Paper- XIII

Expt. No. 1 **Date: -**

Determination of R:S ratio.

Aim: - To determine the R:S ratio of bacteria

R:S ratio is defined as the ratio of microbial population per unit weight of rhizosphere soil (R), to the microbial population per unit weight of the adjacent non-rhizosphere soil (S).

R:S ratio i.e. the ratio between the microbial population in the rhizosphere (R) and in the soil (S) to find out the degree or extent of plant roots effect on soil microorganisms. R: S ratio gives a good picture of the relative stimulation of the microorganisms in the rhizosphere of different plant species.

It is obvious that the rhizosphere micro flora predominates as compared to non-rhizosphere ones. Therefore, quantitative estimation is required to determine the R : S ratio and assess the rhizosphere effect .

Requirements

1. Sterile nutrient agar plates
2. Sterile 9 ml d/w tubes

Procedure

1. Take 5 test tubes and transfer 9 ml distilled water in each flask, plug them properly, label 1-5 and autoclave at 15 Ib/inch^2 for 30 minutes.
2. Collect small amount of soil from 5 different places of a desired field and mixed to make one lot.
3. Weigh 1 g of soil sample and transfer into test tube 1 containing 9 ml sterilized water. It gives the dilution 1: 10 (i.e. 10^{-1})
4. Shake the tube and transfer 1ml soil suspension from 10^{-1} dilution into tube 2 containing 9 ml sterilized water to get dilution 10^{-2}. Mix the suspension gently.
5. Similarly serially transfer 1 ml soil suspension from 10^{-2} dilution into tube 3 containing 9 ml water to get the final dilution of 10^{-3}. Mix the suspension gently.
6. Serially make dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}
7. Aseptically pour 1 ml soil suspension from 10^{-3} to 10^{-5} dilutions into nutrient agar plates.

8. Gently shake the plate so as to spread soil suspension uniformly on the medium.
9. Incubate the plates at 25 ± 1 ^{o}C for 24-48 hours.
10. Pick up each colony, prepare smear and perform Gram's staining for differentiation.
11. Count CFUs of bacteria from rhizosphere and non-rhizosphere soil.

Colony forming units (CFUs) /g dry soil =

$$\frac{\text{Average number of colonies}}{\text{Dry weight of the soil}} \text{ X Diluionfactor}$$

12. Divide the values of CFUs of rhizosphere microorganisms by that of non-rhizosphere microorganisms.

Result:

$$\frac{\text{CFUs of rhizosphere microorganisms}}{\text{CFUs of non-rhizosphere (i.e. soil) microorganisms}}$$

Conclusion: -

As average is more than one, bacteria are more in rhizosphere zone than non-rhizosphere zone.

Expt. 2 **Date: -**

2a) Demonstration of Ammonifying bacteria from soil

Aim: - To demonstrate ammonifying bacteria from soil.

Theory: -

Formation of ammonia from nitrogen containing organic compounds, amino acids and proteins is called ammonification. Amino acids are deaminated under aerobic condition and are called oxidative deamination.

$$\text{Amino acids} \xrightarrow{\text{Deaminase}} \text{Keto acids} + \text{Ammonia}$$

Microorganisms: Micrococcus, Proteus, Pseudomonas spp,

The ammonia formed is detected by adding Nesseler's reagent which gives yellow colour.

Requirements: -

1. Soil sample
2. 4 % peptone water
3. Nesseler's reagent

Procedure: -

In 4 % peptone water (100 ml), one gram soil is added and incubated at 37^0 C for 24 hours. Then 3 ml culture from this flask is taken in a test tube and one ml Nesseler's reagent is added to it.

Observation: - Yellow colour is observed.

Result: -

Yellow colour developed after addition of Nesseler's reagent indicates ammonia production. Thus test is positive.

Conclusion: -

Ammonifying bacteria from soil are isolated. They use peptone as a protein source. They degraded protein and ammonia is formed.

