized distilled water in a 250 ml flask,
- Mix soil well and heat the sample at 80ºC for 15 min,
- Agitate it again and again,
- Take 1 ml of soil suspension and pour it into 9 ml of sterilized distilled water and further dilute up to 10-7 for each sample separately,
- Take 100 µl of diluted suspension at different levels as per the population of the bacteria in the soil (10^{-3}, 10^{-4} &10^{-7}) and spread it with the help of an 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.

ISOLATION OF *PSEUDOMONAS* SPP.

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 - 3g; $K_2H.PO_4$ - 5g; $MgSO4.7H_2O$ - 2g; Glycerol -

15g ; Agar - 15g; Distilled water - 1000ml

Procedure:

- Take 10.0 g of rhizospheric soil of a plant and mix it into 100 ml of sterilized distilled water in a 250 ml flask,
- Mix soil well and keep for 30 min,
- Agitate it again and again,
- Take 1 ml of soil suspension and pour it into 9 ml of sterilized distilled water and further dilute up to 10-7 for each sample separately,
- Then take 100 µl of diluted suspension at different levels as per the 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±1ºC in the BOD incubator.

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

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, Petri plates.

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

Preparation of 0.02M (20m M) Phosphate buffer (Make up the volume of the solution by adding 1000ml of distilled water and adjust the pH to 7.0); $Na_2H.PO_4$ - 1.704g/liter; $KH_2.PO_4$ - 1.088g/ litre

Procedure:

- Collect the 10 plants from the field and bring them to the lab,
- Wash the collected plants with tap water and keep them 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 a known quantity of sample (1.0g) and crush it in 9.9 ml of buffer in a sterile pestle and mortar. Dilute serially triturated suspension up to 10-7 in potassium phosphate buffer solution,
- Pour 100 µl suspension of different dilutions 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 25ºC.

Observations: *Erwinia carotovora* subsp. *carotovora* reduces the tetrazolium to insoluble red formazan and colonies (about 1.5mm diameter) develop a pink to red/purple center. Single colonies of subsp. *atrospectica* remains colorless 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.

PURIFICATION OF PHYTOPATHOGENIC BACTERIA

Spreading dilution on solid agar

- Take poured 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 onto the medium in Petri plates from the last 2 or 3 different dilutions,
- In each plate spread the drop uniformly with a sterile angled L-shaped glass spreader,
- Incubate the Petri plates in a BOD incubator at 28±2ºC 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 hour 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 from the 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 them in an inverted position at 28±2ºC and examine them 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 45ºC,
- 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 Petri plates,
- Label the plates and incubate at 28±2ºC for 2-6 days in an 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 onto the nutrient agar slants. Touch the wire loop of the inoculation needle on a well isolated colony and streak it on an agar slant in a tube. The cultures obtained from a single colony need 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.

ISOLATION OF BACTERIOPHAGES

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, Petri plates containing nutrient agar medium, pipette, tips, 24h old culture of *Xanthomonas citri*

Nutrient sucrose agar medium: Beef extract - 3g; Peptone - 5g; Sucrose - 10g; Agar - 15g; Distilled water - 1000 ml (*Do not add agar if nutrient broth is desired).

Procedure:

- Collect canker-infected leaves of citrus from an orchard,
- Wash the leaves thoroughly with the tap water,
- Cut the leaves into 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 mixed broth culture onto the NSA medium in Petri plates by spreading method,
- Incubate the Petri plates 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 transferred 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 of infected soil in 250 ml of a 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 the 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. The supernatant is tested for the presence of phages,
- Inoculate 1 ml broth culture on NSA medium in Petriplates by spreading method,
- Incubate the Petri plates at 25ºC 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.

MORPHOLOGICAL CHARACTERIZATION OF BACTERIA (SHAPE/ FLAGELLATION/ SPORULATION)

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: 24h 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.

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 discernible against a coloured background.

Requirements: 24 hour old culture of bacteria, 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 into the drop of stain and mix with the sterile inoculating loop,
- Put slide vertically against drop of suspension of an 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 discernible against dark coloured background (Fig 8).

SIMPLE STAINING (POSITIVE STAINING) OF BACTERIA

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

Requirements: 24 hour old culture of bacteria, 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 it 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 visible (fig 9).

GRAM STAINING OF BACTERIA (Schaad *et al*., 2001)

Gram reaction is essential for the primary division of bacteria. The gram staining differentiates gram (-ve) and gram (+) ve bacteria based on the chemical composition of the cell wall of bacteria. Gram staining involves the use of a primary stain (crystal violet), the trapping reagent (iodine solution), a decolouring agent (95% alcohol) and a 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 hours old. Simple staining, basically charged dyes are used to stain negatively charged bacterial cell.

