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University of Baghdad
College of Science
Department of Biology

Practical soil and aquatic microbiology 2021-2022

المرحلة الرابعة - الدراستين الصباحية والمسائية

الفصل الدراسي الثاني

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Soil microbiology

Lab 1

Soil is the material found on the earth's surface composed of organic and inorganic material. The soil varies due to its structure and composition. Inorganic materials, or those materials that are not living, include weathered rocks and minerals. As rocks are broken down, they mix with organic materials, which are those materials that originate from living organisms. For example, plants and animals die and decompose, releasing nutrients back into the soil.

Types of Soil:

- There are **three** basic types of soil: sand, silt and clay. But, most soils are composed of a combination of different types.

Water content:

The water in the soil plays a significant role, as it influences the metabolic activities of microbiota. Soil water is divided into two types:

1- **Free water (Gravitational water)** this water is not bounded by colloidal material, which is affected by gravitation, so it tends to drain quickly.

2- Bound water It's a thin water film on soil particles surface, because it's bounded by colloidal material, this type of water is not affected by gravitation, so it tend to drain slowly.

Water activity (a_w):

It's useful to express qualitatively the degree of water viability, microbiologist generally uses a_w, which is less amount of water required for microbial activities and growth. The water activity of a solution is 1/100 the humidity of a solution (expressed as a percentage).

Different methods can determine the water contant of sample, the most current assay method is:

Drying method

Procedure:

1. Weight 10gm of soil in previously weighted clean Petri dish.

2. Dehydrate soil in a hot air oven at 105° C for 3-4 hrs.

3. Reweight dried soil sample and determined water contant of the sample according to the following.

The water content of soil sample = weight of moist soil - the weight of dry soil

Weight of dry soil sample =l 0 - water content

$$\text{Weight of soil sample for } \mathbf{1\ gm\ dry\ soil} = \frac{\text{weight of dry soil}}{\text{weight of moist soil}}$$

Enumeration of soil Microorganisms

Soil microorganisms can be classified as bacteria, actinomycetes, fungi, algae and protozoa. Each of these groups has characteristics that define them and their functions in soil. Fertile soil contains many M.O., but there is no particular isolation lab. procedure can be given quiet and accurate microbial numbers in a soil sample.

Enumeration methods:

1. Direct slide count (Breed Method)

This method is considered the quickest and simplest technique, although there are some disadvantages like:

- Estimate the number of live and dead cells in the sample.
- Bacteria are dominant in the soil, so it's difficult to distinguish other microbial cells that are present, such as microbial spores.
- Can't distinguish microbes from soil particles.

Procedure:

1. Mark square (1 cm diameters) on a clean glass slide.
2. Suspend 1 gm of soil sample in 9 ml of sterile diluted water, mix properly.
3. Spread loop full of soil dilution (0.01) in the square.
4. Stain with a simple stain.
5. Count visible microbial cells in 10 microscopic fields and determine no. of bacteria cells in a soil sample from the equation:

$$\text{No. of cells} = \frac{\text{Number of cells in 10 fields}}{10} \times 5000 \times 100 \times 10$$

- To find the No. of cells in dried wt. of soil sample.

$$\text{No. of cells in 1 gm of } \textbf{dry} \text{ soil sample} = \frac{\text{No. of bacterial cells}}{\text{dry wt of soil sample}}$$

Constant factors:
5000 = no. of fields in 1 cm^2
100 = (0.01) conversion of loop full volume to 1 ml.
10 = Inversion of dilution

2 - **Dilution plate count (Viable Plate Count)**

This technique is widely used for determining approximate viable no. of soil bacteria, and it applies to any M.O. that will grow as colonies, but this method has some disadvantages**:**

▪ It ignores sampling error due to unequal distribution of the cells, especially in the high dilutions.
▪ There is no ideal culture medium for cultivating most soil bacteria.
▪ Antagonistic activities of soil bacteria, such as the production of antibiotics and some
enzymes that inhibit the growth of another M.O.

Colony-forming unite / gm soil = No. of viable colonies x invert dilution factor x?

?= depends on inoculum size

$$\textbf{CFU / gm of dried soil} = \frac{\text{CFU/ gm soil}}{\text{Wt. of 1gm dry soil}}$$

Lab:3

Isolation of soil microorganisms

The most numerous microbes in the soil are the bacteria, followed by actinomycetes, the fungi, soil algae and soil protozoa, but in our study, we are going to focus on bacteria, actinomycetes and fungi.

1. Isolation of Bacteria

We are going to isolate bacteria from the soil by using the Pouring plate method.

1. Add 1 gm of soil to 9 ml D.W to get 10^{-1} dilution, mix properly, farther dilutions depend on the soil type as the following table :

Types of soil	Number of dilutions
Domestic soil	8
Vegetable soil	6
Citrus soil	5
Palm soil	4
Hydrocarbon polluted soil	3
Uncultured soil	2

2. Transfer 0.1 ml from every 3 last dilutions to sterile Petri dishes; after that, pour melted cold soil extract agar (pH 7) into dishes and homogenize the inoculum with the medium by mixing it clockwise and anticlockwise.
3. Incubate plates at 30 °C for 24 hr.
4. Count the bacterial colonies and identified by microscopic and macroscopic examination.

Note: count plates that show only about 30-300 colonies.

CFU / gm soil = No. of viable colonies x dilution factor x ?

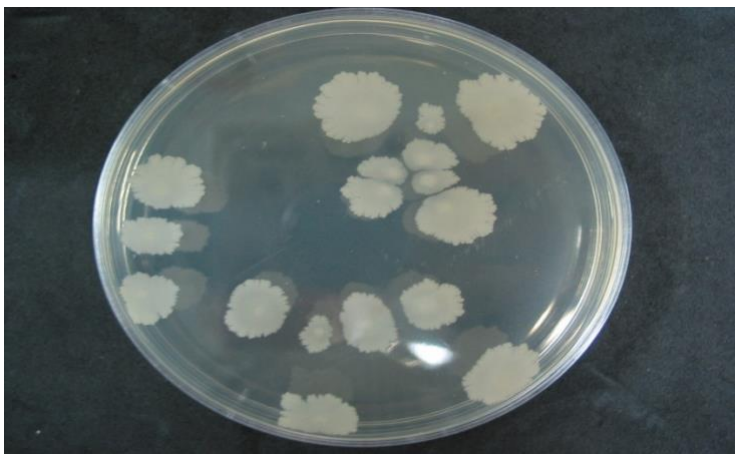
To find the No. of bacterial colonies in dried wt. of soil sample

$$\text{CFU / gm of dried soil} = \frac{\text{CFU}}{\text{Wt. of 1gm dry soil}}$$

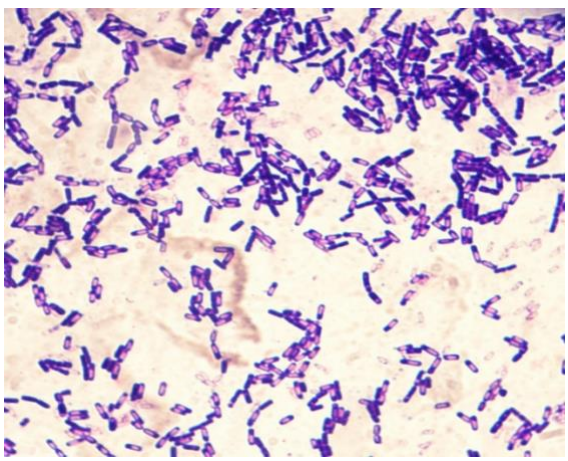
Note: For isolation and enumeration of spore-forming soil bacteria (heat the last sample dilution at 80 ° C water bath for about 15 min), transfer 0.1 ml to Petri dish and continue the same procedure as above.

Bacteria slide preparation

1- Put a small drop of water on the slide and take a touch by loopful from one colony from the Petri dish & mix it softly with the drop of water on the slide and let it dry.
2- Fix the smear by heating 45° over the burner flame (not through the flame) 3 times.
3- Add drop from Crystal Violet (1-1.5min), then wash carefully with Tap water.
4- Add a drop of Iodine (Trapping agent) (1min), then add Alcohol (decolorizing agent) (60sec).
5- Add Safranin (1-1.5min), then wash carefully with tap water.
6- Find a clear field at 10X, 40X, then move to the oil lenses (100X) after adding a small drop of oil on the slide.



