AIM – ISOLATION AND CHARACTERIZATION OF ACTINOMYCETES FOR PRODUCTION AND CONFIRMATION OF ANTIBIOTICS

Requirements – Garden soil or Pure cultures of Actinomycetes, Soil extract agar, Starch Casein Agar, Yeast Extract Malt Extract Agar, Sterile Petri plates, Test tubes, Conical flasks, Centrifuge, Micropipette, Incubator and High Resolution Microscope

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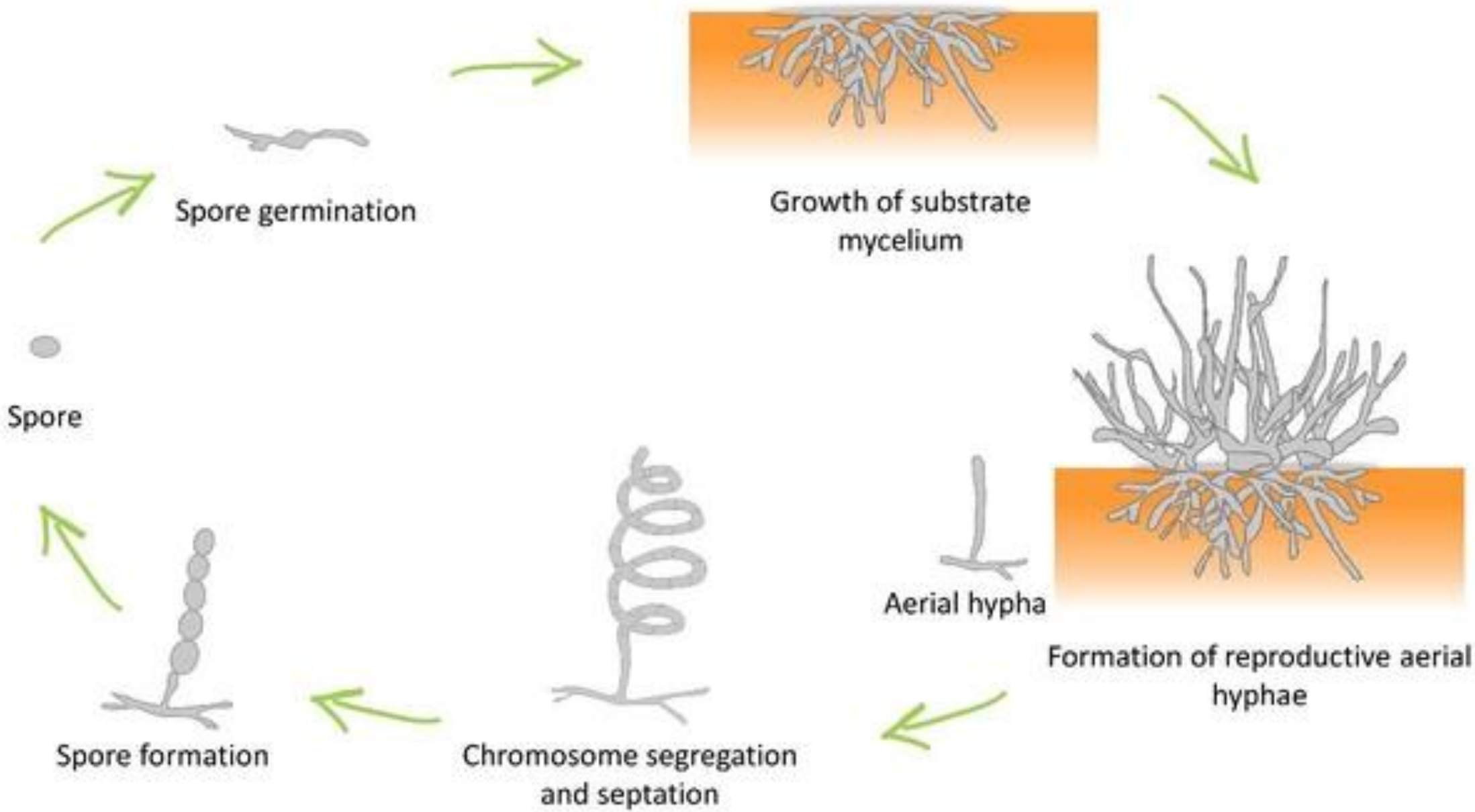
Principle