Requirements: 24 hour old culture of bacteria, grease free microscope glass slide, glass rods, cotton, tap water, cleaning agent, microscopes, blotting sheets, Hucker's 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 - 2g/l; Ethyl alcohol (95%) - 20ml
   Solution B: Ammonium oxalate – 0.8g/l; Distill water – 80ml
   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 - 1g/l; Potassium Iodide - 2g/l; Distilled water - 300.0 ml
   Allow the iodine solution to dissolve for 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). With practice, any of the three colorising agents will yield good results.

4. **Counter stain**

   Stock solution: Safranin - 2.5g/l; Ethyl alcohol, 95% - 100ml
   Working solution: Stock solution - 10ml; Distilled water 90ml

Procedure

- On a grease free slide, dry a thinly 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, e.g. ethyl alcohol, until the solvent flows colourlessly from the slide (about 30 sec) and blot dry. (If decolouriser is used longer, gram-positive bacteria may lose colour,
- Take Rinse in the tap water for about 2 seconds,
- Counterstain with safranin solution for about 10 seconds,
- Wash briefly in tap water. Blot dry and examine it under a 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.

STAINING OF BACTERIAL FLAGELLA

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

Tannic acid solution: Tannic acid - 5g; Ferric chloride - 1.5g; Formalin (15%) - 2ml; Sodium hydroxide (1%) - 0.8g/l; Makeup the volume to with distilled water - 1000ml

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 the remaining 90 ml until a heavy precipitate is formed. Add ammonium hydroxide until precipitates dissolve. Then use from saved 10 ml silver nitrate, and back titrate until a clouding appears and persists. The pH of the solution should be adjusted to 10 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 a YDC medium slant for 24-48 h,
- Use *Erwinia* spp. having peritrichous flagella of pseudomonas marginalised having amphitrichous flagella as control and test bacteria,
- Prepare a faintly cloudy suspension by carefully adding 1.0 ml of distilled water to the 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 (ammoniated silver nitrate solution) for about 10 seconds and immediately wash in distilled water,
- Air dry and examine under the microscope (100x with oil emulsion).

CAPSULE STAINING OF BACTERIA

Capsules are polysaccharide 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.

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 the 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 the remainder of the bacterial cells are red (Fig 12)

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 hands it is better to incubate the culture 2-3ºC below the optimum temperature for growth. The culture should be sampled when it is in the 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 the agar slant with about 1 ml of double distilled water,
- Prepare a dilution series using a suspension of about 109 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 8º,
- 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 the stain by touching the edge of the grid to filter paper.
- Observe under electron microscope.

Observations: Flagella attachment to bacterial cells will be seen.

BIOCHEMICAL AND PHYSIOLOGICAL TEST FOR THE CHARACTERIZATION OF BACTERIA

In biochemical tests, the ability of a bacterium utilizes C or N sources or to produce enzymes/gas is determined. Recently some commercial kits have been available to perform these tests. 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 tests 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 tests used for the biochemical characterization of plant pathogenic bacteria are given below.

UTILIZATION AND DECOMPOSITION OF CARBON COMPOUNDS

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 a 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.

Voges-Proskaur (VP) and Methyl Red test: Glucose - 5g; $NH_4H_2PO_4$ - 0.5g; K_2HPO_4 - 0.5g; $MgSO_4.7H_2O$ - 0.2g; NaCl - 5g; Yeast extract - 5g; Distilled water - 1000ml (Enterobacteriacea subgroups (Erwinia) are differentiated by these tests).

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 up to 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

3-ketolactose production test: This test can differentiate species of Agrobacterium *A. tumefaciens* produces 3-ketolactose, while *A. rhizogens* and *A. vitis* show negative results. 40-48 h old culture of bacteria is inoculated about 1.0 cm diameter on the medium containing a-lactose 1.0%, yeast extracts 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 Benedict's reagent and left at room temperature for one hour.

Observations: If 3-ketolactose is present, a 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.

Acid production from erythritol and D (+) melezitose Basal medium: $NH_4H_2PO_4$ - 1g; KCl – 0.2g; Yeast Extract – 1g; Bromthymol blue 1% (w/v) in 50 % ethanol - 3ml; $MgSO_4.7H_2O$ - 0.2g; Agar – 1.5g, Distilled water – 1000ml; Adjust pH 7.1 before adding agar

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 of melezitose, whereas *A. rhizogens* and *A. vitisshows* negative results.

Acid production from carbohydrates Medium C: $NH_4H_2PO_4$ - 0.5g; K_2HPO_4 - 0.5g; $MgSO_4.7H_2O$ - 0.2g; NaCl - 5g; Yeast extract - 5g; Cysteine hydrochloride - 0.1g; Distilled water - 1000ml

- 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 onto 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.

DECOMPOSITION OF NITROGENOUS COMPOUNDS

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

Medium: $NH_4H_2PO_4$ - 0.5g; K_2HPO_4 - 0.5g; $MgSO_4.7H_2O$ - 0.2g; NaCl - 5g; Yeast extract - 5g; Cysteine hydrochloride - 0.1g; Distilled water - 1000ml

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 a strip of Whatman filter paper in 5% lead acetate solution, air dried and autoclaved. The test tubes are examined for 14 days.

