AGRICULTURAL MICROBIOLOGY: AG MICRO. 1.1

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INTRODUCTION

- Microbiology is a specialized area of biology (Gr. *bios = life + logos = to study*) that deals with the study of microorganisms or microbes.
- In a broad sense, Microbiology can be defined as "a branch of biological science which deals with the bacteria and related forms of life like rickettsiae, viruses, yeasts, molds, algae and protozoa.
- Microorganisms: Organisms are too small (less than 0.1 mm in size) that cannot be clearly perceived (seen) by the human eye are known as microorganisms. E.g. Bacteria, fungi (yeast, mold), protozoa, algae, virus, phytoplasma and rickettsia.
- The human eye is not able to perceive (see) objects with a diameter of less than 0.1 mm size, therefore an object must be required to magnify at least 0.1mm to preferably 0.2mm for clear vision.
- Bacteriology can be defined as the branch of science, which deals with systemic study of unicellular prokaryotic microorganisms, known as bacteria.

The Discovery of Microbial world:

- Microorganisms were probably first living things to appear on earth.
- Roger Bacon, in the 13th century, postulated that disease is caused by invisible living creatures.
- This suggestion was made again in 1546 by a physician 'Girolamo Fracastoro' of North Italy.
- As early as 1658, a monk named Athanasius Kircher (1601-1680) referred to "worms" invisible to the naked eye in decaying bodies, meat, milk, and diarrheal secretions.
- In 1665, Robert Hook , an English Scientist, used a simple lens that magnified objects approximately 30X. He examined thin slices of cork, the bark of oak tree, and found that cork was made of tiny boxes that Hook referred to as "Cells" and all his observations were published in book titled 'Micrographia'

- **Antony Van Leeuwenhoek**: He was a merchant of Holland by profession but he ground lenses and made microscopes as a hobby. He had no formal university education but had a keen mind. The best microscope he made had a magnification of 200 to 300 times.
  - He observed food particles deposited between teeth of his own and colleagues under his microscope. He reported minute objects moving speedily, which he called "Animalcules' (Small animals), which we now know as Protozoa, fungi and bacteria.
  - Leeuwenhoek is considered as father of bacteriology, heamatology, histology and protozoology.

Spontaneous Generation Theory

The origin of life from non-living materials – also called Abiogenesis. (The production of living organisms from other non-living materials).

- ❑ The belief that life could originate from non-living or decomposing matter.
- ❑ The theory was stated by **Aristotal** in 346 B.C.
- ❑ Spontaneous Generation also called abiogenesis, is the belief that all living things originated spontaneously from inanimate/non-living matter, without the need for a living progenitor to give them life.

Louis Pasteur- He finally disproved the Theory of spontaneous generation.