- Actinomycetes are Gram-positive, facultative anaerobic filamentous bacteria characterized by formation of branched filamentous body and produce mold-like spores or conidia
- They are extensively distributed in natural habitat and involved in different biological and metabolic processes
- Almost 90% of actinomycetes genera have been isolated from soil, which are usually harmless
- It has been suggested that *Streptomyces* are recognized as best producer of antibiotics followed by *Nocardia* and *Micromonospora*


Spore germination
Growth of substrate mycelium
Spore
Aerial hypha
Formation of reproductive aerial hyphae
Spore formation
Chromosome segregation and septation

Procedure

A. Isolation of Actinomycetes:

1. Primary Isolation: Prepare Soil Extract medium

(100g garden soil, boil with 100 ml water for 30 min)

↓ Filtration

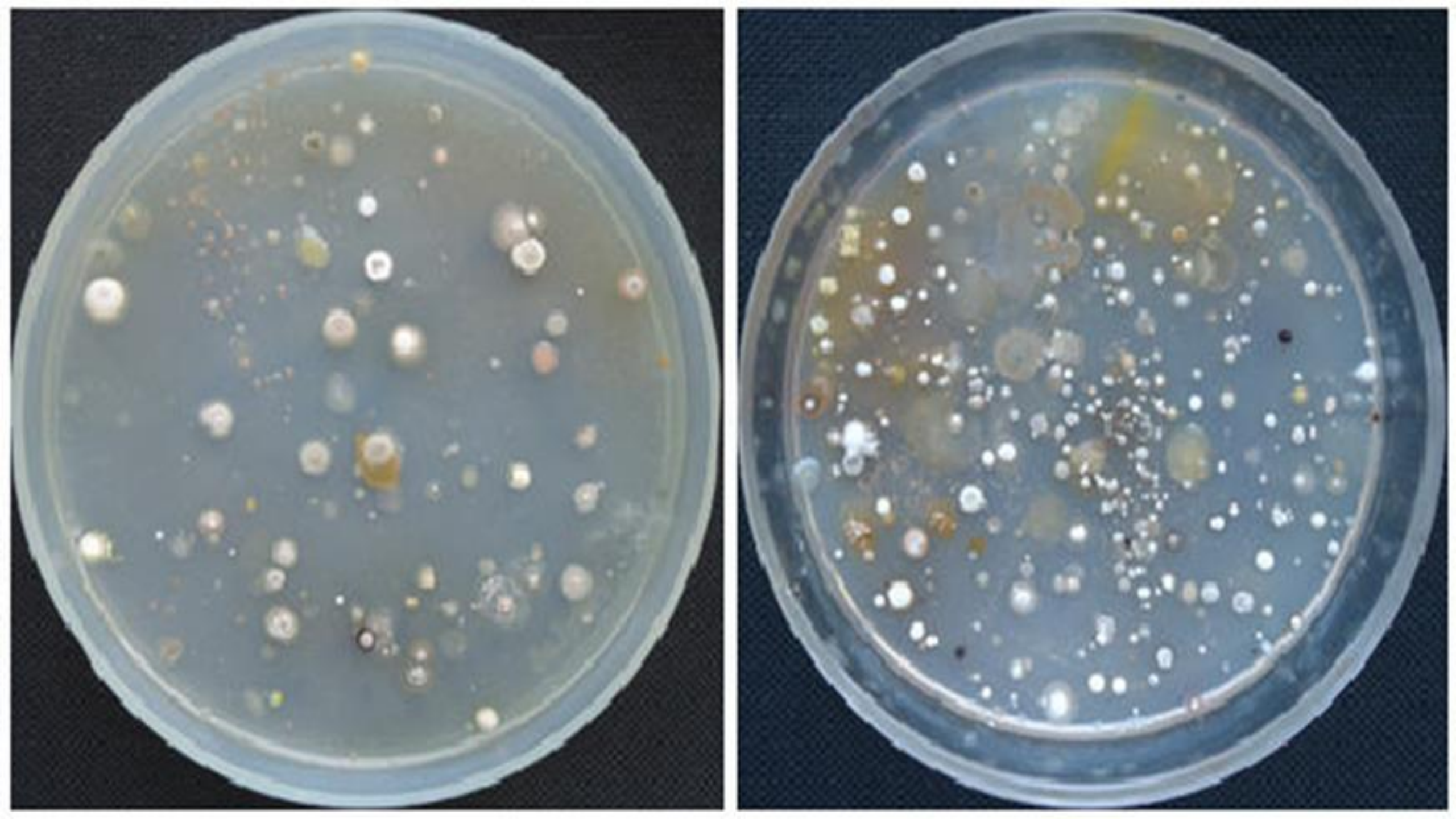
Soil extract

↓ Add few pinches $CaCl_2$

Clear filtrate

↓ Add Glucose 1g and 500mg Dipotassium Phosphate and 15g Agar

Soil Extract Agar Medium (1000 ml) pH 6.8



2. Pure Culture production:

Prepare Starch Casein Agar

(Soluble starch10 g, K_2HPO_4 2 g, KNO_3 2 g, casein 0.3 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.01 g, agar: 15 g, and water 1000 ml and pH 7.0 ± 0.1)