Observations: The paper strip becomes black due to the 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 - 10g; NaCl - 5g; Water – 1000ml; 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 strip (5x50 mm) in a 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 the test strip become pink or red.

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

Medium: Peptone - 10g; Beef extract - 5g; KNO_3 (nitrite free) - 3g; 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 - 1g; NaCl - 5g; K_2HPO_3 (nitrite free) – 0.3g; Agar – 3g; Phenol red – 0.01g; L-arginine – 10g; Distilled water – 1000ml; pH – 7.

Dispense 5ml of the medium in each tube and autoclave. Stab inoculates the medium with 48h growth of the bacterium and

covers the medium with sterile liquid paraffin to a depth of 1cm incubates the tubes for 7days at 25-30ºC and observes daily.

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

Decomposition of Macromolecules

(i) Gelatin hydrolysis (Liquefaction) (Dye, 1968): Proteolytic bacteria decompose gelatin, which produces an extracellular enzyme gelatinase. As a result of decomposition, the gelatin loses its gel forming property.

Medium: Peptone - 10g; Beef extract - 5g; Gelatin - 4g; Agar - 20g; Water - 1000ml; pH - 7.0

Autoclave the medium in a flask and cool at 45ºC. Then pour into the petridishes and 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 by a clear zone (Fig 17).

i) **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 causes partial hydrolysis of erythrodextrin and dextrin.

Medium: Peptone - 10g; Beef extract - 5g; Starch (soluble) - 2g; Agar - 20g; Water - 1000ml; pH - 7.0

Sterilize the medium in a flask by autoclaving and cool at 45ºC. Then pour into the Petri dishes 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 a 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.

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 the Oxidase test streak a 24-48h 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 appears 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*, *P. savastanoi* and *P. viridiflava* are negative (Fig 19)

(i) Catalase test: Catalase is an enzyme that 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.

i) **Fluorescent Pigment production:** The KB medium is used for the detection of fluorescein, a florescent green or blue water soluble chloroform insoluble petridine pigment (i.e. pyoverdine siderophores). After 24-48h growth at 27ºC, colonies are examined for fluorescence with a long wave-length (366 nm) 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 - 20g; K_2HPO_4 – 1.5g; $MgSO_4.7H_2O$ – 1.5g; Glycerol – 10ml.; Agar - 15g; Distilled Water - 1000ml

Observations: After 24-48h 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

ii) **Potato soft rot test:** Cut 7-8 mm, peeled and alcohol-flamed potato tuber, and place in sterile Petriplates. Cover surface of the 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 the rotting of slices after 3-5 days.

iii) **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's (1963) injection infiltration method.

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

iv) **Ferric ammonium citrate broth test:** This test can be used to differentiate the species of Agrobacterium. *A. tumefaciens* produces a reddish brown pellicle at the surface of the medium, whereas *A. rhizogens* and *A. vitisshow* negative results.

Medium: Ferric ammonium citrate - 10g; $MgSO_4.7H_2O$ - 0.5g; K_2HPO_4 - 0.5g; $CaCl_2$ - 0.2g; Distilled Water - 1000ml

PRODUCTION OF ALKALI IN THE MEDIUM

i) Alkali from malonic acid

Basal medium: $(NH_4)_2SO_4$ - 2g; KH_2PO_4 - 0.4g; K_2HPO_4 - 0.6g; NaCl - 2g; Yeast extract - 0.1g; Malonic Acid, Sodium salt - 3g; Bromothymol blue, 1.0% (w/v) in 50% ethanol - 2.5ml; 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.

i) **Alkali from mucic acid and L-tartaric acid Medium:** $NaNH_4PO_4$ - 2g; NaH_2PO_4 - 0.4g; K_2HPO_4 - 0.6g; KCl - 2g; Bromothymol blue, 1.0% (w/v) in 50% ethanol - 2.5ml; 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 27ºC for about two weeks.

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

REDUCING SUBSTANCES FROM SUCROSE

Medium: Peptone - 10g; Agar - 17g; Distilled Water - 800ml

Sterilize the medium and then add filter sterilized sucrose (40g dissolved in 200ml water) and pour it into Petri plates. Inoculate dense aqueous suspension of test bacterium in a 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 35g and $Na_2CO_3.H2O$ 20g is dissolved in 160ml of distilled water and heated to dissolve. Dissolve 3.5g of $CuSO_4.5H_2O$ in 40ml distilled water. Mix both solutions. This mixture can be kept for several months at room temperature.

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

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

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

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 a 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 a grid pattern onto the medium. Incubate the plates at 25±2ºC for 2 days. Then place a drop of (ca. 100 µl) concentrated ammonia in the lid of Petri plates 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.

Utilization of keto-methyl glucoside (methyl a-d-glucoside) Medium: KH_2PO_4 - 2g; NH_4Cl - 10g; $MgSO_4.7H_2O$ (10% conc) - 2ml; K_2HPO_4 - 7g; Casamino acid - 1g; Agar - 15g; Distilled water - 998ml.

