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Isolation of Plant Pathogens

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Isolation: It is a process by which separate the casual organism from the diseased tissue of the host and obtaining it into pure form in culture medium or the process of separating micro-organism from the diseased/infected tissue of host.

Isolation of pathogen is done to

(1) Get organism in pure form to determine the host pathogen relationship.
(2) To study the beneficial or harmful effect of particular microorganism.
(3) To prove the Koch's postulates (proving the pathogenicity).
(4) To study its life cycle, cultural characteristics and physiology of pathogen.
(5) For identification of plant pathogen.

Pure culture:-

- The growth of single species of microorganism on a medium is called pure culture.
- A pure culture is required for studying the morphology and physiology of microbes.
- R. Koch (1875) was the first scientist to introduce the pure culture technique in microbiology.

Mixed culture:-

It is growth of two or more species of microbes in the culture medium.

Plate culture:-

It consists of an organism growing on solid medium contained in a Petri plate.

A slant culture:-

It consists of an organism on the inclined surface of solid medium in a test tube. This is also referred to as nutrient agar slant culture.

Liquid / Broth culture:-

The liquid medium such as nutrient broth, milk or dextrose broth containing the growth of an organism is called broth culture.

Stab culture:-

It is prepared by stabbing a solid medium (N.A.) to a considerably depth with sterilized wire needle such as nutrient gelatin or nutrient agar.

Methods of pure culture

1. The spread plate method.
2. The pour plate method.
3. The streak plate method.

1. Spread plate method:- Serial Dilution Method

Requirements

- Sample
- Six sterile Petri plates containing specific media.
- Six test tubes containing 9 ml sterile distilled water
- Glass spreader
- Nutrient agar slants
- Pipette

Procedure:

- Label all the Petri plates and test tubes form one to six.
- Take one gram of soil or other sample from the bacteria need to isolated and mix in 10 ml sterile water. This will give 10^1 dilution.
- Take 9 ml water in remaining all the tubes
- Add 1 ml solution (soil : water) from the first tube to the second. This will give 10^2 dilution.
- Now the second tube will have 10 ml of solution and the same.
- From the tube (second tube) take 1 ml solution (soil : water) and mix in the third tube. This will give 10^3 dilution.
- The same should be repeated till the last test tube (six tubes). This will give 10^6 dilution.
- Take 0.1 ml of the solution from the each tube and spread the help of spreader on the different labeled Petri plate containing specific media.
- Incubate the plate at suitable temperature
- Observe the plate after 24 hours
- Individual isolated colony represent the cfu (colony forming unit)
- Individual colony can be transfer to some other plate or slant for the pure culture
- Colonies can be counted on the colony counter and total number of the microorganism can be calculated by the formula presented hereunder.

Formula: - No. of cells/ml or g of sample (Cfu/ml)

Cfu/ml = Average no. of colonies X Dilution × volume taken

E.g. one gets 30 colonies in 10^5 dilution.

Then no. of cfu/ml = $30 \text{ X } 10^5 \text{ X } 0.1$

$= 30 \times 10^5 \times 10$

$= 30 \times 10^6$

Pour plate method:-

The principle of this method is to dilute the inoculum in successive tubes containing liquefied agar medium and throughout distribution of cells within the medium. The inoculum then poured into Petri dishes and incubated to get well isolated colonies.

- Mixed broth culture of bacteria.
- Three sterile Petri dishes.
- Three tubes of nutrient agar medium.
- Three tubes for nutrient broth.
- Water bath and thermometer.
- Four nutrient agar slants.

Procedure:-

- Dilute the culture several times by transferring 1 ml of culture to 9 ml of sterile distilled water tubes as shown in serial dilution method .
- Melt 2-3 tubes containing15-20 of the nutrient agar, put them at 45°C in water bath.