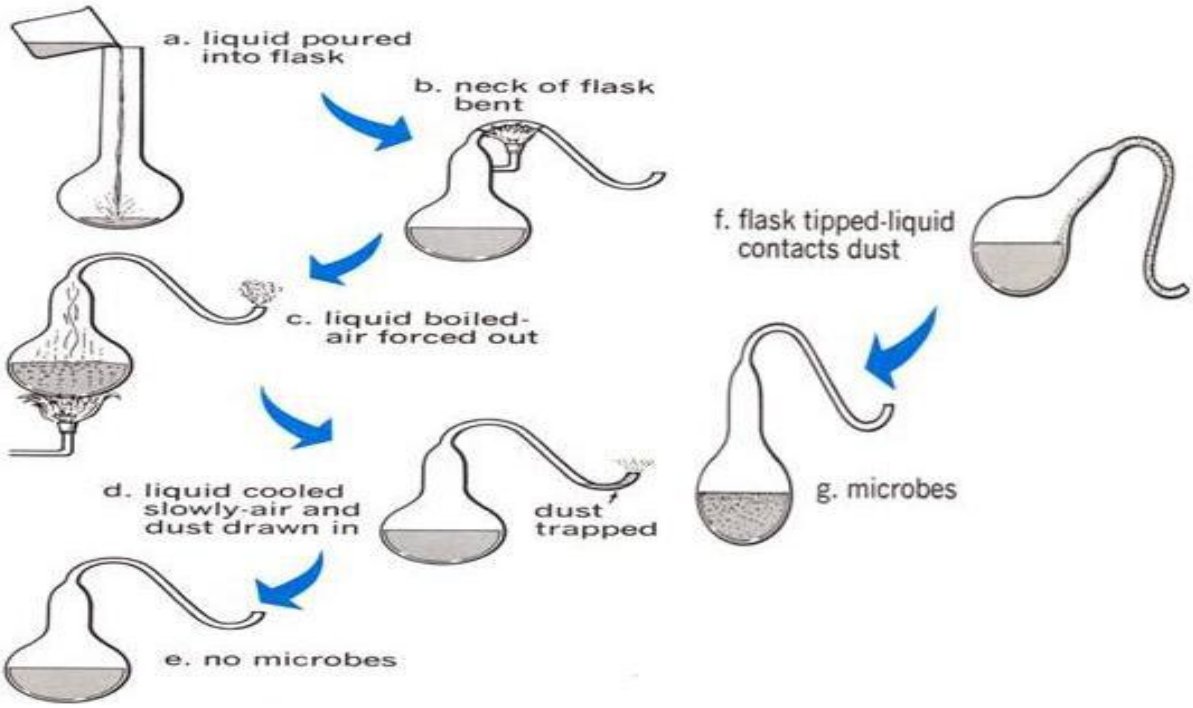
- He boiled meat broth in a flask, heated the neck of the flask in a flame until it bent into the shape of an 'S'.
- Pasteur's Swan-Necked Flasks: He used swan-necked flask. Pasteur created unique glass flasks with unusual long, thin necks that pointed downward.

These swan-necked flasks allowed air into the container but did not allow particles from the air to drift down into the body of the flask.

- Air could enter the flask, but airborne microorganisms could not - they would settle by gravity in the neck. As Pasteur had expected, no microorganisms grew.
- When Pasteur tilted the flask so that the broth reached the lowest point in the neck, where any airborne particles would have settled, the broth rapidly became cloudy with life.
- Pasteur had both refuted the theory of spontaneous generation and convincingly demonstrated that microorganisms are everywhere - even in the air.
- No growth was observed because dust particles carrying organisms did not reach the medium, instead they were trapped in the neck of the flask; if the necks were broken, dust would settle and the organisms would grow; in this way Pasteur disproved the theory of spontaneous generation .

John Tyndall

- ❑ Omission of dust ----> no growth.
- ❑ Demonstrated heat resistant forms of bacteria (endospores).
- ❑ He conducted experiments in a specifically designed box to establish and prove the fact that dust actually contained and carried the microbes (i.e. germs).



Pasteur's Experiment

Scientist who have done research on spontaneous generation theory

Name of Scientist	Experiment
Francesco Redi	Conducted three jar experiment and showed that maggots are larval stage of flies thus destroyed the myth of spontaneous generation
John Needhm	supporter of the theory of spontaneous generation and proposed that tiny organisms, the animalcules arose spontaneously on his mutton gravy
Lazzaro Spallanzani	boiled beef broth for longer period, removed the air from the flask and then sealed the container and disproved the hypothesis of spontaneous generation
Franz Schulze & Theodor Schwann	passed air through hot glass tubes or strong chemicals in to boiled infusions in flasks and disproved spontaneous generation theory
Schroder & T. Von Dusch	Discovered technique of cotton plugging and disproved spontaneous generation theory

Germ Theory of Fermentation

- In past some chemists (Berzelius, Liebig, and Wohler) believed that souring of milk and production of alcoholic beverages were spontaneous chemical processes. They opposed the views that microorganisms caused these changes (first proposed by—Latour, Schwann and Kutzing in 1938).
- Louis pasteur worked on lactic acid fermentation and suggested that specific microorganisms were responsible for specific changes or fermentation. This is called germ theory of fermentation, which laid the foundation of Industrial microbiology.