Bacillus **colonies on plate**



Bacillus **under a microscope**

2. Isolation of Soil Fungi

Fungi are typically divided into:

1- Molds : which are composed of branching filaments termed hyphae that grow by apical extension to form mycelium. They are helpful but could also be harmful to plant and other soil microorganisms.

2- Yeasts are unicellular and oval or round in shape and reproduce mainly sexually by budding.

Isolation Procedure

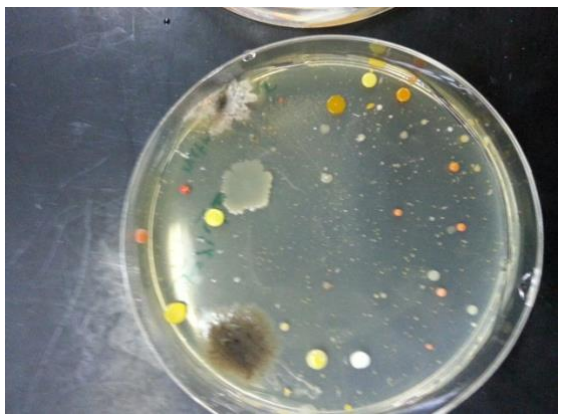
1- Add 1 gm of a soil sample to 9 ml D.W., and then make 10-fold serial dilution by D.W.

2- Transfer 1 ml from the last dilution to Petri-dish, then pour melted Malt extract agar medium (pH 5.5) or Rosebengal agar medium (pH 6) and thoroughly mix.

3- Incubate plates at 28 C^0 for 2-3 days for yeasts or 5-7 days for molds.

4- Examine formed colonies and recognize their color and morphology.

***Penicillium* colonies on plate**



Yeasts colonies on plate

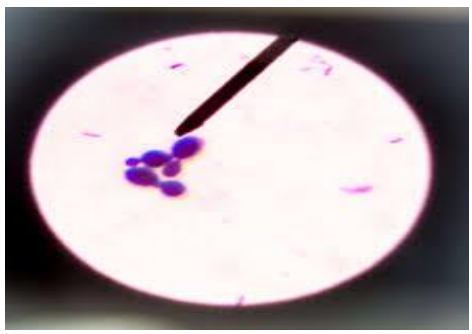
Molds slide preparation

1- Place a drop of Lactophenol cotton blue on a slide.
2- Dig the mold colony from the agar by a loop.
3- Put it over the slide, then put a cover slide over it.
4- Knock carefully at the left angle to spread the colony under the slide cover without breaking it.
5- Find a clear field under 10X & Examine under 40X

Yeasts slide preparation

1- Put a small drop of water on the slide.
2- Take a touch by loopful from **one colony** from the Petri dish
3- mix it softly with the drop of water on the slide, than let it dry.
4- Fix the smear by heating 45° over the burner flame 3 times.
5- Add drop from **Crystal Violet (1-1.5min),** then wash carefully with Tap water.
6- Find a clear field at **10X** & Examine at **40X**.

Alternaria under microscope



Yeasts under microscope

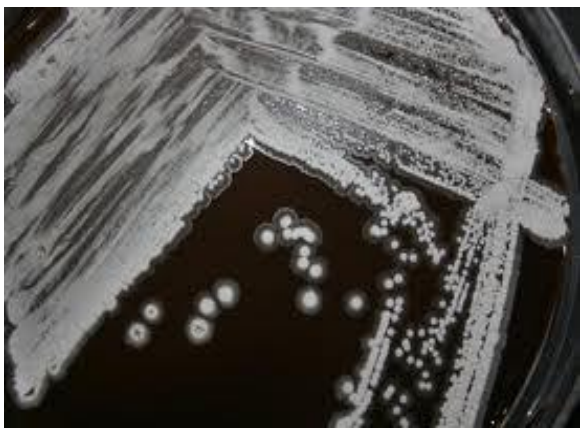
3. Isolation of Actinomycetes

Actinomycetes are a group of gram-positive bacteria that form branched filamentous hyphae like fungal hyphae, but their hyphal diameter is less than fungal hyphae. Actinomycetes give soil its characteristic smell due to their production of volatile substances such as geosmin; Actinomycetes prefer slightly alkaline environments.

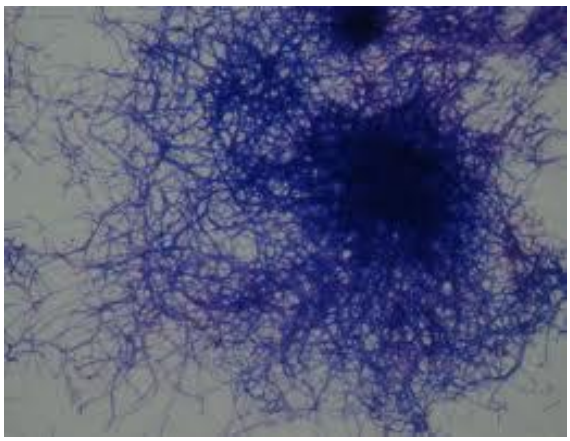
Actinomycetes colonies are powdery mass forms often pigmented with orange or red, or yellow color.

Isolation procedure

1- Add 1 gm of a soil sample to 9 ml of D.W., and then make serial 10-fold dilution with D.W.
2- Spread 1 ml of the last dilution on Jensen's agar plates, pH8.5-9 (Actinomycetes selective medium), incubate at 28^0C for 3-7 days.
3- Prepare slide using gram stain, examine under high power, and observe Actinomycetes thin curly mycelia net.



Actinomycetes colonies on plate



Actinomycetes under a microscope

Lab:4

Role of Soil Microbes in Elements Recycling

Human & other organisms cells chemical composition can be described as a complex blend of organic compounds consisting of different elements such as C, N, O and H in variety combinations, these elements which are an essential component of all organism protoplasm undergo cyclical alteration between inorganic state free in nature and a combined organic state in living organisms.

Microorganisms play a major role in the degradation (decomposition) of organic matter that contributes to elements recycling which maintains their balance & keeps life on the earth from ceasing.

Carbon Cycle

The element carbon is present in all living organisms. It's recycled through various processes. Transformation of carbon occurs constantly and ubiquitously, carbon is introduced into the organic system from its most oxidized state Co_2 & it reduced primarily by photosynthesis and becomes part of photosynthetic organism's components as organic carbon in that temporarily. It is immobilized until the decomposition of cells by M.O.

Role of soil Microorganisms in degradation of carbohydrates

1- Degradation of cellulose

Cellulose is a complex carbohydrate, which is part of plant structure polysaccharides. Soil contains a rich deposit of M.O which produce extracellular cellulase that breaks down cellulose into two to three glucose units called cellobiose and cellotriose, respectively. These smaller compounds are readily degraded and assimilated as glucose.

Examples of microorganisms that degrade cellulose: Bacteria (*Cytophaga* and *Cellulomonas*), fungi (*Aspergillus, Penicillium* and *Trichoderma*) and actinomycetes (*Nocardia*).

Isolation of Soil Cellulytic Microorganism

Procedure:

1- Suspend 1 gm of soil sample in 9 ml of **special isolation broth (**the medium contains all required nutrients except carbon source).
2- Transfer 1 ml of soil suspension to another tube containing the same isolation medium with **a strip of filter paper** (as carbon source).
3- Incubated tubes at 28^0C for 5 – 7days.
4- +ve result for cellulolytic M.O. shown as yellow spots on filter paper strips (The cellulolytic bacterial colonies colored by yellow to orange color due to carotenoid formation).
5- - ve result no color on the strip.
6- Prepare smear of detected spots on a glass slide, stain with gram stain & examine under oil immersion.

2- Degradation of Pectin

Pectin is a homopolymer of D-galactronic acid found in the middle lamella of the plant cell wall(pectin give solidify & support to plant cells by their combination with calcium carbonate).

Microbial pectinolytic enzymes (pectinase) are responsible for the lyses of pectin, which enables M.O to invade tissues of living plants, causing soft rot and wet or dry necrosis & galls in economically important crops such as potatoes, carrot and cucumber.

