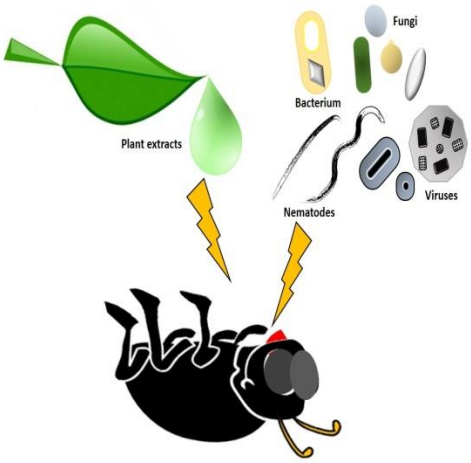
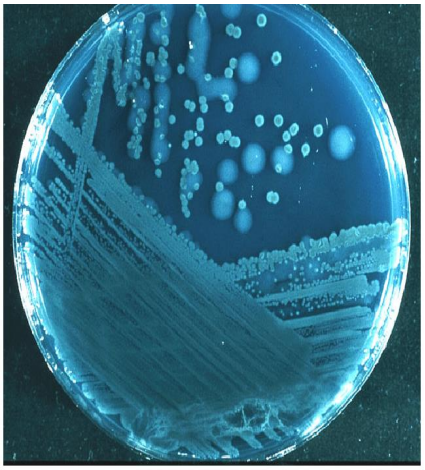
THE NEOTIA UNIVERSITY

ज्ञानम् आत्म प्रदीपाय

Biopesticides and Biofertilizers
Practical Manual
Course No, EC-AGL506





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CONTENTS

PRACTICAL 1: ISOLATION AND PURIFICATION OF *TRICHODERMA* BIOPESTICIDES AND ITS PRODUCTION.

Introduction

- Free living fungus common in soil and root ecosystem, Highly interactive in root, soil and foliar environment, Suppresses the pathogen by different mechanism of biocontrol, Cultures are fast growing at 25-30 degree C. Conidia forming within on week in compact or loose tufts in shades of green or yellow or less frequently white. Yellow pigment may be secreted into the agar, especially on PDA. A characteristic sweet or 'coconut' odour is produced by some species.

Objective

Objective of this experiment is Isolation And Purification of *Trichoderma* Biopesticides and Its Production.

Materials Required

Soil Samples, recently collected and not air dried one, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare Potato Dextrose Agar medium as per requirements.
2. Pour Potato Dextrose Agar medium into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 35 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
5. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
6. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

PDA medium	
Starch (Potato)	20g
D-Glucose	20g
Urea	3g
$MgSO_4.7H_2O$	0.5g
KCl	0.15g
KH_2PO_4	0.08g
$ZnSO_4.7H_2O$	0.01g
Agar	20g
Distilled Water	1000 ml

Observations;

Mass production of biocontrol agent

1. Mix 30 gm molasses and 6gm Brewer's yeast in 1 litre of water. Distribute 60 ml in each conical flask then Autoclave them.
2. Inoculate 8mm mycelia discs of Trichoderma in medium
3. Incubate for 10 days at room temperature
4. Use for multiplication in the fermentor
5. Prepare 50 lit of molasses + Yeast medium and sterilize for 30 min in the fermentor
6. Transfer aseptically 1 lit of Trichoderma
7. Incubate for 10 days
8. Using Haemocytometer
9. 500 ml of fungal biomass + 1 kg of talc powder
10. Air dry and Carboxy Methyl Cellulose (CMC) + Stickers 5gm / kg
11. Store in Polythene bag

Conclusion;

You tube link: https://youtu.be/TruIZMdLMVY

PRACTICAL 2: ISOLATION AND PURIFICATION OF *PSEUDOMONAS* BIOPESTICIDES AND ITS PRODUCTION.

Introduction

- Pseudomonas fluorescens is a common Gram-negative, rod-shaped bacterium.
- It belongs to the Pseudomonas genus.
- P. fluorescens has multiple flagella.
- It has an extremely versatile metabolism, and can be found in the soil and in water.
- It is an obligate aerobe, but certain strains are capable of using nitrate instead of oxygen as a final electron acceptor during cellular respiration.