Expt. 2 **Date**

2b) Demonstration of Nitrification

Aim: - Demonstration of nitrification

Theory / Approach

Nitrification:
Ammonical nitrogen / ammonia released during ammonification are oxidized to nitrates and the process is called "nitrification". Soil conditions such as well aerated soils rich in calcium carbonate, a temperature below 30 ° C, neutral PH and less organic matter are favorable for nitrification in soil.

Nitrification is a two stage process and each stage is performed by a different group of bacteria as follows.

Stage I: Oxidation of ammonia of nitrite is brought about by ammonia oxidizing bacteria viz. *Nitrosomnonas europaea*, *Nitrosococcus nitrosus*, *Nitrosospira briensis*, *Nitrosovibrio* and *Nitrocystis* and the process is known as nitrosification. The reaction is presented as follows.

$2\ NH_3 + 1/2O_2 \longrightarrow NO_2 + 2\ H + H_2O$
Ammonia Nitrite

Stage II: In the second step nitrite is oxidized to nitrate by nitrite-oxidizing bacteria such as *Nitrobacter winogradsky, Nitrospira gracilis, Nirosococcus mobiiis* etc, and several fungi *(eg. Penicillium, Aspergillus) and* actinomycetes *(eg. Streptomyces, Nocardia).*

$NO_2(-) + ½\ O_2 \longrightarrow NO_3$
Nitrite ions Nitrate ions

The nitrate thus, formed may be utilized by the microorganisms, assimilated by plants, reduced to nitrite and ammonia or nitrogen gas or lost through leaching depending on soil conditions. The nitrifying bacteria (ammonia oxidizer and nitrite oxidizer) are aerobic gram-negative and chemoautotrophic and are the common inhabitants of soil, sewage and aquatic environment.

Requirements

1. Nessler's reagent
2. Ammonium sulphate broth*
3. Trommosdorf's reagent**
4. Sulphuric acid
5. Diphenylamine reagent
6. Glass rod
7. Spot plate
8. Culture of Nitrosomonas

*** Ammonium sulphate broth:**

$(NH_4)_2S0_4$	2.00g
$MgS0_4 \cdot 7H_2O$	0.50g
$FeS0_4 \cdot 7H_2O$	0.03g
NaCl	0.10g
$KiP0_4$	1.00 g
Distilled water	1 litre

****Trommsdorf's reagent:**

'Zinc chloride solution (20%)	10ml
Starch	40 g
Potassium iodide	2 g
Distilled water	100ml

Take a small amount of distilled water and add 0.10g of starch into it. Now pour slowly 100 ml of zinc chloride (20%) with a constant stirring. Heat to dissolve the starch and get a clear solution. Add more water to dilute it and add 2 g of potassium iodide. Add more water to make the volume to one litre.

Procedure

1. Ammonium sulphate broth is inoculated with the culture of Nitrosomonas and incubated at 30^0 C for 24 hours.
2. A drop of the culture of Nitrosomonas is transferred from the ammonium sulphate medium to the 3 drops of Trommsdorff's reagent with 1 drop of

dilute sulphuric acid on a spot plate. If nitrites are present, blue-black colour will appear.

3. Further confirmation is done by adding Nessler's reagent. If no colour appears it shows the presence of nitrite. However, if yellow colour appears, it indicates that ammonia is not oxidized into nitrite and hence, nitrite is absent.

Observation

Blue black colour appeared / not appeared

Result

Nitrite is produced / not produced

Conclusion

Formation of nitrite from ammonia by Nitrosomonas has taken place / not taken place.

NITRATE PRODUCTION IN NITRITE BROTH

The-second step of nitrification process in the nitrogen cycle is the formation of nitrate from nitrite.

Requirements

1. Culture medium* Culture of Nitrobacter
2. Sulphuric acid
3. Trommsdorf's reagent Diphenylamine
4. Spot plate
5. *Culture medium:
   $NaN0_2$
   $MgS0_4$ H_2o
   $FeS04 \cdot 7$ H_2o
   $NaC0_3$
   $HP0_4$
6. Distilled water

Procedure

For demonstrating the presence of nitrate, it is necessary to confirm first that no nitrite is present.