Examples of microorganisms degrade pectin: Bacteria (*Erwinia*) and fungi (*Fusarium*)

Isolation of soil Pectinolytic Microorganism

Procedure

1- Suspend 1 gm of soil sample in 9 ml of **special isolation broth** medium.
2- Transfer 1 ml of soil suspension to another tube containing the same isolation medium with **a piece of potato** (as a source of carbon) incubate at 28 ^{0}C for 5 – 7 days.
3- + ve result color change and soften of potato.
3- Prepare slide, stain with gram stain and examine under oil immersion.

3- Degradation of starch

Starch consists of a long chain of ∝ - glucosyl residues, Starch is broken down by amylases, which are produced by various living organisms, ranging from M.O. to plants & humans. Bacteria & fungi secrete amylase outside of their cells to carry out extracellular digestion. They have broken down the soluble, and products such as glucose and maltose are absorbed into their cells.

Amylases are classified based on how they break down starch molecules.

α- **Amylase (alpha-amylase)**: breaking down the bonds at random way, therefore producing varies the size of glucose chains.

β- Amylase (Beta-amylase): reduce the viscosity of starch by breaking the glucose bonds down by removing two glucose units at a time, therefore producing maltose.

Amyloglucosides (AMG) breaks successive bonds from the non-reducing ends of the straight chain, producing glucose.

Amylase producer Microorganism

Although many M.O produce these enzymes, the most common producers are bacteria (*Bacillus, Clostridium* and *Micrococcus*) and fungi (*Aspergillus, Fusarium* and *Rhizopus*).

Isolation of soil amylase producers Microorganisms

Procedure

1- Suspend 1 gm of soil sample in 9 ml of **special isolation broth** medium to isolate starch hydrolysis M.O.

2- Incubate tubes at 28 $C^0$for a week.

3- Transfer adequate volume of the medium to a test tube & add drops of gram's iodine solution.

4- + ve result **yellow color confirm the positive result**, due to microbial hydrolysis of starch.

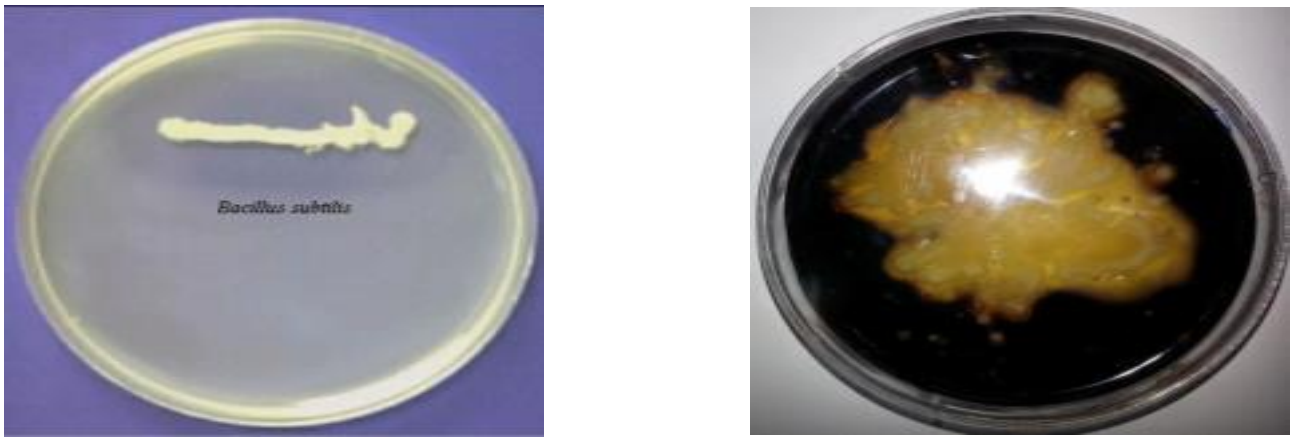
- ve result blue color confirm the negative result.

Another detection procedure

To isolate amylolytic fungi by **spreading** 1 ml of soil suspension on potato dextrose agar plates PDA (with 0.1 mg/ml streptomycin sulfate), incubate at R.T for about 3-7 days.

For isolation of **amylolytic bacteria,** Spread 1 ml of soil suspension on a nutrient agar plate (1 % w/v soluble starch) at 30^0 C for 24 hr.

Starch hydrolyzing colonies will have an area of clearing around them, for confirming, flood plates with gram's iodine (area around the colony appear yellow, while the area still contains starch appear ink-black color.



Positive result to starch hydrolysis

Lab:5

Nitrogen cycle

The nitrogen cycle is the biochemical cycle in which nitrogen is converted into multiple chemical forms, consecutively passing from the atmosphere to soil to organism and back into the atmosphere.

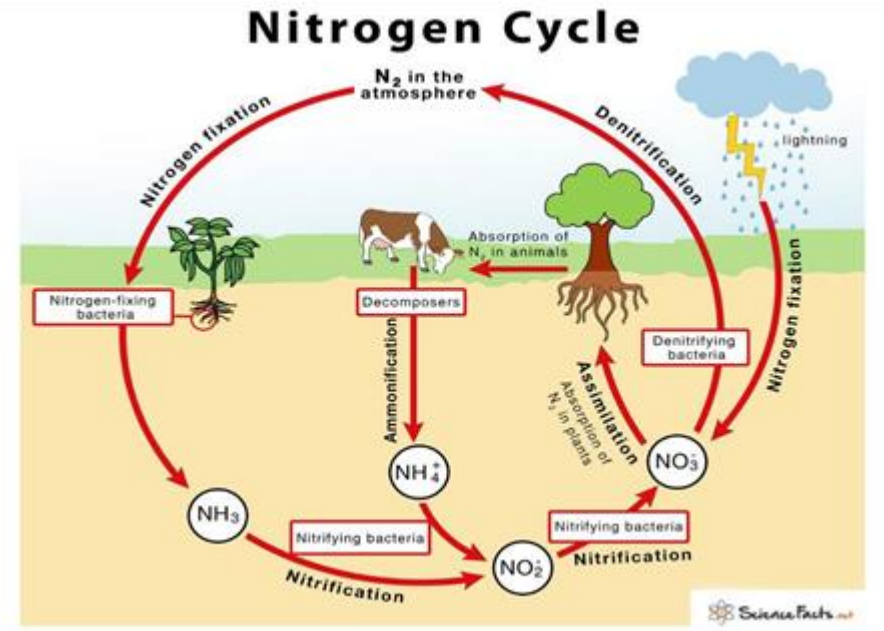
The main stages of the nitrogen cycle are:

1. Proteolysis and ammonification

2. Nitrification

3. Denitrification or nitrate reduction

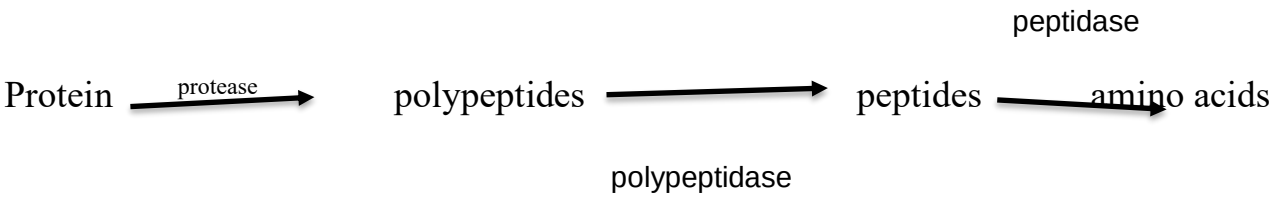
4. Nitrogen fixation



Nitrogen cycle steps

1. Proteolysis and ammonification

Proteolysis is a process in which a protein is broken down partially into peptides, or completely, into amino acids, by proteolytic enzymes. The amino acids undergo deamination and liberate the ammonia; this is called ammonification, which usually occurs under aerobic conditions.



Numerous microorganisms can release ammonia from organic compounds in the soil, such as:

Proteus and *Micrococcus.*

Isolation and detection of ammonification M.O.

- Suspend 1 gm of the soil sample in 9 ml of Sodium casein broth medium (as a source of protein), then incubate tubes at 28 C^{o} for a week.
- Mix 1 ml microbial suspension with a few drops of Nessler reagent in a clean test tube.

positive result

Golden -orange deposit demonstrates releasing of ammonia, as in the picture below.