Objective

Objective of this experiment is Isolation and Purification of *Pseudomonas* Biopesticides and Its Production.

Materials Required

1. Rhizospheric Soil Samples Recently collected and not air dried one.
2. Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare **King's Medium** as per requirements.
2. Pour **King's Medium** into sterile Petri plates and allow them to solidify.
3. Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
4. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
5. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

1. King's B Medium	**g/l**
Peptone	20.00
K_2HPO_4	1.50
$MgSO_4$	1.50
Glycerol	10 ml
Agar	20.00
Distilled water	1000 ml
pH	7.2 ± 0.2

Observations;

Mass Multiliplication

Step 1: Preparation of mother culture

Step 2: Multiplication of culture in conical flask

Step 3: Liquid fermentation of P.fluorescens using King'B medium.

Step 4: Incubate for 7 days

Step 5: Use haemocytometer

Step 6: 500 ml culture with 1 kg of talc powder

Step 7: Air dry and add supplementary nutrients and sticky agents

Step 8: Store it in polythene bag

Conclusion;

You tube link : https://youtu.be/28MM6wqwT_A

PRACTICAL 3: ISOLATION AND PURIFICATION OF *BACILLUS* BIOPESTICIDES AND ITS PRODUCTION.

Introduction

It's a gram +ve bacteria, Rod shaped, Flagellated, Cell size 4 to 10 micro meters, it can form endospores (To survive extreme environment conditions of temperature. Optimum temperature range is 25 to 35 degree Celsius. They produce colonies which are dry, flat and irregular with lobate margins.

Objective

Objective of this experiment is Isolation and Purification of *Bacillus* Biopesticides and Its Production.

Materials Required

Soil Samples, recently collected and not air dried one, Autoclave, Laminar Air flow chamber, BOD Incubator, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments; Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare **Nutrient Agar Medium** as per requirements.
2. Pour **Nutrient Agar Medium** into sterile Petri plates and allow them to solidify.
3. Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
4. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
5. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

2. Nutrient agar (Bacteria)	**g/l**
Beef extract	3.00
Peptone	5.00
Sodium chloride (NaCl)	5.00
Agar	20.00
Distilled water	1000 ml
pH	7.0 ± 0.2

Methodology

1. Spread Plate Method;

   The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

Observations;

Mass Multiplication

The nutrient broth is prepared in fermentor and sterilized at 15 lb pressure for 15 minutes. Then the mother culture is added @ 1lit/100 lit of the medium and incubated at room temperature for 2 days. The medium containing the bacterial growth of *B.subtilis* is used for mixing with talc powder.

Conclusion;

You tube link: https://youtu.be/alTgt0h5jOE

PRACTICAL 4: ISOLATION AND PURIFICATION OF *METARHYZIUM* BIOPESTICIDES AND ITS PRODUCTION.

Introduction

- Fungi represent a diverse group of insect pathogens.
- The insects attacked by the fungus die shortly after the fungus begins to develop in the haemocoel.
- An **entomopathogenic fungus is fungal species that** can act as a parasite of insects and kills or seriously disables them. These fungi usually attach to the external body surface of insects in the form of microscopic bodies (usually asexual, mitosporic spores also called conidia).

Objective

Objective of this experiment is Isolation and Purification of *Metarhyzium* Biopesticides and Its Production.

Materials Required:

Soil Samples, recently collected and not air dried one, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure:

1. First prepare Potato Dextrose Agar medium as per requirements.
2. Pour Potato Dextrose Agar medium into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 35 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
5. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
6. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

PDA medium	
Starch (Potato)	20g
D-Glucose	20g
Urea	3g
$MgSO_4.7H_2O$	0.5g
KCl	0.15g
KH_2PO_4	0.08g
$ZnSO_4.7H_2O$	0.01g
Agar	20g
Distilled Water	1000 ml

Observations;

Mass production of Metarhizium anisopliae (green muscardine fungus)

Metarrhizium anisopliae *is* a widely distributed soil inhabiting fungus. The spore of *M. anisopliae can be* formulated as dust and sprayable formulation.