1. Culture medium is prepared and sterilized it.
2. Medium is poured in plates and inoculated with pure culture of Nitrobacter.
3. Plates are incubated for a week.
4. 3 drops of Trommsdorf's reagent with 1 drop of H_2S0_4 (1:3.) in 7 fold culture is added in nitrite medium. No blue-black colour appears that shows nitrites are not present.
5. Test for nitrate is carried out by mixing 2 drops of sulphuric acid with 1 drop of diphenylamine and 1 drop of the culture is added on the plate. Blue-black colour appeared that shows nitrate production.

Observation

Blue black colour appeared / not appeared

Result

Nitrate is produced / not produced

Conclusion

1. Formation of nitrate from nitrite by Nitrobacter has taken place / not taken place.

Expt. No. 3 **Date: -**

Isolation and study of Rhizobium species from root nodules of leguminous plant

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Aim: - To Isolate and study Rhizobium species from root nodules of leguminous plant.

Theory: -

Numbers of microorganisms are able to use molecular nitrogen. The conversion of molecular nitrogen into nitrogenous compounds is known as nitrogen fixation. (N_2 , NO, NO_2, NO_3, NH_3).

Rhizobium species fix nitrogen in the root nodules of leguminous plants called as symbiotic nitrogen fixation.

Requirement: -

1. Healthy root nodules of leguminous plant
2. Sterile Petri plate
3. Sterile forceps / scalpel
4. Sterile distilled water
5. Sterile glass rod
6. Mercuric chloride solution 0.1 %
7. 70 % ethanol

Yeast extract manitol agar

Composition

K_2 HPO_4	0.50 gm
$MgSO_4$.7H2O	0.20 gm
NaCl	0.10 gm
Mannitol	10.0 gm
Yeast extracts	1.00 gm
PH	6.8
Agar-agar	25.0 gm
Congo red 1 % solution	2.5 ml
Distilled water	1000 ml

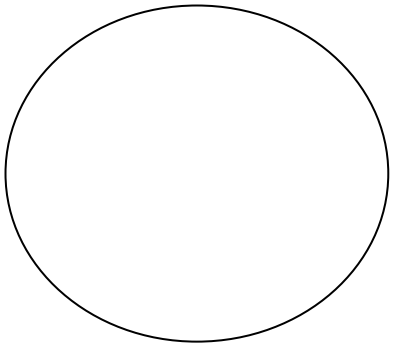
Dissolve all the ingredients in distilled water and mix in agar solution. Make the volume to 1000 ml and autoclave it. Congo red solution is to be sterilizing separately and added to the medium at the time of pouring.

Procedure

1. Uproot the roots of leguminous plant and bring to laboratory.
2. Wash the root system under running tap water to remove adhering soil particles.
3. Select healthy pink, undamaged and fresh root nodule and wash in water.
4. Immerse the root nodules in 0.1 % HgCl2 solution for 5 minutes for surface sterilization.
5. Repeatedly wash the nodules in sterile water for 3-4 times to get rid off the sterilizing agent.
6. Place the nodules in 70% ethyl alcohol for 3 minutes.
7. Repeatedly wash (2-3 times) the nodules in sterile water.
8. Crush the nodules in 1 ml sterile water with sterile glass rod.
9. Streak on YEMA plate.
10. Incubate the plates at 26-300C FOR 2-8 days.
11. Observe the plates after 3-4 days incubation and regularly afterward for the development of rhizobial colonies.
12. Development of large gummy colonies on YEMA plates is an indication of rhizobial colonies

Observation: -

Colony characters	**Colony No.1**	**Colony No.2**	**Colony No.3**
Size			
Shape			
Colour			
Margin			
Elevation			
Opacity			
Consistency			
Gram nature			



Result: - Rhizobium species are isolated from the root nodules of the leguminous plants.

Conclusion: - Rhizobium species are isolated from the root nodules of the leguminous plants.

Expt. No. 4 **Date: -**

Isolation of Non-Symbiotic Nitrogen fixing bacteria from the soil

(Azotobacter species)

Aim: - To isolate Non-Symbiotic Nitrogen fixing Azotobacter species from the soil.