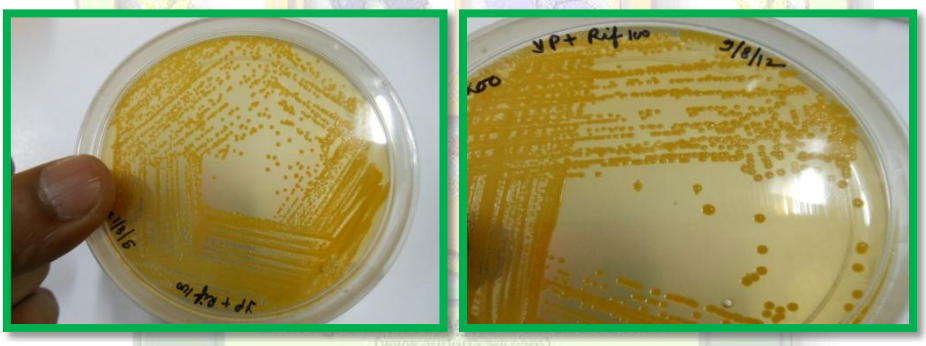
- Allow the melted medium to cool, approximately to 50° C.
- Inoculate 1 ml of highest dilution in to cool melted agar.
- Mix contents of the tube well by rotating the tube between palms of the hand.
- Pour the inoculated nutrient agar tube in to sterile Petri dish.
- Similarly second dilution is also poured into sterile Petri dish.
- Allow the agar to solidify at room temperature.
- Mark the plate, invert and incubate at 37° C for 24 hours.
- Proceed further in the manner similar to the streak plate method.

2. Streak plate method:-

It is also called as the rapid qualitative isolation method. It is essentially a dilution technique that involves streaking a loopful of culture over the surface of an agar plate. Although many types of procedure are performed the four ways of quadrant streak is commonly adopted.

Procedure:-

- Take loopful of a mixed culture and make streak on the surface of nutrient agar medium in the Petri dish.
- Similarly with the same loop, after flaming it without taking culture again rotate the plate streak with the help of wire loop.
- Again rotate the plate after flaming the wire loop streak on the surface of nutrient agar medium on the Petri dish.
- Flame the wire loop. Rotate the plate again at 45° angle again streak for the fourth time on the surface of nutrient agar on Petri plate.
- Invert the Petri dish and incubate it at 37°C temp. for 24 hrs to 36 hrs.
- Observe and record the observation.
- Transfer the well separated colony on nutrient agar slant from the Petri dish to get pure culture.



Plates Showing Growth of Bacterial Cells After Streaking

1. Isolation of the fungi from the infected/diseased host

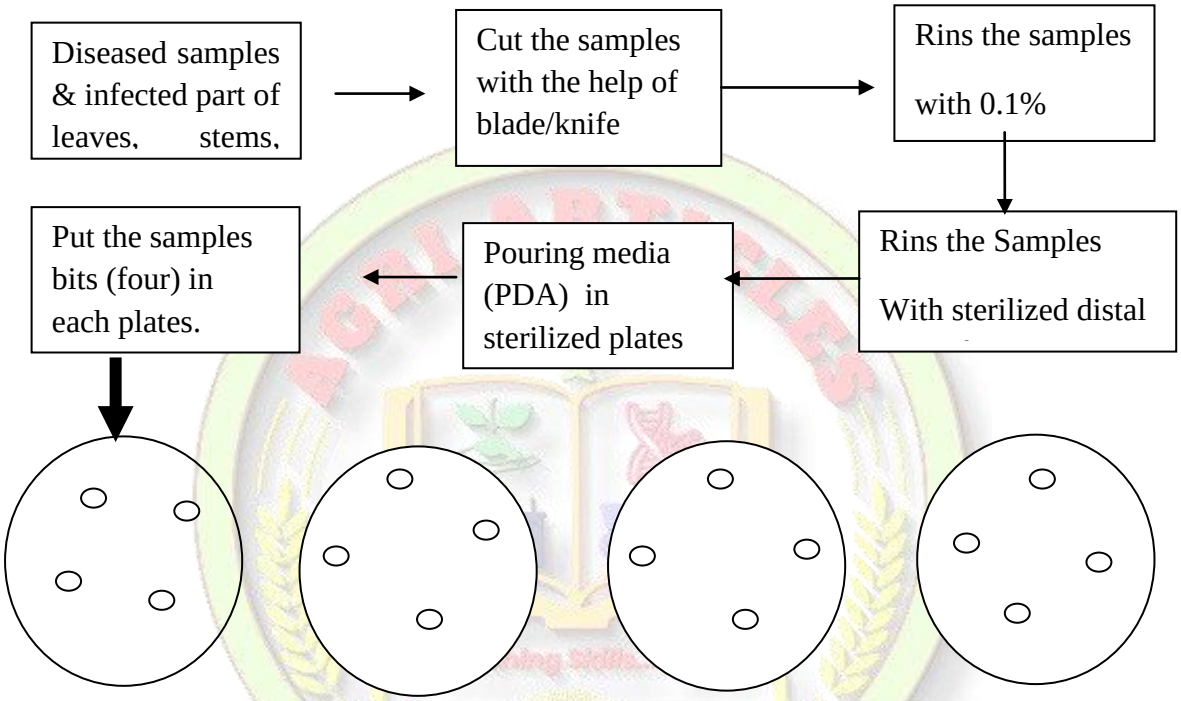
- This method is also called as tissue method and is followed for the isolation of pathogen particularly fungal pathogen from the host tissue when the pathogen is situated deep in the tissue.
- In this method, piece of tissue are transplanted/placed on sterilized agar plates or test tube slants for activating the fungal growth or diseased tissue may be kept in moist chamber.