2. Nitrification

Oxidation of ammonia (produced from the degradation of organic compounds in ammonification) to nitrate by a specialized group of strictly aerobic chemolithotrophic.

Oxidation of ammonia occurs in two steps:

In the first step: ammonia is oxidized to nitrite (NO_2^-) (nitrosofication)
The most involved microorganisms in this process are: *Nitrosomonas* and *Nitrosococcus*

Isolation & detection of Nitrosofication M.O

1. Suspend 1 gm of the soil sample in 9 ml of Allen I broth medium (Contains $(NH_4)_2 SO_4$ as ammonia source) and incubate tubes at 28 C^o for a week.
2. Mix 1ml microbial suspension with an equal volume of reagent A and reagent B let them react for a few seconds.

Positive result: The formation of a red colored deposit indicates releasing of NO_2 due to the nitrification process.

Negative result

Positive result

2. In the second step: NO_2 oxidized to nitrate(NO_3^-), the *Nitrobacter* mainly carry out the second step.

Isolation and detection Nitrite Oxidizing microorganisms

Follow the procedure as in previous but use Allen II broth as a medium containing $NaNO_2$ as nitrite source. To detect released NO_3, mix 1 ml of microbial suspension with drops of nitrate reagent, which consists of Diphenylamine (DPA) and sulfuric acid (H_2SO_4).

Positive result

The formation of a blue deposit indicates releasing NO_3, as in the picture below.

Note: If the deposit is not observed, that does not mean the absence of nitrifying microorganisms in the taken soil sample; rather, it means ammonia oxidation continued.

3. Denitrification or nitrate reduction

Under anaerobic conditions, reduce nitrate to nitrite, ammonia and return to the atmosphere as nitrogen gas (N2↑).

The reaction was mediated by the nitrate reductase enzyme that used nitrate as an electron acceptor in anaerobic respiration.

This process is performed by bacterial species such as *Pseudomonas* and *Clostridium.*

Isolation & detection of denitrification M.O

Suspend 1 gm of the soil sample in Allen l8 broth medium (the medium containing KNO_3 as a source of NO_3) in a test tube. Fill tubes completely with medium to create anaerobic conditions and incubate at 28 C^o for a week.

Results

Read the result by the following procedures:

• **NO_3 detection reagent**: Formation of blue deposit detects the absence of denitrifying microorganisms.
• **NO_2 detection reagent**: Formation of red colored deposit detects reduction of NO_3 to NO_2.
• **Nessler reagent**: Formation of golden–orange deposit detects complete reduction of NO_3 to ammonia.

Lab:6

4. Nitrogen fixation

Nitrogen fixation is a process by which nitrogen gas is converted into ammonia or related nitrogenous compounds in soil by soil microorganisms.

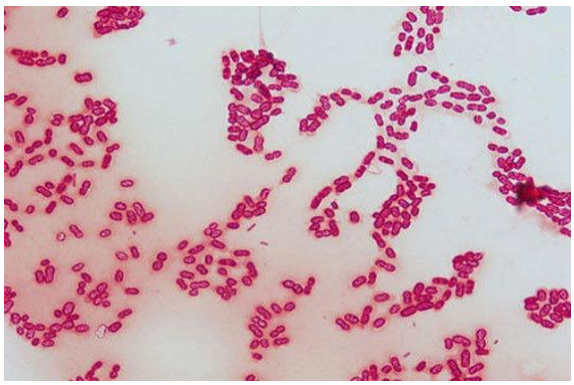
Nitrogenase is the most important enzyme involved in nitrogen fixation.

Two kinds of nitrogen-fixing microorganisms are recognized:

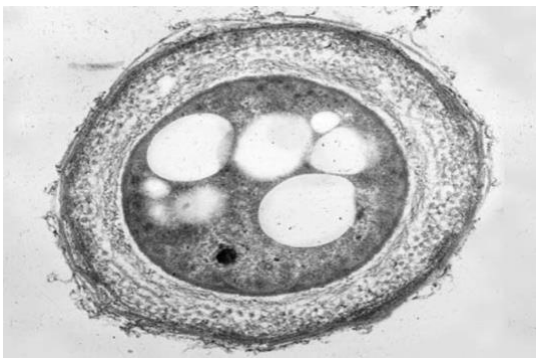
1. **Non-symbiotic N_2 fixer**: Those can convert N_2 to cellular nitrogen independently of other living organisms, so-called free-living nitrogen-fixing bacteria.

The main involved M.O.

Azotobacter: large gram-negative motile rods that may be ovoid or coccoid in shape (pleomorphic), aerobic and cells form cysts as the culture ages.



***Azotobacter* under microscope**



***Azotobacter* cyst**

(Macroscopically)

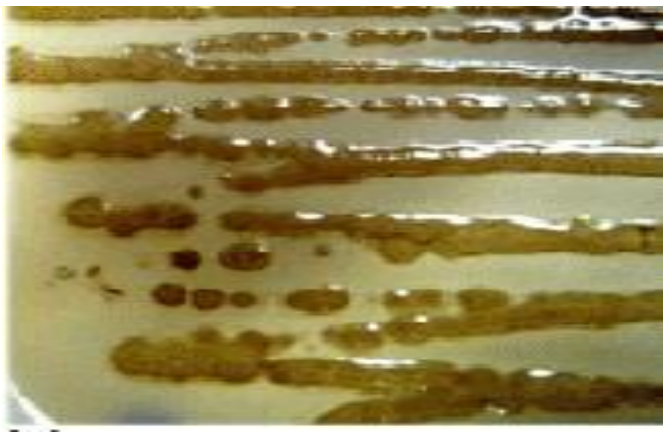
Azotobacter has grown on solid agar media as large convex, mucoid colonies with white, brownish color, colorless, and any other color depending on bacterial species. As soon as culture aged, brownish colonies were pigmented with dark chocolate color.

Brownish colonies of Azotobacter

Isolation of *Azotobacter*

1. Suspend 1 gm of the soil sample in 9 ml of D.W.

2-Spread 1 ml of soil suspension on Ashby's agar medium pH 7.6 (selective *Azotobacter* medium), including sodium molybdenum, incubate the plates at $28C^0$ for a week.
Azotobacter is characterized as mucoid and brownish large colonies as in the picture below.



2. Symbiotic N2 fixers

Symbiotic N_2 fixers microorganisms live in the roots of legume family plants, and the fixation process results from a mutualistic association between legumes plants and bacteria.

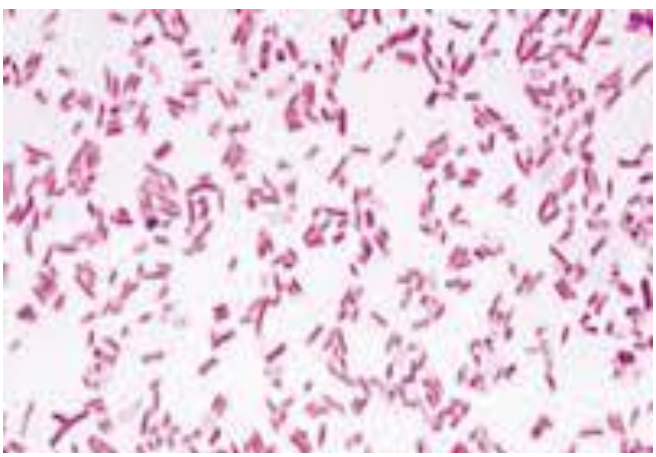
The main symbiotic N2 fixer bacteria is *Rhizobium*.