Materials required

- Coconut water, Flat glass bottles, Cotton plug, Pressure cooker, Injection syringe, Bunsen burner Laminar flow chamber, Mixer grinder

Methodology

- Take glass bottles containing 40 ml of coconut water. Plug these bottles with cotton plug and sterilize in autoclave for 20 minutes at 15 psi.
- The bottles are inoculated with 1 ml suspension containing spores of the fungus with the help of a sterile injection syringe.
- Sterilize the bottles with the help of burner. Spores are inoculated in bottles with the help of syringe in a laminar flow chamber.
- Keep inoculated bottles till the surface of medium is fully covered by the olive green sporulated fungus. Whole culture is grinded thoroughly in mixer. Keep culture in cool and dry place in packets.

Conclusion;

You tube link: https://youtu.be/dU8NM8v4lRs

PRACTICAL 5: IDENTIFICATION OF IMPORTANT BOTANICALS

You tube link: https://youtu.be/KIDasd5DHu8

PRACTICAL 6: QUALITY CONTROL OF BIOPESTICIDES

Introduction

- **Pesticide** use has certainly contributed towards improving agricultural production, in terms of both **yield and quality**, thus **increasing agricultural income**, particularly in developed countries.
- However, careless use of pesticides without adhering to the safety norms and recommended practices has posed **serious health risks to humans**, **other living organisms**, and **the environment**, from **on-farm workers**' exposure and release of chemicals into the air and water, to commodities containing pesticide residues.
- There has been a growing demand for **food safety** and quality in recent decades, as reflected in the tight **safety regulations** on imports of products and **strict regulations** on the amount of pesticide residues on commodities.
- Moreover, increasingly high standards regarding product quality are continuously being set. Public awareness about the adverse effects of pesticides on the **safety of foods** and on the **environment** has increased in recent years, and the search for **alternatives** to widely used chemical pesticides, including **biopesticides**, has become a priority

Regulation of Biopesticides

- Bio-pesticides are **potential alternatives to synthetic chemical pesticides**. It was known that, biopesticides are living natural enemy organisms and/or their products including plant and microbial products and/or their byproducts and they could reduce pest populations
- In the present decade, biopesticides are **widely acceptable** and demanded for **sustainable agriculture** and for **production of safer foods**.
- It was significantly considered that, biopesticides are **eco-friendly**, **target- specific**, **easily biodegradable** and **safer alternatives**.
- **Regulatory measures:** As the bio-control agents are living organisms, it is very important to have effective regulatory measures. The quality control of commercial bio-agents must be strictly enforced by the Government. In this connection, **the Directorate of Plant Protection Quarantine and Storage**, **Department of Agriculture and Cooperation, Ministry of Agriculture,** GOI have issued guidelines/data requirements for registration of bio-pesticides in the country. As per this, all the units have to meet the Indian standards and technical specifications to be eligible for registration under the **Insecticides Act, 1968**.

Why Quality Control is required?

<u>The registration data must include its</u>

a) Composition and description
b) Biological properties and quality control standards at different stages
c) Specificity of containers/packaging, delivery system, label information for market products,
d) Tolerance limits, residues, safety to non-target organisms
e) Taxonomic identification

f) Detail production technology
g) Efficacy and biological impact
h) Contamination, and shelf-life under storage and regular use

Quality control (QC)

- Quality control needs to be **ensured** at all levels of **mass production** of natural enemies, viz. host/prey insects, natural enemy and plants or their products.
- Quality control is needed in **all the sectors**, i.e. rearing, culturing, preserving, storage methods, containers, transportation and releasing techniques.
- Therefore, Quality control (QC) is of paramount importance in order to ensure that products are delivered that complies with pre-determined **specifications** and delivers the **efficacy** within the prescribed conditions for use.
- Quality control does not only refer to the **final end-use product**, but also to the **production and the production processes**.
- Definitions of quality control used for microbials will preferably be similar to the ones used for natural enemies and other beneficial biocontrol agents

Quality Control Objectives

- Properties of incoming raw materials comply with the manufacturer's specifications.
- There is consistency between production runs and products.
- End-use products meet criteria set by registration authorities.
- Product performance meets the end-user's perception of quality in relation to price, and leads to repeat purchases of the product.