Procedure:

1. Select a tissue from the most advanced portion of the disease part.
2. Cut the disease piece from the infected leaf of square or rectangular shape.
3. Cut the infected portion with a blade in such a way that each piece should have a part of infected/diseases/necrotic/infected part showing symptoms and three parts showing healthy part.

4. In order to eliminate the contaminants from the outside of the host surface disinfection is necessary. It is carried out by 0.1% mercuric chloride solution for 30sec to 1min or 2.5% sodium hypochlorite solution for 2-3 min. This should be done in the controlled condition so that contaminants cannot land on the tissue after disinfection.
5. Wash with 3-4 times with sterilized distilled water after surface disinfection to remove excess of disinfectant.
6. Transfer of diseased piece on media (Potato dextrose agar medium) previously filled Petri plates or slant for the active growth of the tissue.
7. Place the plate at appropriate place in incubator after proper labeling.

Process:



2. Isolation of bacteria from the infected tissue

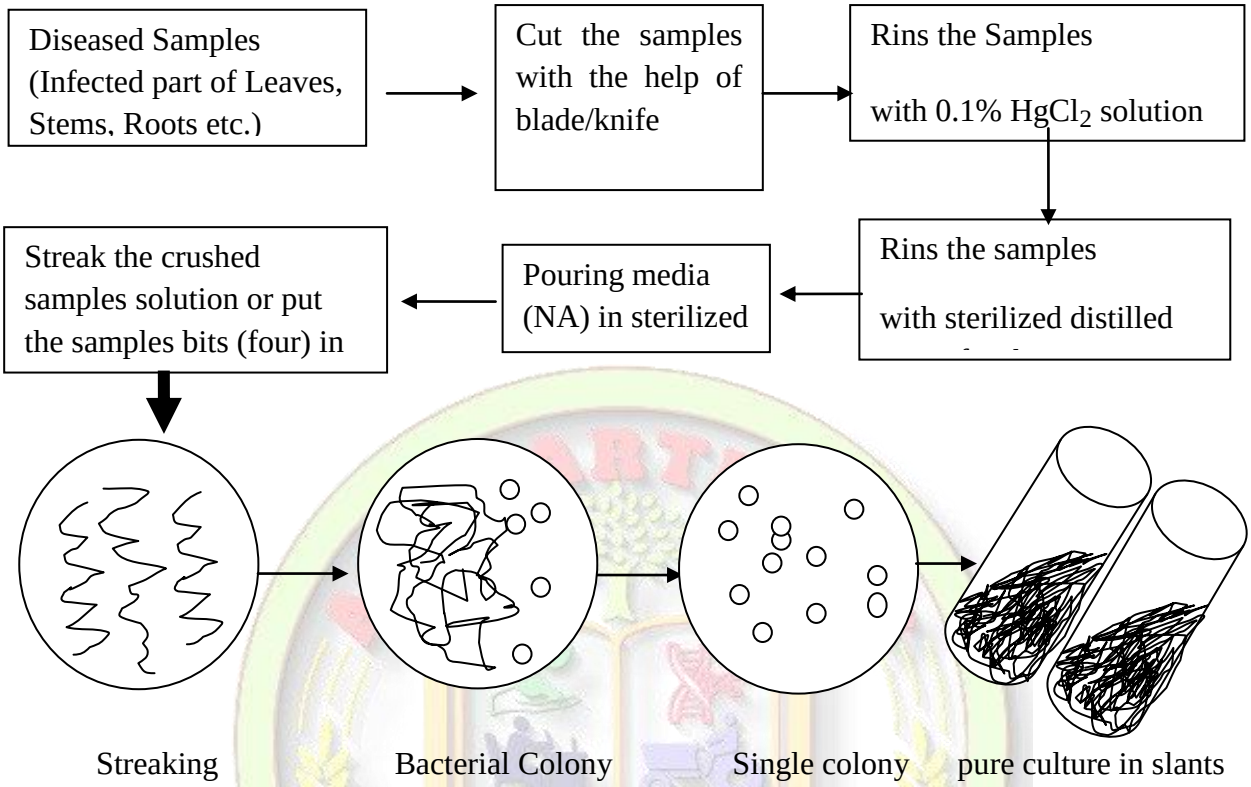
Tissue isolating is not carried out for the bacterial disease as bacteria survive inside the cell. Bacteria do not have positive movement. However, when the bacteria disease infected tissue is cut in the presence of water, many bacterial cells come out of the cell due to rupture of the cells in the form of cloud. This can be observed very easily under the microscope. This cloud of the bacteria cell is known as ooze. If the cell is severely infected and quite large size of the piece is cut and dipped in the water the ooze can be seen in the water without the aid of microscope.

Procedure:

- Select diseased tissues.
- Surface dis-infectant i.e. 0.1% mercuric chloride.
- Washing with distilled sterilized water.