Theory: -

Number of microorganisms are able to use molecular nitrogen. The conversion of molecular nitrogen into nitrogenous compounds is known as nitrogen fixation. ($N_2 \longrightarrow$ NO, NO_2, NO_3, NH_3).

Nitrogen fixation is of two types namely-

1. **Symbiotic nitrogen fixation**
2. **Non-Symbiotic nitrogen fixation**

Non-Symbiotic nitrogen fixation is carried out by free-living bacteria and blue green algae. Some bacteria are capable of fixing nitrogen in presence of oxygen i.e. aerobically e.g. Azotobacter spp. Some bacteria are capable of fixing nitrogen anaerobically e.g. Clostridium spp.

The enzyme required for nitrogen fixation is called nitrogenase, which is sensitive to oxygen. The cells of Azotobacter species are large, flat and round. These are strict aerobic and posses exceptionally high respiratory rate. Hence nitrogenase enzyme is protected from oxygen. Azotobacter species are heterotrophic Gram negative, capsulated and mesophilic. These are large rods, even cocci showing considerable variation in shape and size. They are also yeasts like, often motile with peritrichous or polar flagella. Sometimes they observe Gram positive. Endospores are not formed but thick walled microcysts are produced. Azotobacter species are differentiated on the basis of characters like pigmentation, motility, cyst formation and biochemical characteristics. Two types of pigments are produced by azotobacter.

1. **Water insoluble: -** These may range from yellow to darkness. With age colonies show good pigmentation.
2. **Water-soluble: -** These may range from green to purple.

Water insoluble pigments

Name of the species	Colour of the pigment
1. Azotobacter chroococcum	Brown and later becomes black
2. Azotobacter beijerinkii	Yellow

Water-soluble pigments

Name of the species	Colour of the pigment
1. Azotobacter vinelandii	Green
2. Azotobacter paspaeli	Green

Azotobacters are isolated by using the medium, which is free of combined form of nitrogen (Nitrogen free medium). First enrichment of bacteria is done to increase the number in liquid medium; later isolation is done on solid medium.

Requirements: -

1. Soil sample
2. Sterile Nitrogen free mannitol broth (For enrichment)
3. Sterile Nitrogen free mannitol agar plate
4. Gram staining set
5. Nitrogen free manitol broth (NFMB)
6. Nitrogen free manitol agar (NFMA)

Composition

Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.2 g
$FeCl_3$	0.005 g
$MnSO_4.7H_2O$	0.005 g
Distilled water	1 lit.
PH	6.8
Agar	2.5 %
$CaCO_3$	10 g

Note: $CaCO_3$ sterilized separately and then added in medium

Procedure: -

Part –I The enrichment of Azotobacter species.

One-gram soil sample is added in sterile Nitrogen free mannitol broth. Flask is incubated at room temperature for about a week; pellicle is formed on the surface of the broth. A loopful of pellicle is taken and Gram staining is done.

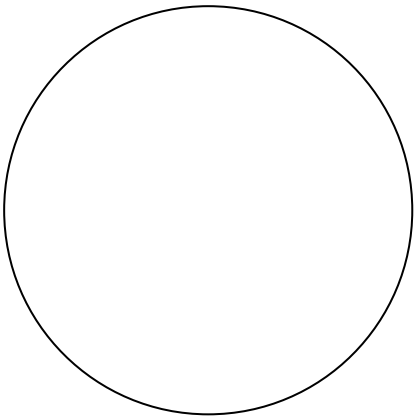
Part – II Isolation and identification of Azotobacter species.

From the pellicle, loopful is streaked on Sterile Nitrogen free mannitol agar plate. The plate is incubated at room temperature for 24 to 48 hours. Then colony characters are studied and Gram staining is done. Loopful of the suspension is incubated in sterile glucose, lactose, sucrose and mannitol tubes and one loopful is streaked on starch agar plate and incubated at room temperature for 24 to 48 hours.