↓

Inoculation of culture from mater plate

↓ **Incubated at 28°C for 7 days**

↓

Pure Culture of Individual Species

↓

Morphological Identification

B. Morphological characterization

1. Macroscopic method

- Colony characteristics, pigment production, absence or presence of aerial and substrate mycelium

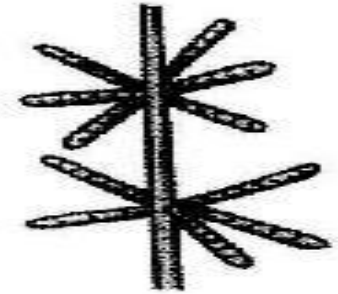
2. Microscopic cover slip culture method

- To examine the arrangement of spores and sporulating structures by inserting sterile cover slip at an angle of 45° in the agar medium
- Remove the cover slip carefully using sterile forceps and place upward on a clean glass slide
- Fix the bacterial growth on the cover slip with few drops of absolute methanol for 15 min and wash with tap water then flood with crystal violet reagent for 1 min followed by washing and blot drying
- Examine the cover slip under the microscope by using oil immersion lens (100×)
- Identification of the actinomycetes was done on the basis of macroscopic and microscopic examination as suggested by Bergey's Manual of Systematic Bacteriology, 2nd Edition, Vol 5, The Actinobacteria, Part A