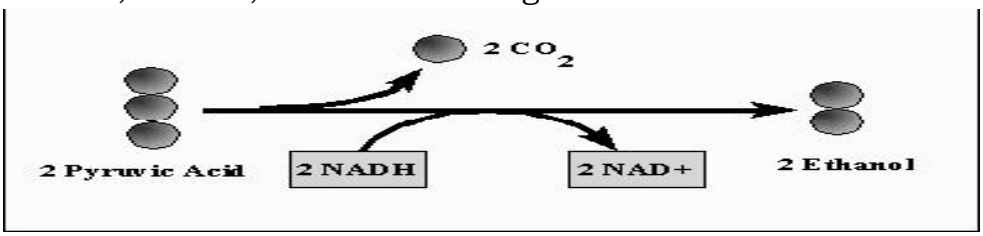
Fermentation: "The anaerobic oxidation of compounds by the enzyme action of microorganisms".

Goal: To reduce pyruvate, thus generating NAD^+

Where: The cytoplasm

Why: In the absence of oxygen, it is the only way to generate NAD^+ and ADP.

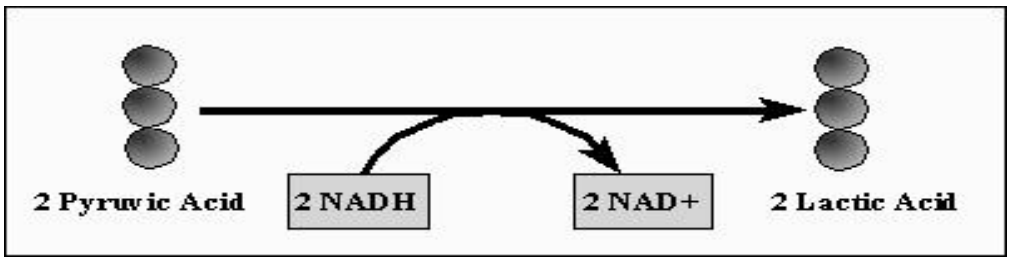
1. Alcoholic Fermentation - occurs in yeasts in many bacteria· The product of fermentation, alcohol, is toxic to the organism



2. Lactic Acid Fermentation

- Occurs in humans and other mammals
- The product of Lactic Acid fermentation, lactic acid, is toxic to mammals
- This is the "burn" felt when undergoing strenuous activity.
- The only goal of fermentation reactions is to convert NADH to NAD+ (to use in glycolysis).

In fermentation - 2 ATP's produced, aerobic respiration - 36 ATP's produced .



Germ Theory of Disease

- The Germ Theory of disease states that the microorganisms (germs) can invade other organisms and caused disease. Even before Pasteur had proved by experiment that bacteria are the cause of some diseases, many observant students had expressed strong arguments for the germ theory of disease.

Koch's Postulates:

1) A specific causal organism (microorganisms) can always be found in association with a given disease.
2) The organism can be isolated and grown in pure culture on artificial media in the laboratory.
3) The pure culture will produce the same disease when inoculated into a susceptible host (animal).
4) It is possible to re-isolate the same organism in pure culture from the experimentally infected host (animal) and compared with original one.