Quality control is important and should be an integral part of the mass production.

Quality control can be divided in

a) Production control
b) Process control
c) Product control

Methods QC

1. Quality Control (Production, Process and Product)
2. Product Quality Control of Microbial Pest Control Products (MPCP)
3. Identity of the Microbial Pest Control Agent
4. Number of Infective Propagules
5. Microbial purity
6. Presence of Toxins
7. Physical, Chemical and Technical Characteristics
8. Efficacy
9. Quality Control and Shelf-Life

Conclusion;

You tube link: https://youtu.be/Y8bDVPLlW44

PRACTICAL 7: VISIT TO BIOPESTICIDES LABORATORY AND FIELD VISIT TO EXPLORE NATURALLY INFECTED CADAVERS. IDENTIFICATION OF ENTOMOPATHOGENIC ENTITIES IN FIELD CONDITIONS

You tube link: https://youtu.be/dU8NM8v4lRs

PRACTICAL 8: ISOLATION AND PURIFICATION OF *AZOSPIRILLUM*

Objective

- *Azospirillum* is an aerobic or microaerophilic, motile, helical, or fibroid, gram negative bacterium. Being an associative symbiotic, this bacterium brings many benefits to many non leguminous crop like cereals, millets, forage crops, and vegetable crops.
- Objective of this experiment is isolation of *Azospirillum* from root surface of crops and study about its morphology.
-

Materials Required:

1. Rhizospheric Soil Samples Recently collected and not air dried one.
2. Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare **Dobereiner's Medium** as per requirements.
2. Pour **Dobereiner's Medium** into sterile Petri plates and allow them to solidify.
3. Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
4. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
5. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

Dobereiner's medium (*Azospirillum)*	**g/l**
Malic acid	5.00
KOH	4.00
Yeast Extract	5.00
Mn SO_4H_2O (1%)	1.00
$MgSO_47H_2O$ (10%)	1.00
NaCl (10%)	2.00
K_2HPO_4 (10%)	4.00
$NaMoO_4$ (0.1%)	0.20
$CaCl_2$ (10%)	1.00
$FeSO_4.7H_2O$ (5%)	1.00
1 m NH_4Cl	5.00
Bromthymol Blue	3.00 ml

Methodology

Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

Observations:

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Azospirillum **Bacteria**	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil (g)}}$$

Conclusion;

You tube link: https://youtu.be/jpuNYpvBmDM

PRACTICAL 9: ISOLATION AND PURIFICATION OF *AZOTOBACTER*

Objective

- The most widely studied of the free living, or non-symbiotic, biological agents of bacteria fixation is undoubtedly the gram negative soil bacterium (*Azotobacter*).
- The objective is isolation of *Azotobacter* from soil and study about its morphology.

Materials Required:

Soil Samples, recently collected and not air dried one, Autoclave, Laminar Air flow chamber, BOD Incubator, Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. First prepare **Jensen medium** as per requirements.
2. Pour **Jensen medium** into sterile Petri plates and allow them to solidify.
3. Sieve the soil through 2 mm sieve, weigh two 10g samples, keep one sample in an oven over night at 35 degree C. Weigh this sample to find out the percentage of moisture in soil.
4. Add the other 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
5. Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
6. Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.
7. Azotobacter colonies appear as flat, soft, mucoid and milky colonies.

Jensen's nitrogen free medium (*Azotobacter*)	**g/l**
Sucrose	20.00
Dipotassium hydrogen ortho-phosphate (K_2HPO_4)	1.00
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.10
Sodium chloride (NaCl)	0.50
Sodium molybdate di hydrate	0.001
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.01
Calcium carbonate ($CaCO_3$)	2.00
Agar	20.00
Distilled water	1000 ml
pH	7.0 ± 0.2

Methodology

Spread Plate Method

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position

Observations:

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Azotobacter Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil}}$$

Conclusion;

You tube link: https://youtu.be/9a_CUhfv8LA

PRACTCIAL 10: ISOLATION AND PURIFICATION OF ***RHIZOBIUM***

Introduction

The Most important system by which molecular nitrogen is fixed biologically results from a symbiotic association between a soil bacterium and a legume plant. Rhizobium, infect legume seedlings through root hairs and stimulate the formation of tumor like nodules on the roots. The nodules comprised of both plant and microbial tissue, are capable of using atmospheric nitrogen.