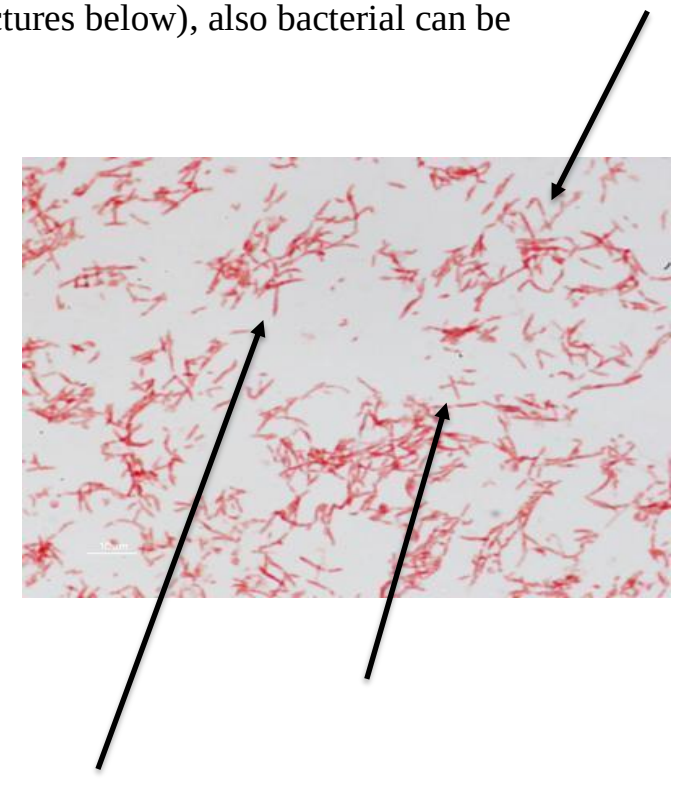
Which are G-ve rods to pleomorphic, motile with variably placed flagella.

Rhizobium invades susceptible plant roots and forms visible nodules (as in the pictures below), which live and fix N2 directly from the air.

Isolation of *Rhizobium* from soil

1. Cut roots of any legumes wash with tap water.
2. Select and cut visible large pink nodule, transfer to clean glass slid, crush it carefully by pressing it by forceps, until seeing a milky suspension then mix with water drop, stain with gram stain, examine under oil immersion *Rhizobium* appears to look like Latin Letters Y X Z T (as in the pictures below), also bacterial can be detected as G-ve bands.





Lab:7

Water sampling

1- Sampling from a tap or pump outlet

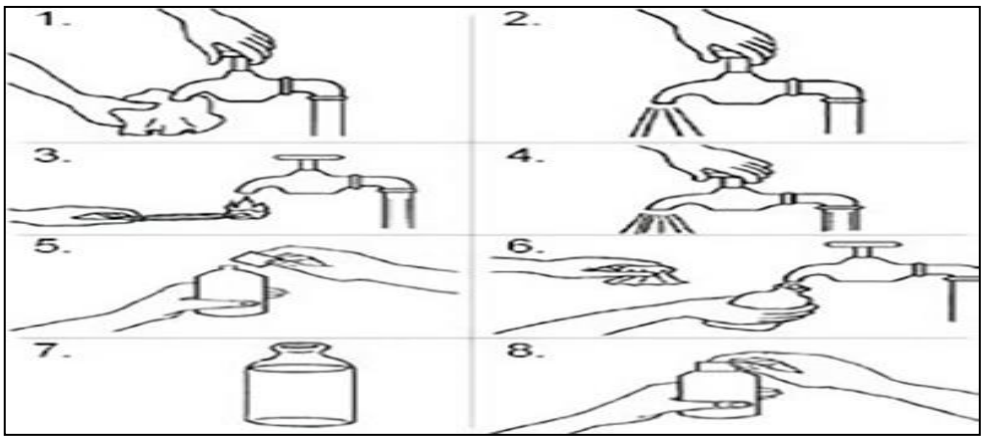
To obtain a representative sample of water, the sampling procedure described below and illustrated in Figure should be followed. The steps are:

1. Clean the tap/outlet using a clean cloth to remove any dirt.
2. Turn on the tap and let the water run at maximum flow for 1 to 2 minutes; then turn it off.
3. Sterilise the tap outlet for a minute with the flame from a cigarette lighter or an ignited alcohol-soaked cotton-wool swab.
4. Turn on the tap again and allow the water to flow for 1 to 2 minutes at a medium flow rate.
5. Open a sterilised bottle by carefully unscrewing the cap.
6. Immediately hold the bottle under the water jet and fill.
7. While filling the bottle, hold the cap face downwards to prevent entry of dust, which may contaminate the sample.
8. Screw on the cap. A small air space should be left so that the contents can be shaken more easily before analysis.

Note :

1- If the water is chlorinated, ensure the bottle contains sodium thiosulfate in order to stop the action of the disinfectant and maintenance the initial count of bacteria .

2- Place the bottle in transport box and returne to laboratory within 6 hours

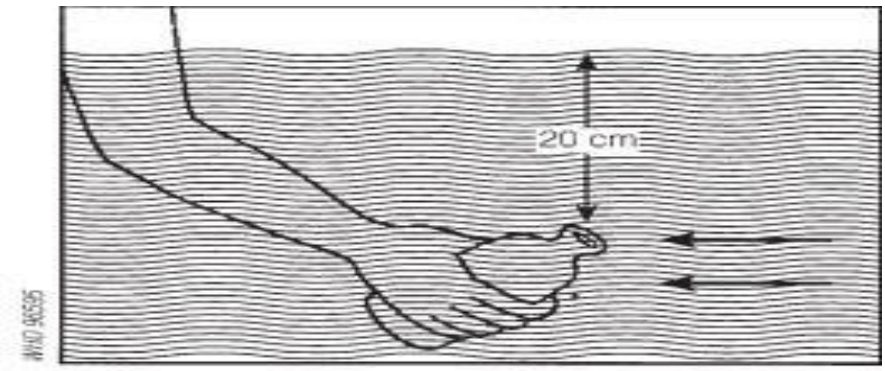


Procedure for sampling water from a tap. (WHO)

2- Sampling from a watercourse or reservoir

1- Fill the bottle by holding it by the lower part and submerging it to a depth of about 20 cm, with the mouth facing slightly upwards. If there is a current, the mouth of the bottle should face towards it .

2-The bottle should then be capped.



Sampling of water from surface water (rivers, ponds, etc.). (WHO)

3- Sampling from dug wells and similar sources

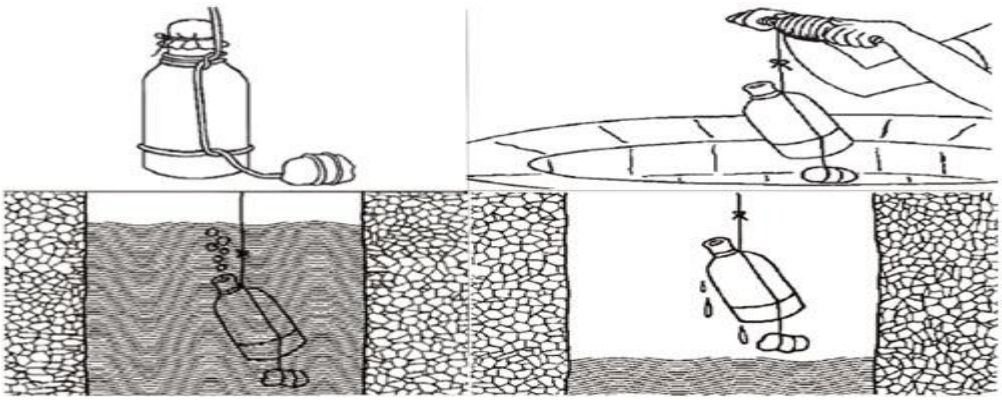
1-Prepare the bottle with a piece of string and attach a clean weight to the sampling bottle.

2-Take a 20 m length of clean string rolled around a stick and tie it to the bottle string.

3-Open the bottle , lower the bottle, weighed down by the weight, into the well, unwinding the string slowly. Do not allow the bottle to touch the sides of the well.

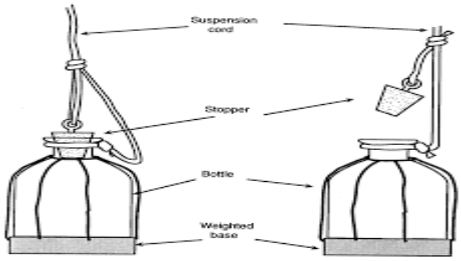
4-Immerse the bottle completely in the water and lower it well below the surface but without hitting the bottom or disturbing any sediment.

5-Raise the bottle when it is judged to be filled, rewind the string on the stick to bring up the bottle. If the bottle is completely full, discard some water to provide an air space , cap the bottle.



