PRACTICAL SOIL MICROBIOLOGY

Third stage

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Soil microbiology

Lab 1

Soil is the loose surface material that covers most land. It consists of inorganic particles, organic matter, water and air. It provides a medium in which plants grow, a habitat for living organisms and is also their source of water and nutrients. Soil is derived from a parent material, generally bedrock, that has been modified through time through the action of physical, chemical and biological processes. As bedrock varies greatly from place to place, so do the soils derived from it. Soils vary greatly in their chemical and physical properties.

Soil types

Soil consists of particles of various sizes, such as sand, silt and clay and the combination of different percentages of sand, silt and clay particles is known as the soil texture. There are **four basic** types of soil: sand, silt, clay and loamy soil which is a combination of sand, silt and clay such that the beneficial properties of each are included.

Water content

Soil water is an important component of the soil which influences soil organisms and plant growth. Soil water is divided into three types:

1- **Free water (Gravitational water)** is water that drains freely through soil under the force of gravity. This type of water is not readily available to plants; so it quickly moves downward through the soil.

2- **Capillary water** is water that is held in the soil pores by capillary forces, which are the result of the attractive forces between water molecules and soil particles. This water is available to plants and can be taken up by roots through the process of capillary action. Capillary water is held more tightly in the soil than gravitational water, so it is not lost as quickly to drainage.

3- **Hygroscopic water** is water that form a thin film surrounding soil particles because it is held tightly to soil particles by adhesion properties. This water is not available to plants.

Water activity

Microbiologists use water activity (a_w) to measure the availability of water for biological reactions, which is less amount of water required for microbial activities and growth. Different methods can determine the water content of a solution or solid, the most common 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 content 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:

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

- 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:2

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 dilutions
Vegetable soil	6 dilutions
Citrus soil	5 dilutions
Palm soil	4 dilutions
Hydrocarbon polluted soil	3 dilutions
Uncultured soil	2 dilutions

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 invert dilution factor x ?

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

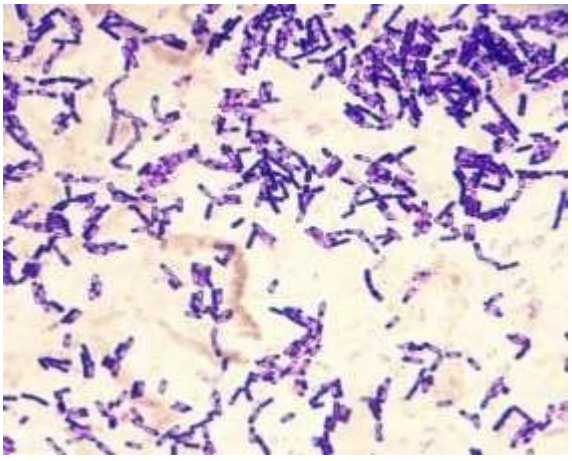
$$\text{CFU / gm of dried soil} = \frac{\text{CFU}}{\text{Wt. of 1gm dry soil}}$$

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 in the center of the slide and take a touch by loopful from one well isolated 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 the burner flame (Heat fixation).
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) (few sec).
5- Add Safranin (1-1.5min), then wash carefully with tap water.
6- Find a clear field at 10X, 40X, and 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 a sexually by budding.

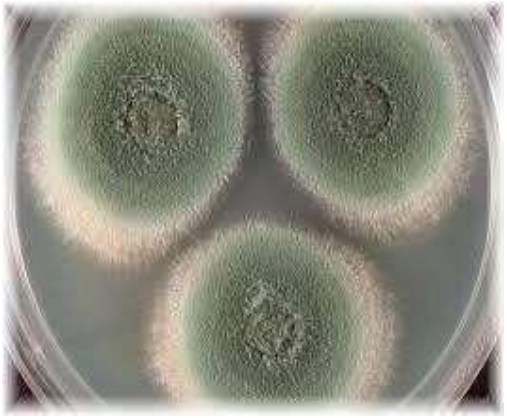
Isolation Procedure

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 0.1 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**