Root Nodules

Objective

The Objective of this method is to provide a procedure for the isolation of Rhizobium species from nodules found on the roots of legume plants and study about their morphology.

Materials Required

Root systems of nodule-bearing legume plants freshly collected ones, Sterilized water, Sterilized Petri dishes, Ethanol (70%), Sterilized forceps, Scalpel or Razor blade, Inoculating loop, YEMA medium

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

1. Wash the soil from the roots and nodules of legume plant chosen for study. Note the appearance, distributions and abundance of nodules.
2. Carefully out, do not tear a nodule from the roots so that small portions of the root remains attached. If possible, select a plum firm nodule preferably pinkish in colour. Wash under running water to insure the removal of all soil particles.
3. Put the nodule into a Petri dish containing 0.1 % $HgCl_2$ and leave it immersed for 5 minutes.
4. Transfer the nodule to a sterile Petri dish containing sterile water. Use sterile forceps to make the transfer. Wash the nodule in the sterile water.
5. Transfer the nodule to another Petri dish containing 70% ethanol for 3 minutes. Remove to another Petri dish of sterile water and rinse thoroughly, and then transfer to a second plate of sterile water for final rinsing.
6. Add 0.1 ml of sterile water to each of six sterile Petri dishes. Remove the nodule from rinse water to Petri dish No.1 and Crush it with flamed forceps. Mix the nodule tissue with the water.
7. Transfer two loops full of the suspension in the No.1 Petri dish to Petri dish No.2 and mix it with the sterile water previously added. Repeat this loop dilution progressively for plate nos.3, 4, 5 and 6.
8. Add 15 ml of YEMA medium (melted in a boiling water bath and held at 42 degree C for pouring) to plates Nos. 2 to 6 inclusive. Mix the agar and dilutions thoroughly by swirling, and incubate at 28 degree C for 7 days.

9. At the end of one week representative colonies of Rhizobium should have developed and isolation can be made for further use or storage. Select the mucoid, or flat, watery colonies that are most abundant in the loop dilution plates. Choose an isolated colony among the domain, Rhizobium-like colonies derived from a single nodule, and transfer aseptically to several slants of the same medium. Label, incubate, and store for further testing.

YEMA medium Composition; (Yeast Extract Mannitol Agar)

Mannitol	10 g/l
K2HPO4	0.5 g/l
MgSo4.7H2O	0.2 g/l
NaCl	0.1g/l
Yeast Extract	0.5 g//l
Agar	20 g/l
Dis.Water.	1000ml

Methodology:

Pour Plate Method

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplates to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify.

Observations:

Present you results as

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Rhizobium Bacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Root}}$$

Conclusion:

You tube link: https://youtu.be/e8zgAIdEa84

PRACTCIAL 11: ISOLATION AND PURIFICATION OF *P-SOLUBILIZERS*

Introduction

- Phosphorus solubilizing microorganisms (bacteria and fungi) enable P to become available for plant uptake after solubilization.
- Several soil bacteria, particularly those belonging to the genera *Pseudomonas* and *Bacillus* and fungi belonging to the genera *Penicillium* and *Aspergillus* possess ability to bring insoluble soil phosphates into soluble forms by secreting acids such as formic, acetic, propionic, lactic, glucolic, fumaric and succinic.

Objective

Objective of this experiment is isolation of PSB from Soil and study about its morphology.