Observation and result: -

1. Colony characters are as shown in table
2. After Gram staining, Gram-negative rods and cysts are observed.
3. Glucose, lactose, sucrose and mannitol tests are positive.
4. On starch agar plate dilute iodine is added. Colourless zone is observed around the colonies, indicating starch hydrolysis.

Colony characters	**Colony - 1**	**Colony - 2**
Size		
Shape		
Colour		
Margin		
Elevation		
Opacity		
Consistency		
Gram nature		



Conclusion: -

From observation and result, it is concluded that isolated bacterium is Azotobacter species.

Expt. No. 05 **Date: -**

5a) Microbiological analysis of milk Direct Microscopic Count (DMC)

Aim: -

To analyse the milk for microorganisms by Direct Microscopic Count (DMC) method.

Theory / Approach: -

Microbial count of milk can be determined by direct microscopic examination of milk in a stained smear. In order to express the count quantitatively the area of the microscopic field must be known. The diameter of the oil immersion field is 0.16 mm.

Area of oil immersion field = Πr^2

$= 3.14 \text{ X } (0.08)^2$

$= 0.02$ sq. mm

Thus in 1 cm^2 area number

of microscopic fields = 100 / 0.02

= 5000

The 10 microscopic fields are observed for number of microorganisms and average number of microorganisms per field is calculated. This number is multiplied by 5000 which gives number of organisms in 0.01 ml of milk sample.

Advantages of DMC: -

1) It is more rapid. More samples can be examined, as less work is required.

2) The equipments necessary are much less.

3) The slide may be preserved as permanent record.

4) Different morphological types can be distinguished. This information is of great value in determining the source and nature of contamination.

Disadvantages of DMC: -

1) This method may be the source of considerable error unless the food contains a high count. This is because a large factor is used for converting the number of organisms per field to the number per ml of food.

2) This method does not differentiate dead from living organisms.

Requirements: -

1) Milk sample
2) Slide
3) Microscope
4) Methylene blue / Crystal violet
5) Xylene

Procedure: -

1) 1 sq. cm area is marked on a clean grease free slide with glass marking pencil.

2) 0.01 ml food sample is spread on opposite side of the marked area.

3) Sample is air-dried and heat fixed.

4) Flood the smear with xylene for 1 minute to remove fat from the milk sample.

5) Smear is stained with methylene blue solution for one minute and gently washed with tap water, air dried and 10 fields are observed under oil immersion lens .

Observation: -

Field no.	Number of organisms
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
Total	

Average no. of organisms per field = total / 10

=

No. of organisms in 0.01 ml food sample = Aver. No. X 5000

=

Result: -

Number of organisms in 0.01 ml food sample is _____.

Expt. No. 5b **Date: -**

Methylene Blue Reduction Time Test (MBRT Test)

Aim: -

To determine the quality of given milk sample by Methylene Blue Reduction Time Test (MBRT).

Theory: -

This test is an indirect method for the determination of load of microorganisms in milk. If number of microorganisms is more, they oxidize components of milk (Proteins, Carbohydrates and Lipids) faster. Therefore the quality of milk is bad. If the number of microorganisms is less, quality of milk is good.

In this test the metabolism (Oxidation of compounds) of microorganisms is detected. When Methylene blue is added in the milk, it is reduced to colourless compound called as Leuco dye. Thus the blue colour disappears when methylene blue is reduced.

Methylene blue ⟶ Leuco Methylene blue

(Blue in colour in oxidized form) (Colourless in reduced form)

Due to oxidation of milk components by microorganisms hydrogen atoms are released which are accepted by methylene blue. It gets reduced. The rate of reduction depends upon the rate of oxidation by microorganisms. More the number of metabolising microorganisms more are the rate of reduction of methylene blue and thus blue colour disappears fast and vice versa.

The oxidation – reduction potential plays an important role in this test. The oxidation – reduction potential of fresh milk is +300 mv, but it becomes less due to oxidation of components by microorganisms.