- Crush the tissue in the small plate, tube etc. with the help of sterile mortar or any other sterile object.
- These crushed tissues can be streak (line) with the help of wire loop on a Petri plate, filled with solidified Nutrient Agar media.
- To streak, a loop is first dip in the crushed tissue and then aseptically touches on the surface of media in the plate and loop is gradually moved on Petri plate in such a way

that it will make zigzag line on the plate. Care should be taken to avoid piercing the media with the loop while making streak. This is known as streak plate method.

- Plates are incubated in inverted position for 24 hours at approximately 37° C temperature in incubator.



3. Isolation of Bacteria From The Soil

Microorganism including fungi, bacteria, vascular arbascular mycorrhiza, etc can be isolated from the soil by serial dilution method. In serial dilution, one gram soil is taken and is mixed in 10 ml sterile water. Similarly, one ml of the solution from first tube is taken and dispensed/added in another tube filled with 9 ml of sterilize distilled water. This will make total volume of the tube 10 ml and the solution will be 1:100 time diluted. Similarly, if the solution is diluted six time this will give 10^{-6} fold dilution of the sample. Hundred micro-litters to One ml of the solution is taken and spread on the Petri plate filled with the desired media. This is known as **Spread Plate Method.** Plates are incubated in inverted position for 24 hours at approximately 37° C temperature in incubator. This will dilute the microorganisms and will give very clear colonies of the bacteria at sufficient distance to further transfer in another tube or plate for the purification. Even if the colonies are appear in the media very close to each other making isolation difficult, these may further be diluted to 10^{-7} - 10^{-8} fold.