Materials Required & Equipments

- Rhizospheric Soil Samples Recently collected and not air dried one.
- Pikovskaya's (PVK) medium
- Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

- First prepare Pikovskaya's (PVK) medium as per requirements.
- Pour Pikovskaya's (PVK) medium into sterile Petri plates and allow them to solidify.
- Weigh the soil sample 10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
- Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
- Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

Pikovskaya's (PVK) Medium

1. Pikovskaya's (PVK) medium	**g/l**
$C_6H_{12}O_6$	10.00
$CaHPO_4$	5.00
$(NH_4)_2SO_4$	0.50
Sodium chloride (NaCl)	0.20
$MgSO_4.7H_2O$	0.10
KCl	0.20
yeast extract	0.50
$MnSO_4.H_2O$	0.002
$FeSO_4.7H_2O$	0.002

Distilled water	1000 ml
Agar	20.00
pH	**7.2 ± 0.2**

Methodology

1. Spread Plate Method;

The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

2. Pour Plate Method

In pour plate method, successive dilutions of the inoculum (serially diluting the original specimen) are added into sterile petriplates to which is poured melted and cooled (42ºC - 45ºC) agar medium and thoroughly mixed by rotating the plates which is then allowed to solidify.

Observations;

Result

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Cyanobacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil (g)}}$$

Conclusion;

You tube link: https://youtu.be/KR4nvTH3yVM

PRACTICAL 12: ISOLATION AND PURIFICATION OF *CYANOBACTERIA*

Introduction

- Cyanobacteria can be found in almost every conceivable environment, from oceans to fresh water to bare rock to soil.
- Aquatic cyanobacteria are probably best known for the extensive and highly visible blooms that can form in both freshwater and the marine environment and can have the appearance of blue green paint or scum.

Objective

Objective of this experiment is isolation of *Cyanobacteria* from Water/Soil and study about its morphology.

Materials Required & Equipments

- Water Sample or Soil Samples Recently collected.
- BG 11 medium
- Sterilized pipettes and Petri dish, Balance, Non Absorbent Cotton, Test tubes, Beakers (500ml), conical flasks (250 ml), Measuring Cylinder, Shaker and Squeeze bottle with Dis. Water, 90 ml sterile water blanks.

Equipments: Autoclave, Hot air Oven, Laminar air flow chamber and BOD incubator.

Procedure

- First prepare BG 11 medium as per requirements.
- Pour BG 11 medium into sterile Petri plates and allow them to solidify.
- Weigh 10ml water /10g soil sample into the 90 ml water blank, shake for 20-25 minutes on the magnetic shaker.
- Make serial dilutions of this sample through sterile water blanks as mentioned under bacteria.
- Add 1ml of each dilution on to the agar plates, rotate the plates for even spreading of inoculum and incubate at 28 degree C for 3 to 4 days in inverted position.

BG 11 Medium Composition	
Magnesium Sulphate	0.025 g
Calcium Chloride	0.05g
Sodium Chloride	0.20g
Dipotassium Hydrogen Phosphate	0.35g
A5 trace elements stock solution	1.0 ml
Distilled Water	1000 ml

Methodology

Spread Plate Method;

- The spread plate technique is used for the separation of a dilute, mixed population of the microorganisms so that individual colonies can be isolated. In this technique, a small volume of dilute microbial mixture is transferred to the center of an agar plate and spread evenly over the surface with a sterile L-shaped bent glass rod, while the petridish is spun, at some stage, single cells will be deposited with the bent glass rod on the agar surface. Incubate the agar plate at 37ºC for 24 hours, in the inverted position.

Observations;

Result

Microorganisms	Dilution	Number of Colonies/Plate	Number of cells/g of soil
Cyanobacteria	10^{-4}		
	10^{-5}		
	10^{-6}		
	10^{-7}		

Calculations;

Calculate the number of cells of soil by applying the formula;

$$\text{Number of cells/g} = \frac{\text{Number of colonies X Dilution factor}}{\text{Wt of Soil (g)}}$$

Conclusion;

You tube link: https://youtu.be/yKCW1raolJw

PRACTCIAL 13: MASS MULTIPLICATION AND INOCULUMS PRODUCTION OF BIOFERTILIZERS

Introduction of Production Technology

- The procedure involves isolation, mass culture production and preparation of inoculants along with inoculants quality control.
- The individual organism can be mass multiplied using specific media either as small scale or as large-scale commercial production procedure using fermenters.
- The desired growth of organisms is then mixed with carrier materials and sealed in culture packets.
- The entire procedure is carried out under aseptic condition to avoid contamination from other undesired organisms.
- The quality of inoculant is regularly checked prior to distribution of individual biofertilizer culture.