Procedure for sampling water from a well.(WHO)

Other device used for sampling of well water surface water such as river



Device used for water sampling from well and depth of surface water such as river

Van Dorn apparatus

Lab:8

Aquatic Microbiology

Water is the elixir of life , it is an essential part of protoplasm and create a state for metabolic activities to occur smoothly. Therefore, there is no life can exist without water, also there are thousands of m.o. which live in water so can cause diseases.

Water receives m.o. from air, soil, sewage, organic wastes, dead plants and animals…etc.

Aquatic Microorganisms….. large number of m.o. are found in water (bacteria, fungi, algae, protozoa, several human and animal viruses) are transferred by water.

Water monitoring for microbiological quality is primarily based on testing of **indicator organisms.**

Indicator Microorganism : A nonpathogenic microorganism whose presence suggests the presence of enteric pathogens , such as :

1-Coliform Organisms

2-Clostridiumespecially***Clostridium perfringens***

3-Fecal streptococci, especially***Streptococcus faecalis***

4-Certain species of anaerobic bacteria***, Bifidobacteriumbifidus.***

5- Bacteriophages

These species are easily isolated from water by the use of relatively simple methods of selective cultivation and are readily identified. The first two species are used frequently as indices of fecal pollution in both water and foods.

Detection and measurement of fecal indicator organisms

There are two main methods used to detect and measure indicator bacteria in water , ther are :

-The most probable number (MPN) multiple tube method

-The membrane filteration method

1- fecal coliform

Fecal coliforms bacteria are the naturally occurring bacteria found in the digestive tracts of warm-blooded animals , coliforms are members of the family Enterobacteriaceae which includes: *E.coli*, *Enterobacter*, *salmonella* and *klebsiella pneumonia.* This group is facultative anaerobes, G-ve, non-spore former, rod ,ferment lactose with gas formation within 48hr at 30ºC .

The coliform groups are present in water due to fecal contamination i.e. discharge of feces by human and animal in water. These bacteria make up about 10% of intestinal m.o. of human and animal . Fecal coliform counts should be zero per 100 mL of sample (0/100 mL) in all water supplies, piped or unpiped, treated or untreated.

* Most probable number**M.P.N**.

The standard multiply tube fermentation technique (most probable number**M.P.N**.) are used for coliform detection and enumeration.

(This method involves 3 routine standards tests):

1) The presumptive test.
2) The confirmed test.
3) The complete test.

1. Presumptive test

A serial dilutions of fermentation tubes (macConkey broth),are inoculated with water samples. These tubes are incubated for 24 - 48hr at 37ºC.

Procedure

1. Make serial dilutions (10^{-x}) of water samples as the following:

Sewage water	**9** dilutions
River water	**8** dilutions
Stagnant water	**6** dilutions
Brooklet water	**5** dilutions
Tank water	**4** dilutions
Drinking water	**3** dilutions

2. Suspend **1ml** of water sample in **9ml**D.W.(10^{-1}),then transfer 1 ml of firstdilution to another tube to prepare the second dilution(10^{-2}).

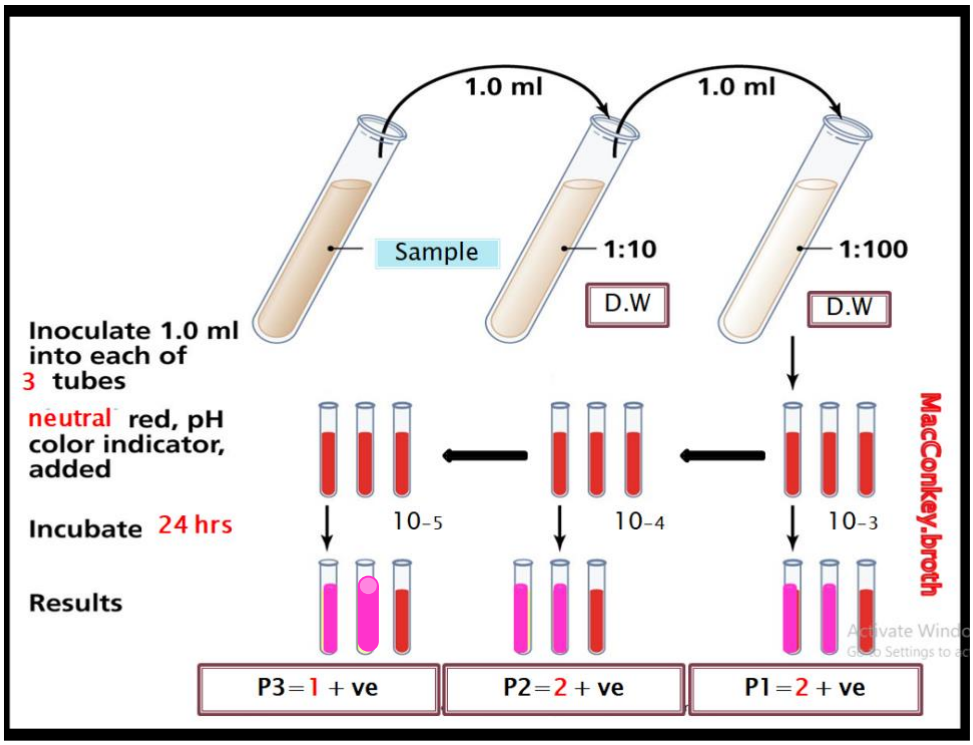
3. Prepare the last 3 dilutions for each water sample in **macConkeybroth** tubes rather than D.W.

ex…. if the water sample require 5dilutions (Brooklet water), the 1st and the 2nd dilutions use **D.W.** , while the 3 last dilutions use macConkey broth(suspending 1 ml of 2nd dilution in 9 ml macConkey broth (10^{-3}) and the other two remaining dilutions prepare in same way(10^{-4} and 10^{-5}).

4. Incubate MacConkey tubes for 24hr at 37ºC.

5. read the result as the following:

(+ve)….. coliform change the color of macConkey broth from **red** to **pink** due to lactose fermentation(as macConkey broth contain neutral red indicator. change to **pink** in acidic environment).



6. Enumerate **+ve** test tubesfor each P1,P2 and P3.thenfind the MPN value of coliform by using **MPN standard table**.

No. of cells / ml =M.P.N.value × reverse of mid dilution×?

The number of coliform present is not absolute, but is a statistical estimate.

2. **Confirmed test**

The positive tubes in Presumptive test does not mean that fecal coliform are detected? other coliformsalso can give false positive presumptive test because they also can ferment lactose.Thereforea confirmed test is necessaryto identify the presence of fecal coliform.

Procedure

Transfer 1ml of any +ve tube of presumptive test to another**macConkey broth**tubes (contain Durham tubes) incubate for 24hr**at 44.5ºC**.

+ve result…… for **fecal coliform**: color change to+gasin durham tube.

3. Complete test

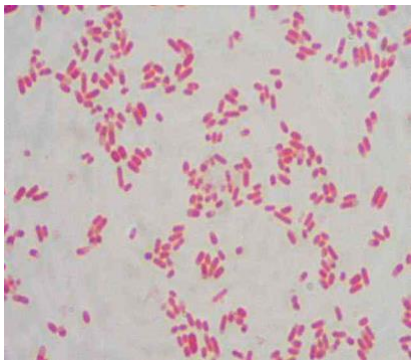
The complete test is use to be as certain about the presence of fecal coliform in water.

Procedure

1. Streak a loop full from the +vetubes on macConkey agar or Eosin Methylene Blue agar (EMB).
2. Incubate plates at 37 ºC for 48hr.
3. Macroscopically ……..coliform colonies on macConkyagarare(pink with dark center and opaque) while on EMB agar(colonies nucleated with metallic sheen).

MacConkey agar…….. (LF&LNF)	**EMB**……Green metallic sheen

4. Make a slide from colony on glass slidewith Gram stain.



E.coli (Microscopically)

Lab:9

2-*Clostridium*

Clostridia… are obligate anaerobic, gram-positive, endospore-bearing, large rods , with rounded ends.The majority of Clostridia are harmless and helpfulsaprophytes . some of them live in intestinal tract of human and animal, other cause serious diseases of humanand animals.

The advantage of testing the presence f Clostridia especially***Clostridiumperfringens***in water samples, is suggestingthe **type of pollution**: an **old fecal** pollution, because the survival time of pollution indicator organisms in water (***Clostridium perfringens***, resistant spores can survive in water).