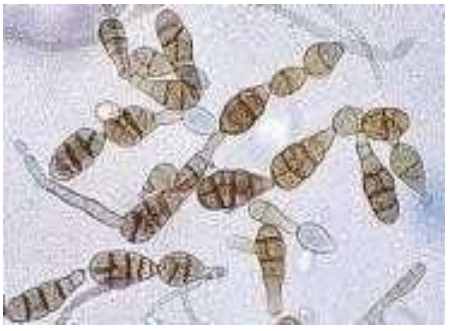
Yeasts colonies on plate

Molds slide preparation

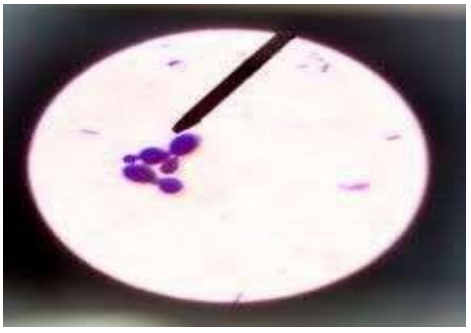
1- Place a drop of Lactophenol cotton blue on a slide.
2- Dig the mold colony from the agar by a loop.
3- Put it over the slide, and then put a cover slide over it.
4- Knock carefully at the left angle to spread the colony under the slide cover without breaking it.
5- Find a clear field under 10X & Examine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

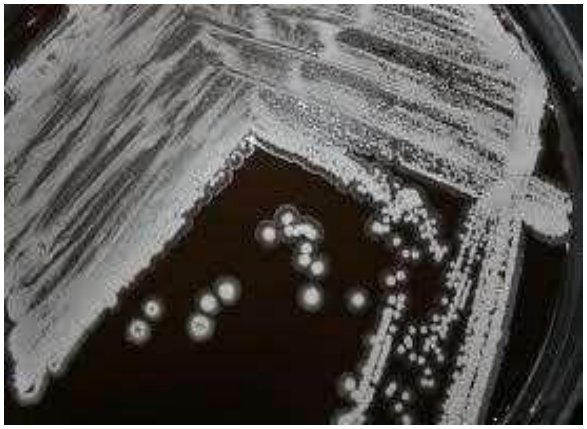
3. Isolation of Actinomycetes

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.

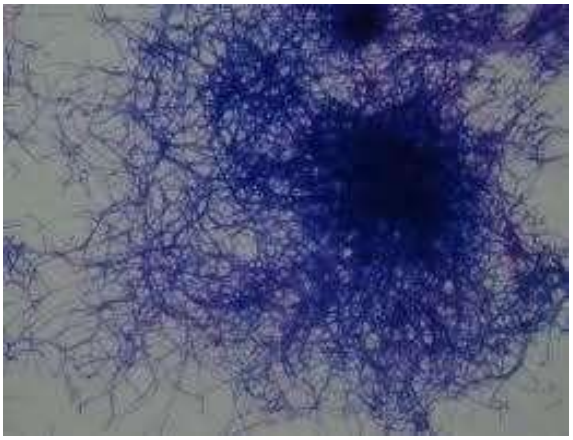
Actinomycetes colonies are powdery mass forms often pigmented with orange or red, or yellow color.

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 0.1 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:3

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 microorganisms

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.
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 calciumcarbonate).

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 microorganisms

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) incubates 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 is a polymeric carbohydrate consisting of numerous glucose units joined by glycosidic bonds and it broken down by amylases, which are produced by various living organisms, ranging from microorganisms, plants & humans. Bacteria and fungi secrete amylases outside of their cells to carry out extracellular digestion. When they have broken down the insoluble starch, the soluble end products, such as (glucose or maltose) are absorbed into their cells.

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

α- **Amylase (alpha-amylase)**: Breaking down the α-1, 4 glycosidic bonds in starch at random way, therefore producing vary the size of glucose chains.

β- Amylase (Beta-amylase): Break down the α-1, 4 glycosidic bonds in starch and removing two glucose units at a time, therefore producing maltose.

-Amylase (Gamma amylase): Breaking down the α-1, 4 glycosidic bonds and the α-1, 6 glycosidic bonds in non-reducing ends of the starch molecule to yield glucose.

Amylase producer microorganisms

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 producer microorganisms

<u>Procedure</u>

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

Lab4:

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.