Need for Basic Facilities-

- There should be modern microbiological laboratory with sufficient space, electrical outlets, sinks, airlines, and temperature control and storage facilities. The rooms should be maintained properly to make it contamination free. The basic equipment are laminar air flow, autoclave, BOD incubator, dry sterilizing oven, rotary shaker, fermenter, sealer, balances, microscopes, and high grade chemicals and glass wares. Supply of gas, water and electricity without interruption should be ensured. The production of biofertilizers should be handled by technically qualified and experienced persons.

Production Technology

- **Isolation** and selection of efficient and competitive strains.
- **Media** for broth culture.
- Multiplication of **mother culture** in the appropriate broth.
- Selection and preparation of broth material.
- Mass production and packing of the **liquid/Carrier based** /Granular /Encapsulated formulations biofertilizers.
- Storage and dispatch to farmers.

STEP 1: ISOLATION

STEP 2: MASS PRODUCTION

STEP 3: PREPARATION OF CARRIER MATERIALA AND BROTH CULTURE

STEP 4: PREPARATION OF INOCULANTS PACKET

Schematic diagram for mass scale production of bacterial Biofertilizers

Carriers for Bacterial inoculants

Preparation of carrier material

Mixing Broth with Carrier (Curing)

Packing and Storage

Quality Standard

Conclusion;

You tube link: https://youtu.be/9W7EIfvtmh0

PRACTCIAL 14: ISOLATION OF AM FUNGI- WET SIEVING METHOD AND SUCROSE GRADIENT METHOD

Introduction

- **Arbuscular mycorrhizal** (AM) fungi (AMF, Glomeromycota) are important beneficial soil microorganisms establishing **mutualistic associations** with most food crops.
- These associations **increase plant nutrient uptake and tolerance to root pathogens and drought**.
- **AMF** are obligate biotrophs and colonize host roots to obtain **sugars** in exchange of mineral nutrients, absorbed and translocated through a fine network of **extra radical mycelium (ERM)** spreading from colonized roots into the soil.
- The function as auxiliary absorbing systems that transfer mineral nutrients, such as **phosphorus (P), nitrogen (N), sulfur (S), potassium (K), calcium (Ca), iron (Fe), copper (Cu), and zinc (Zn),** from the soil outside the roots to the host plants.

Objective

Objective of this experiment is isolation of AM fungi from Soil by **wet sieving method and sucrose gradient method** and study about its morphology.

Materials Required & Equipments

- Soil Samples Recently collected.

Equipment:

- Sieves with various mesh size (1 mm, 100 μm and 50 μm. Other sizes such as 500μm and 250μm are preferable.), Sterilized Pipette or needle, Petri dish, Balance, Conical flask, Funnel, Glass tubes, Dissecting microscope, Compound microscope: etc.

Wet Sieving Method

- Also known as wet sieving and decanting method (Gerdemann and Nicolson, 1963). Developed to isolate different size of spores.
- The soil near the root system is collected and an aqueous suspension is passed through different sieves to collect spores of different sizes.
- The wet sieving and decanting is one of the popular technique when compare to other techniques. This technique is used for sieving the coarse particles of the soil and retaining AMF spores and organic particles on sieves of different sizes.10 g of soil was mixed with 100ml of water in the 500 ml conical flask. The soil mixture was agitated vigorously to free the AMF spores from soil and allowed to settle for 15-45 minutes and the supernatant was decanted through standard sieves. By using a dissecting microscope, spores were picked by means of pipette or needle.
- Earlier, Gerdemann (1955) devised the first useful technique for extracting spores from soil. A soil sample was suspended in four times its volume of water, heavier particles were allowed to settle for a few seconds, then the liquid was decanted through a sieve with 1mm mesh. Whatever passed through this sieve was then poured through another sieve with 0.25 mm mesh. Material retained by this sieve was washed and transferred to a petridish, and the spores picked out by hand under a dissecting microscope.