The presence of this bacteria in the absence of the others, particularly ***E. coli,***suggests pollution that may had presence for a considerable**long time (old pollution)**, but if both (Clostridia &*E. coli*) founded in water suggest relatively a few hours or days**(recent pollution)**.

(1) Presumptive test

1. Full a glass tube of tested water sample without any serial dilution (heat the tube in water bath for 10 min at 80 °C?).

2. Transfer 1ml of heated water sample to tubes with selective medium **differential reinforced Clostridia medium (DRCM)** supplemented with sodium sulfateandferrous citratein equal volumes to great reducing state , incubate tubes an aerobically (anaerobic gar) at 37 °C for 48 hr.

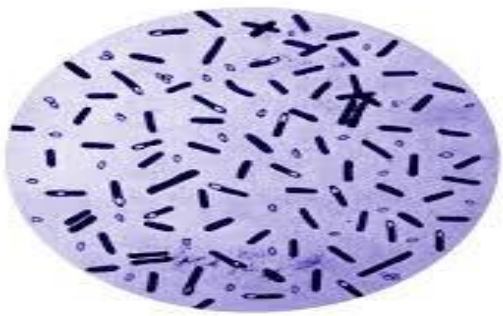
3. +ve tubes detected by the formation of **black deposit** of ferrous sulphate**FeS**.

(2) Confirmed test

The confirmed test is done to confirm the presence of ***Clostridiumperfringens***in positive tubes by using **Litmus Milk**(stormy fermentation phenomenon), since ***Clostridium perfringens***ferment sugar in litmus milk(lactose to lactic acid)lead togreats extremely acidic condition which lead to coagulation of milk protein(Casein) and the gas that formed through sugar fermentation pushprotein clot to the surface

Procedure

1. Transfer 1ml of +ve tubes to preheated test tubes filled with litmus milk medium (precaution: be carefully do not expose samples to air).
2. Incubate tubes an aerobically at 37° C for 48 hr.
3. Detect +ve tubes for stormy fermentation.
4. Make a slide, stain with gram stain and examine under oil immersion.



Microscopically***Clostridiumperfringens***

Membrane filtration

For the membrane filtration of water, place a membrane filter over the **carbon disk** by using forcep sterilized under the flame.. A **vacumn** system allows the complete separation of **filtrate** through the membrane filter.

.

Transfer of membrane filter: Then, place a membrane filter over the prepared petri dish containing absorbent pad saturated with a liquid nutrient medium. Finally, incubate the Petri plate for 24-48 hours at 35-37 degrees Celsius temperature.

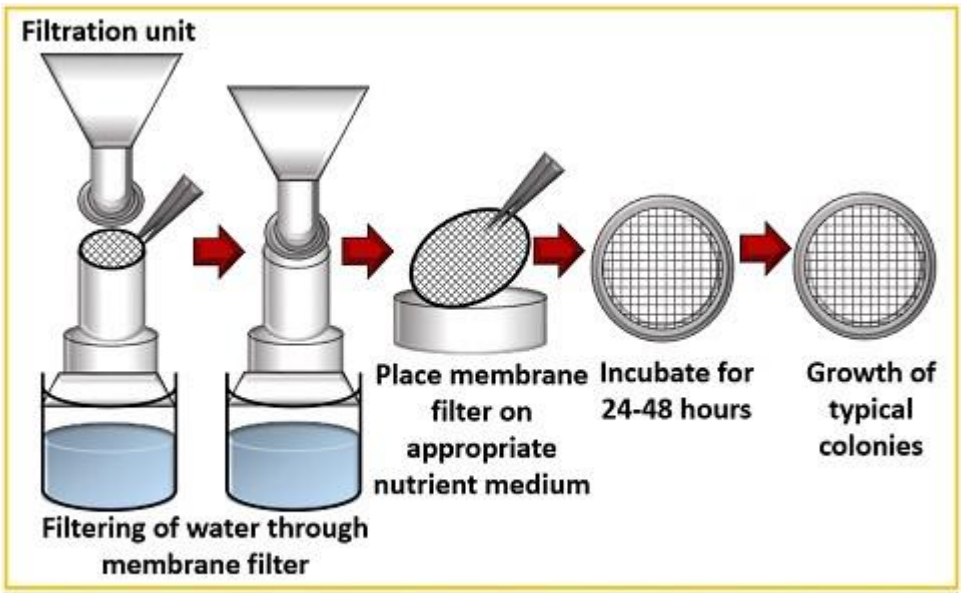
Observation: For the **quantitative analysis**, we need to count the number of colonies directly on the colony counter.

Advantages

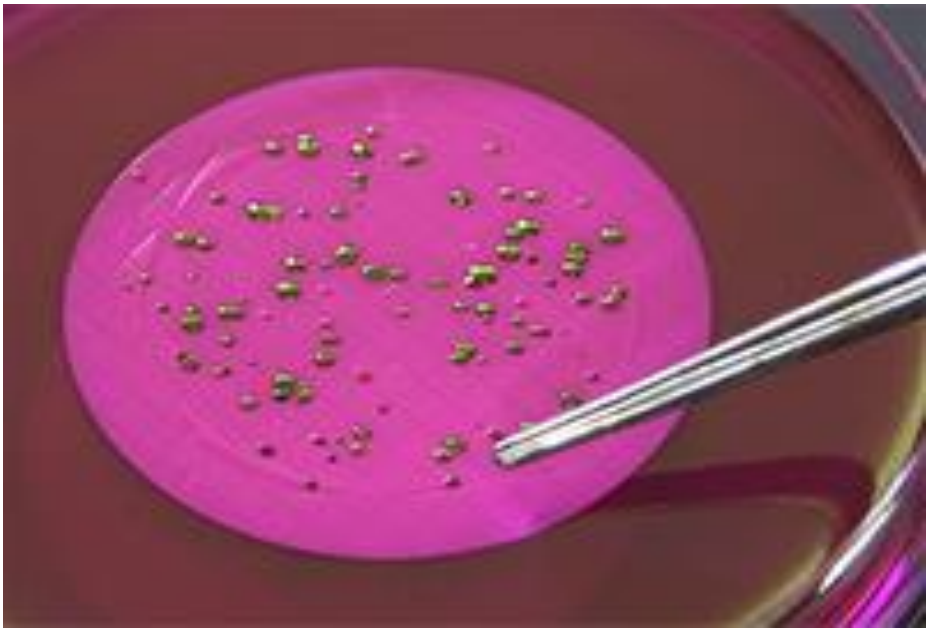
- MF technique is an alternative to the most probable number method.
- One can test a large volume of a water sample through the millipore filtration method.
- Membrane filtration consumes less time than the MPN method, as it only involves four to five steps to get the results.
- One can enumerate the number of bacteria present in the water sample directly by using the colony counter.

Disadvantages

- Membrane filtration method is not applicable to test the turbid water.



METHOD OF MEMBRANE FILTRATION



3-Fecal Streptococci

The fecal streptococci found in the feces of humans and other worm- blooded animals:

(a)- *Streptococcus faecalis*

(b)- *Streptococcus bovis*

(c)- *Streptococcus equines*

Because of **limited survival time outside** the intestinal tract their presence indicates **very recent pollution.**

Fecal streptococci data verify fecal pollution and many provide additional information concerning the recency and probable origin of pollution, In combination with data on coliform.

(FC/ FS) Ratio

The relation of the fecal coliform and fecal streptococci density may provide information on the potential source of contamination, (N. of **Fecal coliform /N.** of **Fecal streptococci) ,**if the ratio greater than 1 indicates human fecal pollution (domestic wastes), while if the ratio less than 1 indicates worm –blooded animals pollution .

Most probable number (M.P.N.) of fecal streptococci

1- Presumptive test

Procedure:

1- add **0.5** ml of water (different types of water) to 3- sterile tubes with **4.5** ml of **Azide dextrose broth** mixed well (**10^{-1}**) .

2-Transfer **0.5** ml from previous tubes to another 3- tubes of Azide dextrose broth mixed well (**10^{-2}**).

3- Finally transfer **0.5** ml from (**10^{-2}**) to another 3- test tubes with Azide dextrose broth and mixed well (**10^{-3}**).