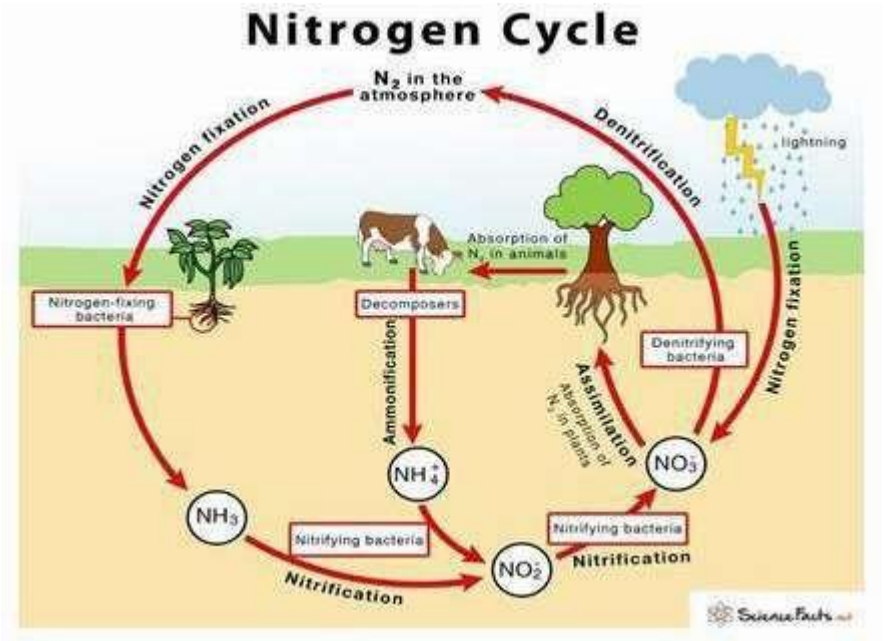
The main stages of the nitrogen cycle are:

1. Proteolysis and ammonification

2. Nitrification

3. Denitrification or nitrate reduction

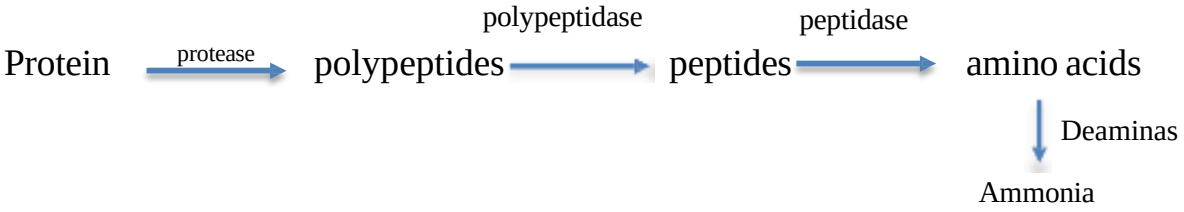
4. Nitrogen fixation



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 fromorganic compounds in the soil, such as:

Proteus and *Micrococcus.*

Isolation and detection of ammonification microorganisms

- 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° 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 microorganisms

1. Suspend 1 gm of the soil sample in 9 ml of Allen I broth medium (Contains $(NH_4)_2SO_4$ 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 microorganisms

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.

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.

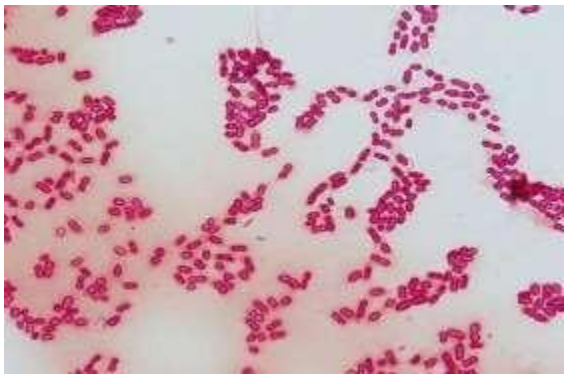
Nitrogenase is the most important enzyme involved in nitrogen fixation.

Two kinds of nitrogen-fixing microorganisms are recognized:

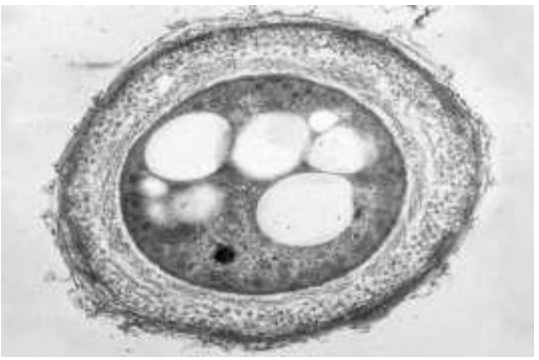
1. **Non-symbiotic N_2 fixer**: Those can convert N_2 to cellular nitrogen independently of other living organisms, so-called free-living nitrogen-fixing bacteria.

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 N2 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 bacterium is *Rhizobium*

That is 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.