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.

Glucose dehydrogenase activity assay Medium:

Mannitol - 10g; L-glutamate - 2.0g; $MgSO_4.7H_2O$ - 0.2g; K_2HPO_4 - 0.2g; Yeast extract - 0.25g; Glucose - 20g; Agar - 15g; Distilled water - 1000 ml; pH - 7.0

Streak the test bacteria on the medium. Colonies are overlaid with 4 ml of 0.5% agar containing a 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 indicates glucose dehydrogenase activity due to the production of 2-gluconate from glucose.

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

Medium: Arbutin - 5g; Peptone - 10g; Yeast extract - 3g; Glucose - 1g; Ferric citrate - 0.5g; Agar - 12g; Distilled water - 1000 ml; pH - 7.0

Poly-β-hydroxybutyrate PHB accumulation

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

1) Nutrient agar with 5% sucrose,
2) Mineral medium: $(NH_4)_2SO_4$ - 0.2g; L-glutamate - 0.2g; $MgSO_4.7H_2O$ - 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 the 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 coupling 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 the slide in water, blot and dry the slide,
- Examine the slide under oil immersion with a 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: $NH_4H_2PO_4$ - 1g; KCL - 0.2g; MgSO4.$7H_2O$ - 0.2g; DL-Poly β-hydroxybutyrate sodium salt - 5g; Proteose peptone number 3 - 20g; Nile blue solution (1%) - 1ml; NaOH - 4.5ml; Distilled water - 900ml; Agar - 17.5g; 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 fluorescence bright orange under a long wave (366 nm) ultraviolet radiation. Colonies of fluorescent pseudomonads will not be florescent in this medium, and the florescent granules are not visible microscopically.

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 with 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 0,5,10 and 20 min after enzyme sample is added to pectin solution.

Study of pigment production by phytopathogenic bacteria

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

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

Yeast Glucose Chalk Agar: Yeast extract - 10g; $CaCO_3$ - 20g; Glucose - 10g; Agar - 20g; Distilled water - 1000ml

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 campestris* pv. *campestris* 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 - 20g; Glycerine - 10ml; $K_2H.PO_4$ - 1.5g; $MgSO_4.7H_2O$ - 1.5g; Agar - 20g; Distilled water - 1000ml; pH - 7.2

Autoclave the medium in a flask and pour the sterilized medium into the Petri plates. Inoculate the 48 h old culture of *Pseudomonas fluorescens* onto the Petri plates with an inoculation needle. Incubate the Petri plates 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 ultraviolet light.

Characterization of biovars of *Ralstonia solanacearum*: For the differentiation of the biovars of *Ralstonia solanacearum* based on 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 a 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 a 0.22 µm membrane filter except dulcitol which is sterilized by separate autoclaving. Add 1% of carbon sources into the basal medium, after mixing, dispense 3 ml of a 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 take a few days longer to give a clear positive result.

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) database, the user can save the pattern to a custom database for future reference. 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 tests 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 tests. There are over 4x1028 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 tests, which provides greater fingerprint discrimination and larger database.

Procedure: The test procedure is fast and simple, involving 5 steps, and requiring only 2-3 minutes hands on time/ 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 l50ml 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.

PATHOGENICITY TEST OF PHYTOPATHOGENIC BACTERIA

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, scissors, 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 each plant,
- The number of infected points per leaf and the severity of the symptoms 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 lesions 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

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 (108 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**

Pathogenicity test of *Ralstonia solanacearum* in tomato plant

Requirements: 48 hour old bacterial culture of *R. solanacearum*,35-40 days old palnts of tomato, scissors, 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 them in 10 ml of sterile distilled water or saline solution (0.85%NaCl) to

produce a turbid suspension of (108 to 10 9 cfu/ml),

- Make slight injury with the help of a scalpel on the root and pour bacterial suspension at the root zone in the soil,
- Water the plants from the bottom to prevent washing the bacteria out of the soil after inoculation.

Observation: The inoculated plants should be observed daily basis for 21 days. 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.

$$\text{Wilt intensity (\%) (I)} = \{(n1xv1) \div (V \times N)\}\ 100$$

The where n1 = number of plants with respective disease rating; v1 = disease rating (1, 2,3, 4 or 5); V= the highest disease rating and N= the number of plants observed

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 106cfu/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 102 to 10 9 cfu/ml,
- Inoculate the plants by using a micropipette tip/ syringe for at least 8-10 plants for each concentration of bacterial culture,
- The injection point should be sealed after inoculation by application of a small amount of petroleum jelly over the puncture,
- Keep the plant at a high relative humidity for better results.

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

Differentiation of virulent and avirulent colonies of bacteria.