Straight

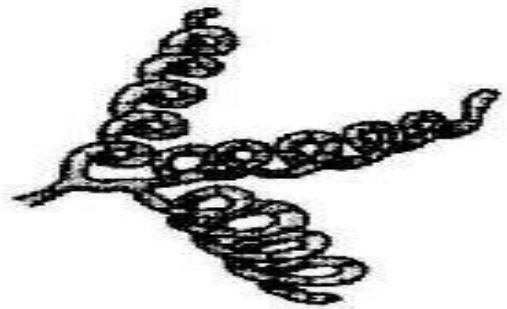
Flexous

Fascicled

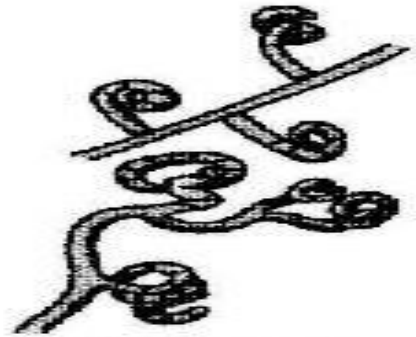


Monoverticillate, no spirals

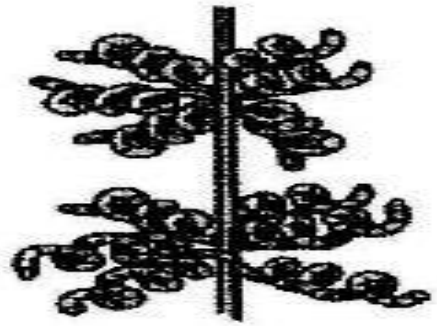
Open loops, primitive spirals, hooks



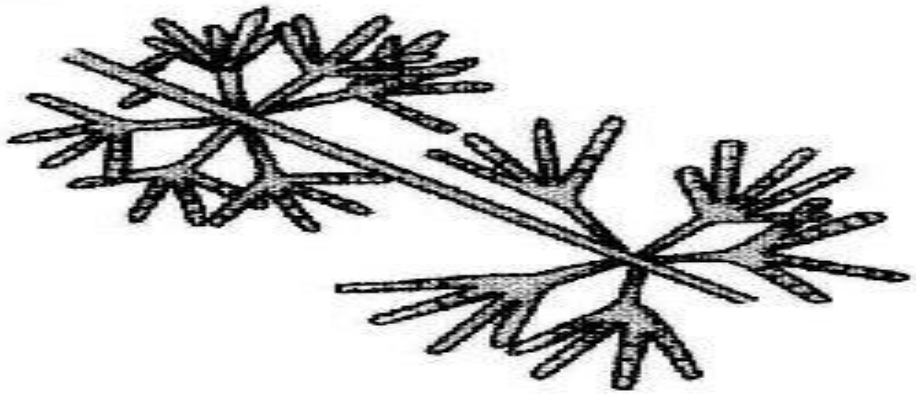
Open spirals



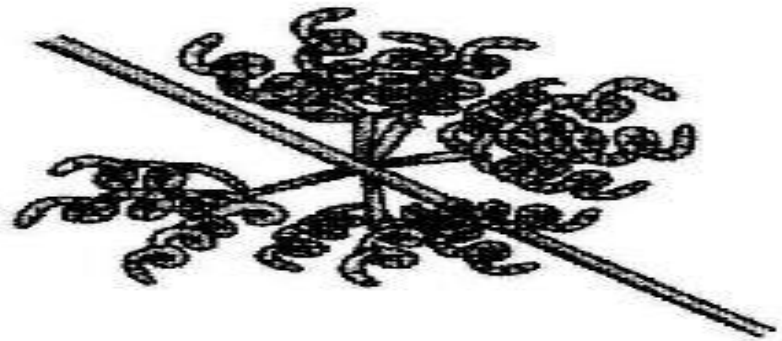
Closed spirals



Monoverticillate, with spirals



Biverticillate, no spirals



Biverticillate, with spirals

C. Primary Screening for Antibiotic production:

Prepare Yeast Extract Malt Extract Agar Medium

Peptone 5.0 Yeast extract 3.0 Malt extract 3.0 Dextrose 10.0 Agar 20.0 Final pH (at 25°C) 6.2±0.2 g/ L

↓

Streak a loopful suspension of the test Actinomycetes on the surface of the agar medium towards periphery of the petri plates