- Technique given by Gerdemann (1955) was slightly refined by Gerdemann and Nicolson (1963) who used the following series of sieves: 1.0 mm ; 710 μm; 420 μm; 250 μm; 149 μm; 105 μm; 74 μm; and 44 μm.
- They found that most of the desired spores fell in the 420- 149 μm range, and they used this fraction for their study.

Sucrose Gradient Method

- Developed by Daniel and Skipper (1982); commonly used technique for AM spore extraction. Requires prior sieving and decanting.
- This gradient centrifugation method is result of many modifications, right from Ohms (1957), Mosse and Jones (1968), Mertz et al. (1979) etc.
- Spores were purified by re-suspending the sieving in the 40% sucrose solution and centrifugation was carried out. Centrifugation was carried out at 1750 rpm for 5 minutes. The supernatant was removed and poured into the sieves. The spores that hold on the sieves are carefully rinsed with tap water. The spores were collected by using dissecting microscope.

Procedure:

- 10 to 50 g of freshly collected soil sample is put into 1 to 2 liters of plastic beakers.
- Usually rhizosphere soils are rich in AM fungal spores. Beaker size can be changed depending on the soil sample size.
- Soil is suspended with about 500 ml to 1 litter of tap water.
- Soil macro-aggregates should be crushed with hand.

- After 10-30 seconds of settling down of soil particles, the upper layer of soil suspension is poured into the sieving.
- The procedure should be repeated until the upper layer of soil suspension is transparent.
- Usually AM fungal spores are collected on 100 µm. Some small spores are on 50 µm. To collect large spores such as *Gigaspora margarita, 250* µm sieve is efficient.

Observations;

Conclusion;

You tube link: https://youtu.be/YKgmelyCc68

PRACTICAL 15: MASS PRODUCTION OF AM INOCULANTS

Introduction

- Being obligate symbionts AM fungi could be mass produced only in the presence of living roots.
- Since AM fungal associations are universal and have been reported in almost all terrestrial plants, these can be reproduced on a wide range of host plants.
- There are several techniques reported for mass production of AM inoculum.

A. *In Vivo Culture*

- AM fungi are grown on roots of green house plants and chopped mycorrhizal roots, often mixed with growth media containing hyphae and spores, are used as source of inoculum.
- Soil could be replaced by inert substances such as vermiculite, perlite, sand or a mixture of these for crude inoculum production.

Mass Production of VAM

Mass production of VAM inside Bricklined tank

Mass production of VAM inside a cement tank

Method of Production

1. Tank for mass multiplication of AM
2. Sprinkling of water in tank with vermiculite
3. Making of furrows to sow maize seeds
4. Sowing the seeds in furrows
5. View of the maize sown AM pit
6. Vermiculite contained raised AM infected maize plants

B. IN VITRO/ AXENIC CULTURE TECHNIQUES

i) Solution culture

ii) Aeroponic culture

iii) Root organ culture

I) SOLUTION CULTURE

- Involves growing infected roots in aqueous medium enriched with mineral nutrients required for the growth of the roots under controlled biotic and abiotic conditions.

II) AEROPONIC CULTURE

- Involves applying a fine mist of nutrient solutions to colonized roots for AM fungal inoculum production.

III) ROOT ORGAN CULTURE

- Use of a modified agar medium (MS rooting medium)/ liquid medium for creation of increased amount of roots from callus tissue and these roots are infected by AM spores or by surface sterilized root bits obtained from mycorrhizal plant.

Benefits of Mycorrhiza;

Application of VAM Fungi;

Conclusion;

You tube link: https://youtu.be/fRxjqlQ9s18

PRACTCIAL 16: MASS MULTIPLICATION AND INOCULUMS PRODUCTION OF LIQUID BIOFERTILIZERS

Introduction of Production Technology

- The procedure involves isolation, mass culture production and preparation of inoculants along with inoculants quality control.
- The individual organism can be mass multiplied using specific media either as small scale or as large-scale commercial production procedure using fermenters.
- The desired growth of organisms is then mixed with carrier materials and sealed in culture packets.
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Production Technology

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Schematic diagram for mass scale production of bacterial Biofertilizers

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Packing and Storage

Quality Standard

Conclusion;

You tube link: https://youtu.be/9W7EIfvtmh0