Requirements: TTC medium, a culture of phytopathogenic cultures of *Ralstonia solanacearum, Erwinia carotovora, Pseudomonas syringaepv. Phaseolicola, Clavibacter michigenesis* subsp. *insidious, Petriplates*

Medium: Peptone - 10g; Casamino acid - 1g; Agar - 20g; 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 based on characteristics given below-

Bacterium	Virulent strain	Avirulent strain
Ralstonia solanacearum	Irregularly round, fluidal white with a 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 distinguished from less virulent on the amount of reddening present in the centre. The more virulent has less reddening and the less virulent has more reddening.

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 immuno-sorbent assay (ELISA), Immunofluorescence (IF), lateral flow strips test and flow through assays.

Antigen preparation from *Xanthomonas campestris* pv. *campestries*

X. campestris pv. *campestris* (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 sub-cultured 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 be 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.

Media: Dextrose - 20.5g; Sodium citrate (anhydrous) - 8g; Citric acid (anhydrous) - 0.55g; Sodium chloride - 4.2g; Distilled water - 1000ml.

(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, wash twice as above and resuspend each pellet in 3 ml of PBS diluent. These cells are now ready for injection.

i) 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 organisms,

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

- Prepare 5x109cfu/ 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 a final concentration of 1mg/ml,
- Stir slowly for 30 minutes at room temperature,
- Dehydrate the mixture in vacuo with a speed-vac centrifuge.

Antiserum production: Circulating antibodies 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 the latter (secondary) response 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 the 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 a control to detect cross reactive antibodies,
- Mix bacterial cells (105/ml) in a proportion of 1:1 with Freund's complete adjuvant,
- Inject 0.1 ml of cells ($1x10^4$ /ml) containing approximately 100 mg protein at four sites subcutaneously,
- On the 4th day, repeat 1-day injection steps with Freund's incomplete adjuvant. After this step, allow an animal to rest for 14 days so that the primary response subsides to a baseline level otherwise a secondary response may not be achieved,
- On the 18th 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 22nd day, bleed and collect serum,
- On 25th day, repeat the above step of intramuscular injection on another leg of the animal,
- On 29th day, bleed and collect serum and pool the serum with the previous sample,
- The intramuscular 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 days 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 antibodies from hydrolysis by the action of naturally occurring IgG proteases.

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 410 nm 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;

Solution B: 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 of distilled water. Add 8.75g of NaCl and make up the volume up to 1000ml. The pH should be 7.4

(ii) Wash buffer: PBS containing 0.05% Tween 20,

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

(iv) Reagents for alkaline phosphatase conjugates

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

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 -20ºC. Read the yellow colour at 414nm. Colour development can be stopped by the addition of 10 ml of 3M NaOH to the wells.

(V) 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 to 4.0 with 1M NaOH. Store at room temperature.

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

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

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

Immunoblot assay

As Xcc is 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 visualisation of the protein bands and the second half of the gel transferred to the 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 that 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 peak 1, P1Ab-Xcc, and 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.

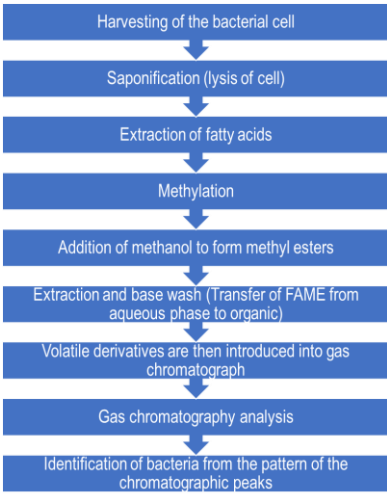
Characterization of Polyclonal antibodies- Xcc: To determine sensitivity and antibody titre. Antigen (10-50 ml) at 102and 105 cells/ml is loaded onto the ELISA plate in different rows and tested with 100ml developed antibody at various dilutions, 1:500, 1:1000, 1:2000 and 1:4000/ well. The ELISA reactivity was examined and the maximum dilutions showing significant reactivity is considered as the antibody titre.

Evaluation of specificity: Various xanthomonads such as *X. campestriespv. campestries* (Xcv), *X. axonopodis* and *X. oryzae* pv. *oryzae* (Xoo) are tested for cross reactivity at 104 and 102 cells/ ml with different doses of antigen (10-50ml/ well). Culture filtrates (100ml) of all these organisms at equal cell concentration of 108 cells /ml are also examined by ELISA.

Fatty 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



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

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 its 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.

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 – 1000ml. 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 – 200µl; pH - 8.0

   Adjust final volume 1000 ml with deionised water.

   1X TE buffer, 10 mM Tris, 1Mm EDTA, pH 8.0 Prepare 1000 ml using
   100X TE Tris- 10ml; pH - 8.0; Distilled water – 990ml
4. GES solutions- Guanidine thiocyanate-EDTA sarkosyl Prepare 100 ml GES solution:
   Guanidine thiocyanate - 186.1g; 0.5M EDTA – 20ml; pH - 8.0
   Add 20ml water
   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.)