↓

Incubate at 28 ± 2ºC for 7 days

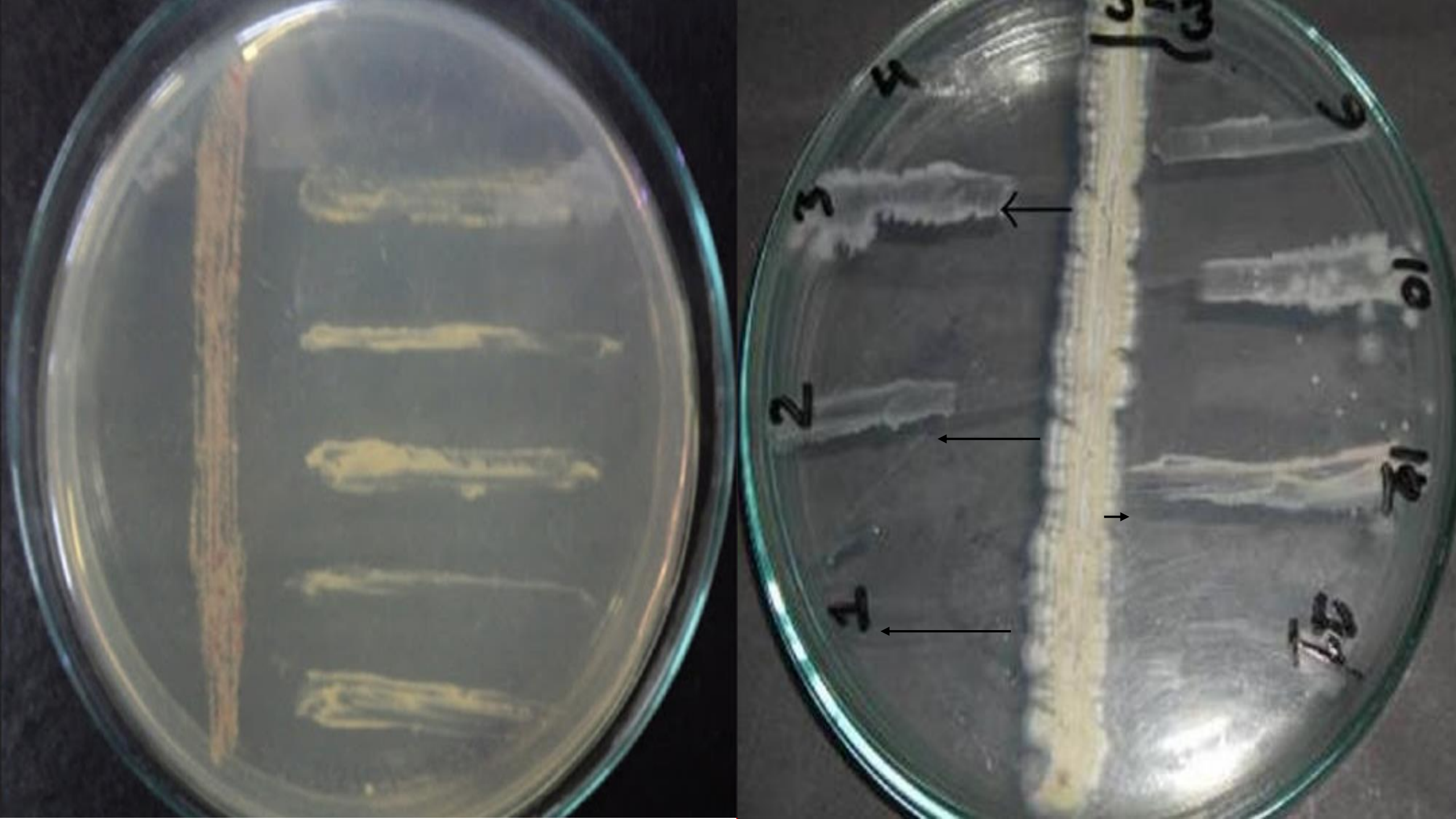
Make another streak of loopful suspension of test organisms at right angle to growing actinomycetes colony

↓

Incubate at 28ºC for 24 hrs.

↓

Record Observation for Total inhibition (TI), Growth Inhibition (GI), growth inhibition and retardation (GIR) and No Inhibition of growth (NIG)