Thus time required for decolourization of methylene blue is calculated and from this quality of milk is determined. As milk is a complete food for

microorganisms, they grow faster in milk and can spoil milk hence microbiological quality of milk is an important factor for the determination of quality of milk. Microorganisms enter in the milk by different sources like milking animal itself, milkmen, milking utensils, soil, water and air etc. According to this test the grades of milk are as follows –

Sr. No.	Time required for decolorization of methylene blue	Quality of milk
1.	6 to 8 hours	Excellent
2.	4 to 6 hours	Good
3.	2 to 4 hours	Fair
4.	Less than 2 hours	Bad

Requirements: -

2. Milk sample
3. 3 sterile test tubes
4. Methylene blue solution (1 : 2,50,000)
5. Incubator adjusted at 37^0 C

Procedure: -

1. Three sterile test tubes are taken.
2. In the first test tube 10 ml milk sample and 1 ml methylene blue solution is added. Tube is shaken well and labelled as ‘Test’.
3. In a second test tube 10 ml milk sample is taken and it is boiled to kill microorganisms, cooled and 1 ml methylene blue is added to it. This tube is labelled as ‘Negative Control’.
4. In the third test tube 10 ml milk sample is taken and 1ml distilled water is added. This tube is labelled as ‘Positive Control’.
5. Tubes are shaken well and incubated at 37^0 C in incubator.
6. The observations are done after every 15 minutes up to complete disappearance of blue colour or up to 8 hours from the tube labelled as ‘Test’. For this observation positive and negative control are compared.

Observation: -

Milk is decolourised within _____hours.

Result: -

The quality of milk is _____.

Conclusion: -

As the decolourization occurred within _____ hours, the quality of milk is _____.

Expt. No. 06 **Date: -**

Isolation of microorganism from common food items: Curd, Bread, Pickle and Spoiled food

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Aim: - To isolate microorganisms from common food items

Theory: - Food microbiology is the study of the microorganisms which inhabit, create or contaminate food. Of major importance is the study of microorganisms causing food spoilage. However "good" bacteria such as probiotics are becoming increasingly important in food science. In addition, microorganisms are essential for the production of foods such as curd, cheese, yoghurt, other fermented foods, bread, beer and wine.

I) Curd

Requirements:

Curd

Test tube containing 9 ml sterile saline

Sterile 1 ml pipette

Sterile glass rod

Gram staining set

Nutrient agar plate

Composition

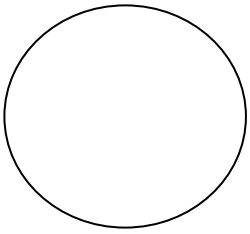
Water	100 ml
Peptone	2.0 g
Yeast extract	01 g
Meat extract	0.3 g
NaCl	0.5 g
Agar	2.5 g
PH	7.2

Procedure:

1. Pipette out 1 ml of curd using 1 ml of sterile pipette.
2. Transfer the curd sample to 9 ml of sterile saline
3. Using sterile glass rod mix the curd sample under aseptic condition
4. Shake the sample vigorously
5. Take one loop full of diluted curd sample under aseptic condition and streak on solidified nutrient agar plate
6. Incubate the plate in an inverted manner at 370 C for 24-48 hour
7. Note the colony characters of the colonies developed on nutrient agar plate
8. Further isolation and identification of microorganism may be carried using selective media

Observation: -

Colony characters	**Colony - 1**	**Colony - 2**
Size		
Shape		
Colour		
Margin		
Elevation		
Opacity		
Consistency		
Gram nature		



Result: - Bacteria are isolated from the curd.

II) Bread

Requirements:

1) Bread sample
2) Test tube containing 10 ml sterile saline
3) Sterile glass rod
4) Gram staining set
5) Nutrient agar plate
6) **Potato dextrose agar**

Composition

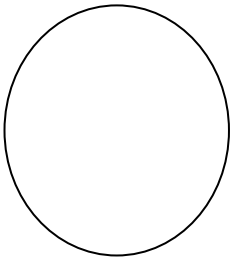
Potato infusion	20 g
Dextrose	2.0 g
PH	5.1
Agar	2.5 g
Distilled water	100 ml

Procedure:

1. Weigh accurately 1 g of bread on butter paper.
2. Transfer the bread sample to 10 ml of sterile saline
3. Using sterile glass rod homogenize the bread sample under aseptic condition
4. Mix and shake the sample vigorously
5. Take one loop full of sample from supernatant under aseptic condition and make streak on solidified nutrient agar plate and Potato dextrose agar plate
6. Incubate both the plate in an inverted manner at 370 C for 24-48 hour
7. Note the colony characters of bacteria the colonies developed on nutrient agar plate and molds on Potato dextrose agar plate
8. Further isolation and identification of microorganism may be carried using
   selective media

Observation: -

Colony characters	**Colony - 1**	**Colony - 2**
Size		
Shape		
Colour		
Margin		
Elevation		
Opacity		
Consistency		
Gram nature		



Result: - Bacteria / fungi are isolated from bread.

III) Pickle

Requirements:

1. Pickle sample
2. Test tube containing 10 ml sterile saline
3. Sterile glass rod
4. Gram staining set
5. Nutrient agar plate

Procedure:

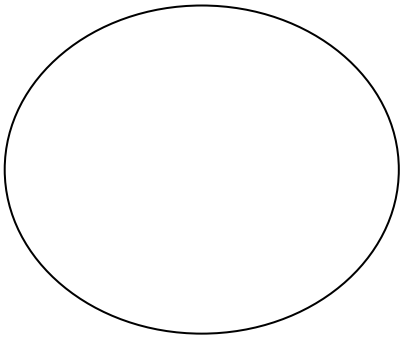
1. Weigh accurately 1 g of pickle on butter paper.
2. Transfer the pickle sample to 10 ml of sterile saline

3. Using sterile glass rod homogenize the pickle sample under aseptic condition
4. Shake the sample vigorously
5. v)Take one loop full of supernatant under aseptic condition and streak on solidified nutrient agar plate
6. vi)Incubate the plate in an inverted manner at 370 C for 24-48 hour
7. vii) Note the colony characters of the colonies developed on nutrient agar plate
8. viii) Further isolation and identification of microorganism may be carried using
9. selective media

Observation: -

Colony characters	**Colony - 1**	**Colony - 2**
Size		
Shape		
Colour		
Margin		
Elevation		
Opacity		
Consistency		
Gram nature		

Result: - Bacteria / fungi are isolated from bread.



IV) Spoiled food

Requirements:

1. Spoiled food sample
2. Test tube containing 10 ml sterile saline
3. Sterile glass rod
4. Staining solution (Gram staining set for bacteria and lactophenol cotton blue for fungi)
5. Nutrient agar plate (for bacteria)

Potato dextrose agar (for fungi)

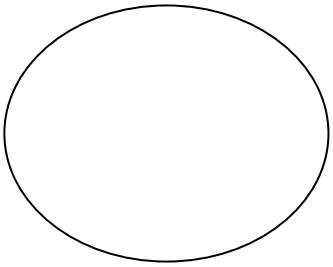
Composition

Water	100 ml
Potato infusion	20 g
Dextrose	2.0 g
Agar	2.0 g
PH	5.1

Procedure:

1. Weigh accurately 1 g of spoiled food (if liquid 1 ml) on butter paper.
2. Transfer the spoiled food sample to 10 ml of sterile saline
3. Using sterile glass rod homogenize the spoiled food sample under aseptic condition
4. Mix and shake the sample vigorously
5. Take one loop full of homogenized sample from supernatant under aseptic condition and make streak on solidified nutrient agar plate and Potato dextrose agar plate
6. Incubate both the plates in an inverted manner at 370C for 24-48 hour
7. Note the colony characters of the colonies developed on both nutrient agar plate and Potato dextrose agar plate
8. Stain the fungal colonies using lactophenol cotton blue
9. Further isolation and identification of bacteria may be carried using selective
   media

Colony characters	**Colony - 1**	**Colony - 2**
Size		
Shape		
Colour		
Margin		
Elevation		
Opacity		
Consistency		
Gram nature		



Result: - Bacteria / fungi are isolated from spoiled food