5. Resuspension buffer -0.15M NaCl, 0.01 M EDTA, pH 8.0 Prepare 1000 ml using:

   NaCl - 8.77g; 0.5M EDTA – 20ml; pH - 8.0

Adjust volume to 1000 ml with distilled water

6. Ammonium acetate 7.5 M Prepare 1000 ml using Ammonium acetate – 578.1g
7. 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)

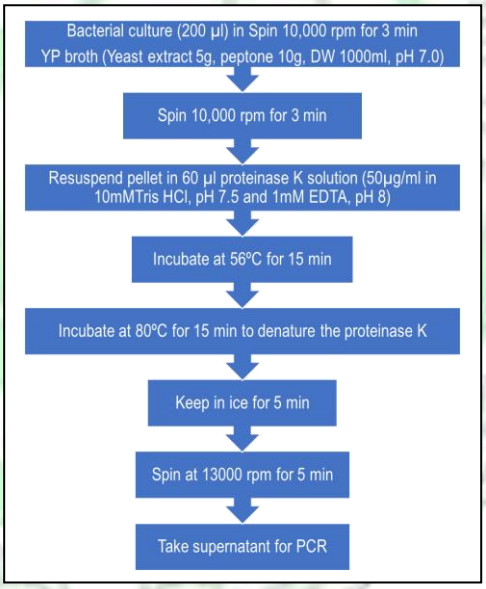
- Take a well grown 24-48 h old culture on an agar plate/broth in a 1.5 ml tube,
- Harvest cells with loop, homogenise/ vortex if grown in agar plates,
- Centrifuge 3 min at 13000 rpm and then remove supernatants carefully and tubes having bacterial pellet should be put on the ice,
- Add 100 µl 1X TE (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 a strong denaturing agent and inactivates endonucleases. Keep on the 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 a homogenous milky solution is obtained,

- Centrifuge approximately 10 min at 13000 rpm until the upper phase is clear,
- Prepared numbered Eppendorf tubes with 400 µl isopropanol ready in a deep freezer at -20ºC,
- Take off 700 µl DNA solution (Clear upper phase) with 1000 µl tips carefully, which the top is cut off (to make the opening wider to avoid DNA from shearing) and transfer to Eppendorf with ice cold,
- Shake gently till a white cloud of precipitated DNA becomes visible Centrifuge for 10 min at 13000 rpm,
- Remove the supernatant carefully and the tubes having pellets,
- 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 the DNA pellet in desiccators or until the 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 at least 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 the 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 is frequent or variable preheat the instrument for longer,
- If the standards have not been changed repeat them,
- It is best to recalibrate the instrument after every 10 sample readings,
- Calculate the concentration of purified DNA (assume ODl= 50µg/ml DNA; use the following formula: ODx50 µg x DF = original DNA concentration in µg/ml). Dilute

Quantification of DNA by Nanodrop spectrophotometer

- If Put 1µl of DNA into a 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) indicates best quality of DNA,
- It is best to recalibrate the instrument after every 10 sample reading,

Isolation of plasmid from *Pseudomonas fluorescens*

Preparation of stock solution

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

Preparation of a 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

- Inoculate the culture in nutrient broth at 37 ºC and 200 rpm,
- Transfer about 1.5 ml culture in a microcentrifuge 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 for 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

Fingerprinting of *Xanthomonas oryzae* 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 polymerase3	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 MgCl2and 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 the 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 product 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 synergelTm). In preparing 0.75% SynergelTm/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 us 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 for loading dye

Bromophenol blue or Xylene cyanol - 25mg; Sucrose - 4g; H_2O - 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.

Detection of *Xanthomonas oryzae* pv. *oryzae* from leaf samples of paddy by PCR Preparation of bacterial ooze

- Cut out 10 very thin (about 1 mm) cross sections of the leaf containing the advancing tip of the lesions,
- Soak the leaf sections in 200 µl of sterile distilled water for at least 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

Detection of *Xanthomonas campestris* pv. *campestris* 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 at least 1 h,
- Streak bacterial ooze in the nutrient sucrose medium and incubate for 72 h in a BOD incubator at 28ºC,
- Pick up the bacterial colonies that appear on the Petri plates i.e. light yellow mucoid raised colonies directly used as DNA templates as discussed in the 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

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 MgCl2	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 units)	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

MULTILOCUS SEQUENCE TYPING (MLST) OF PHYLOGENETIC BACTERIA

Multilocus sequence typing is a valuable molecular technique widely used for epidemiological studies. It is one of the most powerful aspects of MLST is its ability to detect and measure 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 a 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 workflow 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 the identification process so, bioinformatics is 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 the quantity by using bio spectrophotometer 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 MgCl2 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 sequenced on both strands by using the 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 database,
- Assign each sequence of gene allele number and combine allele number for an isolate defined by the sequence type. Group sequence type into clonal complexes using BRUST v3 (http://ebrust,mlst.net). The majority of MLST databases 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 a closer allelic profile including single locus, double locus variants (DLVs) i.e. strain 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 and 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 sequences but which occur several times in a data set are assigned by recombination. The role of recombination relative to the role of point mutations in creating diversity will be measured by the determining ratio of recombination to point mutation (r/m ratio) per allele per nucleotide.