4
3
2
1

D. Secondary Screening and Confirmation of Antibiotic Production:

Prepare Yeast Extract Malt Extract broth

↓ Inoculate actinomycetes culture and incubate at 28^0C for 7 days

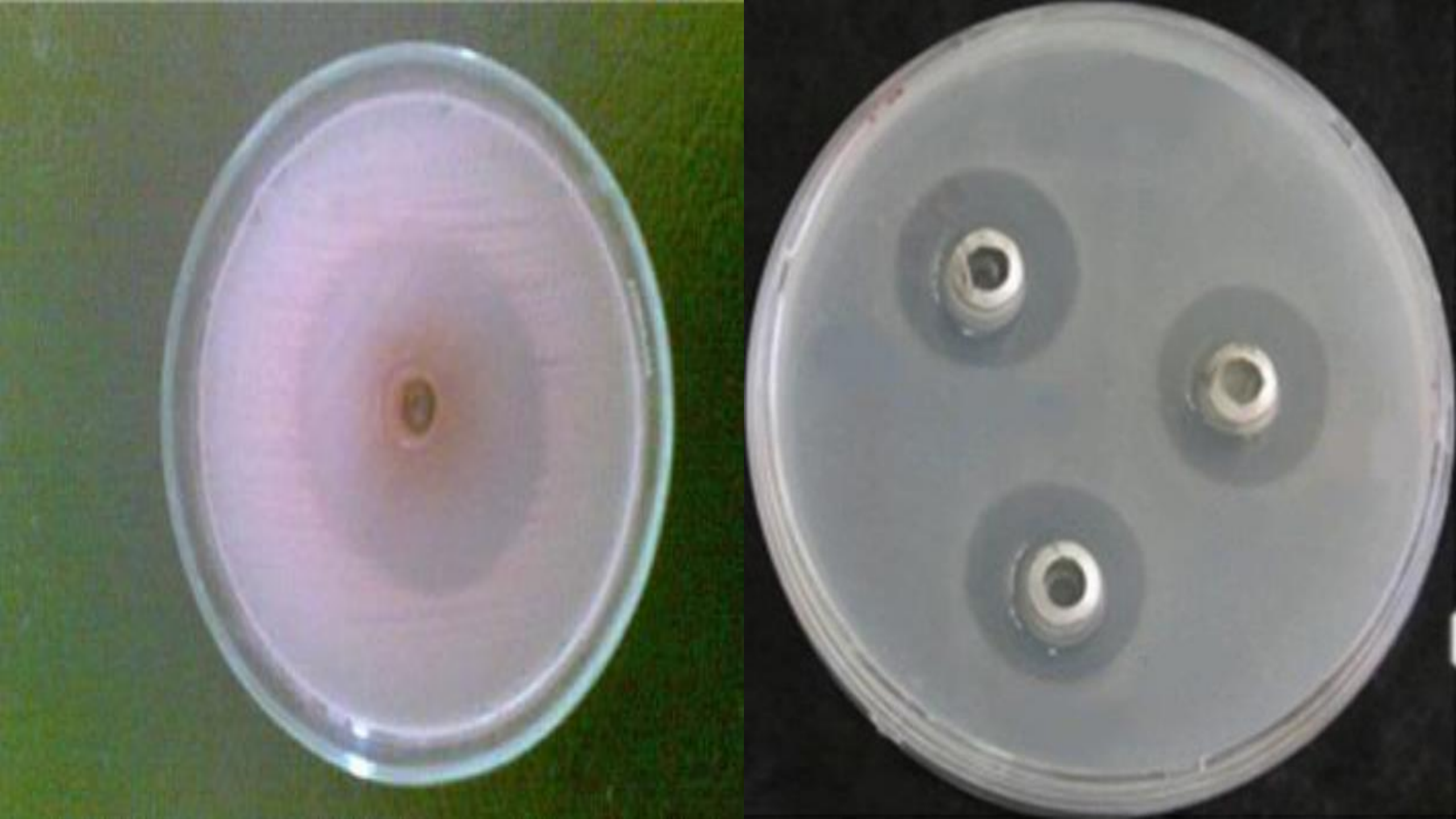
Bulk Culture

↓ Filter and Centrifuge the extract at 10000 rpm for 20 -30 min

Prepare NA Medium and inoculate with the test bacteria by spread plate techniques

↓ Make well in the plate and pour the 20 µl extract in the well

Observe for Size of Zone of Inhibition



Observation

A. Morphological characterization

1. Macroscopic method:

Macroscopic Features	Culture Code			
	A	B	C	D
Colony Characteristics				
Pigment Production				
Aerial Mycelium				
Substrate Mycelium				

2. Microscopic cover slip culture method:

S.No.	Culture Code	Microscopic Observations as per Bergey's Manual
1	A	
2	B	
3	C	
4	D	

B. Primary Screening for Antibiotic Production:

S. No.	Culture Code	Type of Inhibition against Bacteria			
		I	II	III	IV
1	A				
2	B				
3	C				
4	D				

Total inhibition (TI), Growth Inhibition (GI), growth inhibition and retardation (GIR) and No Inhibition of growth (NIG)

C. Secondary Screening for confirmation of Antibiotic Production:

S.No.	Culture Code	Zone of Inhibition against bacteria (mm/cm)			
		I	II	III	IV
1	A				
2	B				
3	C				
4	D				

Result: Depict your results as per the observation received as

1. Total number of isolates recovered from the given soil sample
2. Tentative identification as per Bergey's manual
3. Type of inhibition shown by the isolates against the test bacteria
4. Confirmation of Antibiotic Production by secondary screening

Interpretation: Interpret the result with the help of your teacher

Precautions:

1. All microbiological preparations and activities should be done cautiously
2. Microscopic observations must be done precisely
3. Care should be taken during handling of bacteria, as it should be pure in origin

Thank You.....