Now we have 9-tubes contain **Azide dextrose broth** (10^{-1}), (10^{-2}), (10^{-3}).

Notes : The procedure include a few number of dilutions**?** because this bacteria do not grow easily in media.

4- Incubate at 37^0 C for 24-72 hr.

5- Record the results if the tubes negative or positive (**+ve**: turbid + change in color from purple to yellow.

6- calculate the number of cells for 1 ml (cell /ml) by using the value of (M.P.N.) from the table .

7- Compare the cell number of coliform(previous data) with the cell number of streptococci, to evaluate the ratio of **(FC/ FS)**.

2-Confirmed test

Confirmed test for streptococci is essential to Confirm the the presence of fecal streptococci especially ***Streptococcus faecalis*** .

Procedure:

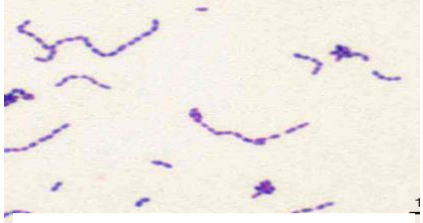
1- Transfer **0.5** ml from +ve results of **Azide dextrose broth** (yellow tubes) to another tubes contain **4.5** ml of **Azide dextrose broth**, mixed well.

2- Incubate the tubes at **45^0 C** for 48 hr**.**

3- + ve result ….turbidity and color change from purple to **yellow** will confirm the presence of ***Streptococcus faecalis***

3- Complete test

Inoculate the selective solid media for Streptococci **Pfiezer selective agar (PSE agars)** with positive result of Confirm test . colonies of ***Streptococcus faecalis*** on this medium have black- brown color encircled by brown zone



Microscopically

Lab:10

Detection of pathogenic bacteria

Pathogenic bacteria come into the water mainly from domestic waste water and most of them cannot stay for a long time because soon they die, so the water-laden feces (Sewage) are the source of disease particularly pathogenic intestinal microorganisms.

The study and investigation of coliform bacteria is important for its close relationship with pathogenic bacteria, it is possible to isolate pathogenic bacteria in the case of presence of coliform bacteria in water , so this test is important always. But we will depend on previous tests have made to the coliform and investigated to other pathological types of bacteria i.e. ***Salmonella , Shigella , Vibrio*** .

Isolation and Identification of S*almonella and Shigella*

Salmonella

G-ve bacteria, bacilli, non- spore former, motile by peripheral flagella, mostly non lactose fermented, produce H_2S.

Distinguishing from other Enterobacteriaceae by:

1- Motility

2- Do not produce capsule

3- non-lactose fermenter through 18-24h when grow on Macconky or S.S.agar so they have pale colonie.

Shigella

G-ve bacteria, bacilli, non- spore former, non-motile, non-lactose fermenter, do not produce H_2S.

Characterized *Salmonella* from *Shigella*

1- Production of H_2S.

2- Produce gas and acidity in sugar solution.

3- Motile

4- Most of them do not have the ability to produce Indol .

Salmonella **….**caused infection of salmonellosis when dealing with contaminated food and drink.

Shigella. ….*Shigella* can cause dysentery through contaminated food and drink when it moves to the large intestine and begins to reproduce, the dysentery accompanied by diarrhea with blood and mucous materials, abdominal pain and neurological disorders.

Enrichment

The important steps to isolate the pathogenic bacteria, because:

1 - Pathogenic bacteria are few in number in the water

2 - Often the selective media toxic to pathogenic bacteria.

Procedure:

1 - Different water samples are taken

2 - Add the water sample to multiplier enrichment media with equal volume of enrichment media.

Enrichment media for ***Salmonella*** and ***Shigella***

1- Selenite broth

This medium allows the rapid proliferation of ***Salmonella*** and it should be incubated within a period of 24 hours; the growth indicated by turbidity and orange color.

Notes :

Increasing the incubation period of selenite broth allows the growth of coliform bacteria, which were inhibited during the early hours of incubation in this medium.

2- GN broth

This medium gives a good growth of the ***Shigella***, it inhibited coliform and fecal streptococci , but if the incubation period is more than 24 hours allows the growth of acidophilic bacteria-like ***Proteus* , *Pseudomonus aeroginosa***.

Note: The growth indicated by turbidity formed in the media.

Growth in Selective media

To separate pathogenic bacteria from non-pathogenic bacteria we should do the following:

1 - The temperatures and duration of incubation should be appropriate for pathogenic bacteria.

2 - Use appropriate media.

Classification of solid selective media:

(1) Differential medium

This media contain or does not contain materials inhibit the growth of microorganisms other unintended to grow on it, but contains materials intended to give the bacteria special

characters that distinguish them from other bacteria, such as **(EMB agar)** allowing for growth of types and genus of bacteria other than the ***E. coli*** but, only ***E. coli*** give the phenomenon of **green metallic sheen**, other bacteria appear in red color.

2) Selective media)

This media contains inhibited materials, allows the growth of bacterial groups without the other, but not necessarily with material distinguish these groups from each other, so it could be selective and differential media at the same time for example:

1- **EMB agar… This media is consider as** selective medium because it contains substance inhibited the growth of G +ve bacteria and allows the growth of G-ve bacteria and it is differential media because it distinguishes the ***E. coli*** from the other enteric bacteria .

2- **Macconky agar… This media is consider** as selective and differential medium ; it is selective because it contain **crystal violet** and the **bile salt which** inhibit the growth of G+ve bacteria and allows the growth of G-ve enteric bacteria ,also it distinguish between their genus, the G-ve lactose fermenter bacteria appear as pink colonies while non-lactose fermenter bacteria appear as pale colonies.

3-**S.S. agar (*Salmonella - Shigella* agar)**

This media is consider as selective and differential medium, because it contains the bile salts which inhibit the growth of G +ve bacteria and it also contains lactose to differentiate between lactose fermenter and non- lactose fermenter bacteria , ***Salmonella*** and ***Shigella*** colonies appear as pale colonies because both of them cannot fermented lactose sugar also this medium distinguishes between ***Salmonella*** and ***Shigella*** , ***Salmonella*** produce of H_2S which reacts with iron, giving the FeS (Black precipitation) which is concentrated in the center of the colonies while ***Shigella*** colonies appear as pale colonies without a black center because it is not produce of H_2S, ,this media contain Neutral red as indicator .

Procedure:

1 - Notes that growth on GN broth appears as a turbid while the growth on Selenite broth appears as a turbid as well as the color changed to orange.

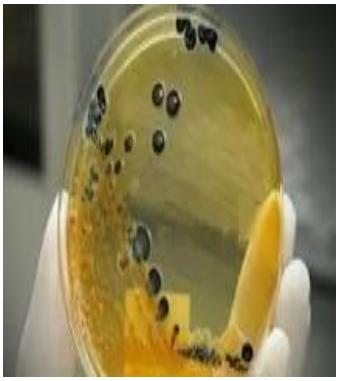
2- **S.S.agar** is prepared and the Petri- dish is divided into two equal halves by which the first half is inoculated from **Selenite broth (turbidity and orange color)** while the second half is inoculated from **GN broth (turbidity).**

3- Incubate the plate at 37C˚for 24h.

4- Notes the appearance of colonies.

5- Pale colonies + FeS ⟶ ***Salmonella***

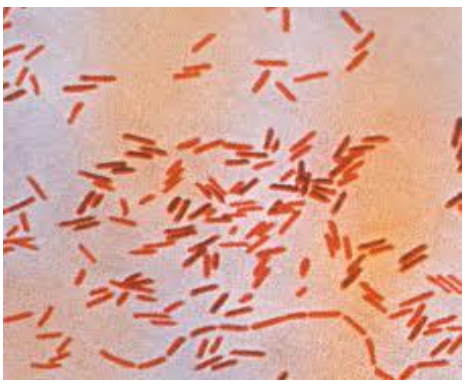
Pale colonies without FeS ⟶ ***Shigella***



Salmonella ***Shigella***

6- Make a slide and stained with Gram stain

Notes: We cannot distinguish between *Salmonella* and *Shigella* under the microscope because they have same features; G-ve , bacilli, non-spore former.



Salmonella and *Shigella* under the microscope