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

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)

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.

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 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 a 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 uppermost and then harvest some growth and transfer it to the liquid medium. The little oil so transferred with the inoculums will float on the top and the culture subsequently recover from under the oil layer with a pipette.

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

Many bacteria survive well in dried soil for longer periods (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 bottle,
- 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 0.1 ml of cell suspension on each soil sample by using a 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 small sample of soil to the suitable broth medium or make a suspension of soil in the broth medium and use this to inoculate in an agar plate.

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

This method is 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 applies to a 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 a longer period. Due to liquid storage, the 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 a dense suspension of cells,
- Transfer the cell suspension to the remaining distilled water. Use a few drops to perform a 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.

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 of preservation is highly suitable to cultures that are continuously in high demand. In lyophilization, two methods of preservation freezing and dying are combined. The overall process involves the 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. However, 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 accession number of the culture on the Whatman No. 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 stick, plug the neck of each ampoule with cotton wool to a depth of 12mm with 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 108cfu/ 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 the 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 the mixture becomes viscous,
- Prepare a slurry of crushed dry ice and 96% ethanol to a depth of 25 mm in a 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 up to an angle of 45º and slip the ampoules into the position. Allow 1 minute for the contents of ampoules to freeze and then return the block to the upright position in the dry ice-ethanol bath for 5 minutes,
- 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 vacuum pump,
- After 5-10 min check the vacuum indicated on the vacuous and ensure that the vacuum is increasing satisfactorily and that no leaks are indicated,
- After 6-8 h when the vacuum 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 vacuum

- Use a pair of scissors 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 a vacuum and must be checked for leaks with a high frequency tester.

6) Sealing of ampoule under nitrogen

- An alternative method of sealing the ampoules under a vacuum is to seal them with an inert gas such as high purity nitrogen before sealing. It is technically easier than sealing under a vacuum and there is less chance of releasing fine particles containing microbes when they are opened. On the other hand, it is easier to detect faulty seals in vacuum 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 up to break the ampoule leaving the cotton wool plug in 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 a suitable temperature,
- The subsequent plate growth provides a 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 them in dark at 4ºC

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 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 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 as 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 outlined 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 to 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 them 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 the 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 that has a hairline aperture through defective sealing may explode if liquid nitrogen has penetrated,
- Thaw the ampoule by immersing it in a 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 transferred 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 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 conduct colonial and cellular purity checks.

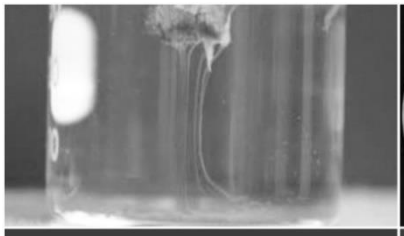


Fig.1: Cloudy mass of bacterial ooze

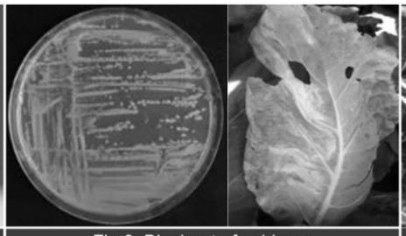


Fig.2: Black rot of cabbage
X. *campestries* pv. *campestries*

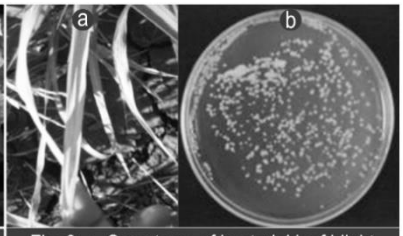


Fig. 3: a. Symptoms of bacterial leaf blight of rice b. *Xanthomonas oryzae* pv. *oryzae*

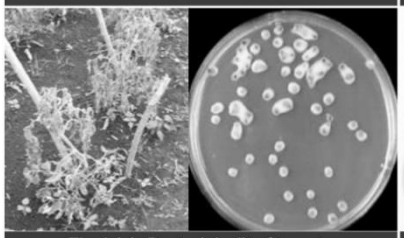


Fig.4:(a). Bacterial wilt of tomato
(b) *Ralstonia solancearum*

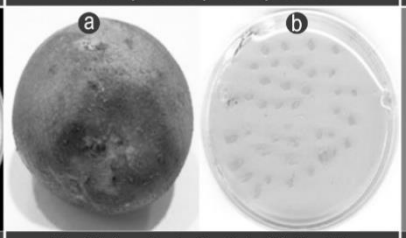


Fig.5:(a) soft rot of potato (b) *Erwinia carotovora* pv. *carotovora* on CVP medium

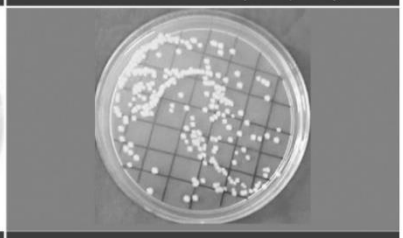


Fig. 6: *Pseudomonas fluorescens* culture

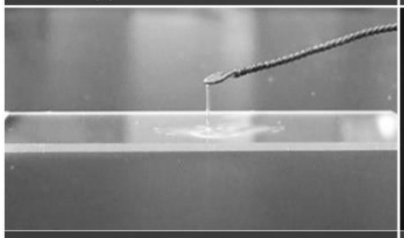


Fig. 7: String of slime showing
Gram (–) ve bacteria

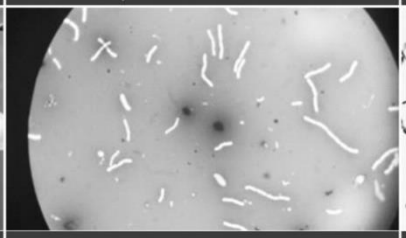


Fig. 8: Negative staining of bacteria

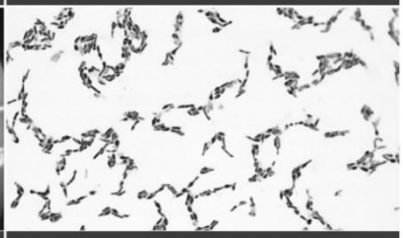


Fig. 9: Simple staining of bacteria

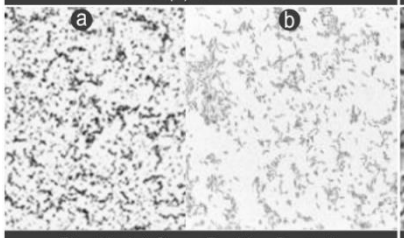


Fig. 10: (a) Gram-Positive Bacteria
(b) Gram-negative bacteria

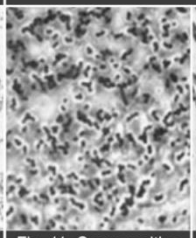


Fig. 11: Gram-positive cocci showing capsules

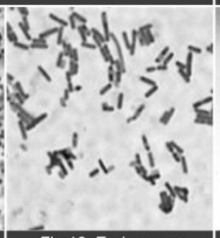


Fig.12: Endospores stained green

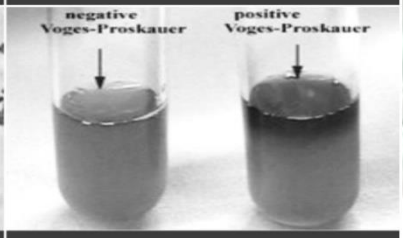


Fig. 13: Voges-Proskauer test

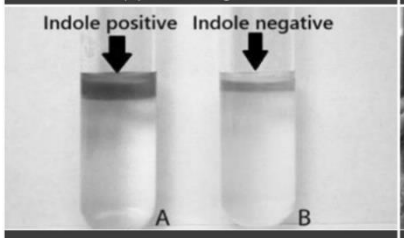


Fig.14: Indole production test

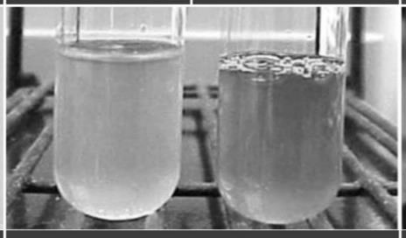


Fig. 15: Nitrate reduction test

Fig. 16:
Arginine test

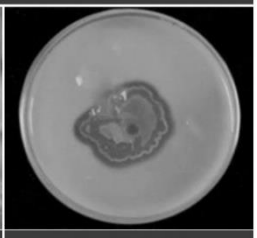


Fig.17: Gelatin
liquefaction test

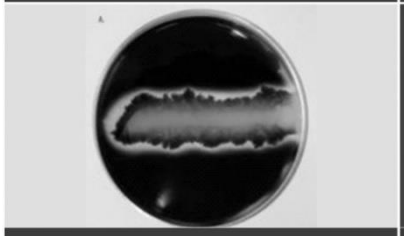


Fig. 18: Starch hydrolysis test

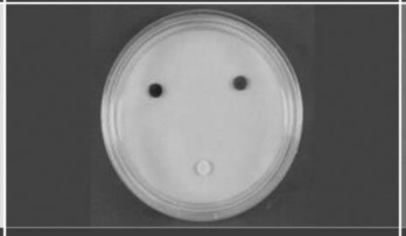


Fig. 19: Oxidase test

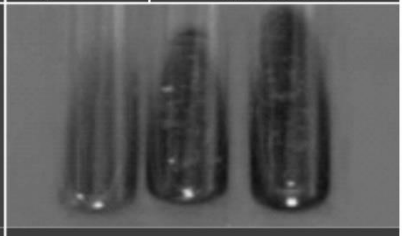


Fig. 20: Citrate utilisation test