- **Immunity:** refers to the state of being immune to or protected from a disease, especially an infectious disease. This state is invariably induced by having been exposed to the antigen on an organism that invades the body or by having been immunized with a vaccine capable of stimulating production of specific antibodies.
- **Antigen:** The protein portion or any part/substance of pathogen which induce disease/infection.
- **Antibody:** An antibody is a water-soluble protein produced from globulins (e.g., γ-globulin) in response to an antigen (foreign protein). Antibodies attack antigens to neutralize them or make them inactive.
- **Chemotherapy:** A therapeutic concept developed by **Paul Ehrlich** (1854–1915) wherein a specific chemical or drug is used to treat an infectious disease or cancer; ideally, the chemical should destroy the pathogen or the **cancer cells** without harming the host.
- **Antibiotics:** A compound produced by a microorganism or prepared partially by synthetic means that inhibits growth or kills other microorganisms at low concentration.
- **Ignaz Philipp Semmelweis:** He was pioneering in the use of **antiseptics** in obstetrical practice.
- **Joseph Lister:** He gave the concept of "serial dilution" for isolation of pure culture. He also gave the concept of "aseptic surgery"
- **Edward Jenner's successful cowpox vaccine (in 1798):** Jenner's epoch-making successful attempts in vaccinating (inoculating) patients with pox vaccine, that ultimately resulted in the development of resistance to the most dreadful smallpox infection.

- **Elie Metchnikoff:** described for the first time that, leukocytes (*i.e.,* type of white blood cells) were able to engulf/ingest the pathogens present in the body. He named these highly specific defenders against bacterial infections, as phagocytes ('cells eater'), and the phenomenon is termed as **phagocytosis.**
- **Paul Ehrlich (1890):** He hypothesized that a foreign material or toxin entering the body would bind to receptors on cells and stimulates the multiplication of receptors, which were then released into blood, where they neutralized the toxin. Thus he gave the concept of **chemotherapy**.

  He discovered : **Arsphenamine [Salvarsan(R)], a** light yellow organo-metallic compound (powder) containing about **30% Arsenic (As)** and minimum side effects, was formerly used in the treatment of syphilis.
- **Alexander Fleming (1928):** Discovered Penicillin from fungus *Penicillium notatum.*
- **Salman Waksman** described the antibiotic streptomycin production by *Streptomyses graseus (Actinomycetes* - soil filamentous bacteria*).*

Important discoveries of Agril. Microbiology

S. A. Waksman published the book "Principles of soil Microbiology" and discovered the antibiotic "Streptomycin" produced by *Streptomyces griseus*, a soil actinomycets (1944).

Rossi (1929) and Cholondy (1930) developed "Contact Slide / Buried slide" technique for studying soil micro flora.

Van Niel- studied chemoautotrophic bacteria and bacterial photosynthesis. **Bortels** demonstrated the importance of molybdenum in accelerating nitrogen fixation by nodulating legumes.

Kubo proved-the role and importance of "leghaemoglobin" (Red pigment) present in root nodules of legumes in nitrogen fixation.

Ruinen (1956) coined the term **"Phyllosphere"** to denote the region of leaf influenced by microorganisms.

Jensen (1942) developed the method of studying nodulation on agar media in test tubes.

Barbara Mosse and J. W. Gerdemann (1944) reported occurrence of VAM (vesicular-arbuscular Mycorrhiza) fungi (*Glomus, Aculopora genera*) in the roots of agricultural crop plants which helps in the mobilization of phosphate.

Alexander Fleming developed the antibiotic "Penicillin" from the fungus *Penicillium notatum* (1929).

Hardy & Associates developed the technique of measurement of nitrogenase activity by "acetylene-reduction test" coupled with gas chromatography and thereby estimation of biological nitrogen fixation.

Dobereiner and associates (1975, Brazil) studied nitrogen fixing potential of *Azospirillum* in some tropical forage grasses like *Digitaria, Panicum* and some cereals like maize, sorghum, wheat, rye etc. in their roots.

He coined the term "Associative Symbiosis" to denote the association between nitrogen fixing *Azospirillum* and cereal roots. Recently this terminology has been changed and renamed as "Diazotrophic Biocoenocis".

Dommergues & associates had discovered / reported nodules on stem of *Sesbania rostrata* which could fix nitrogen and can be used as an excellent green manure crop in low land rice cultivation.

They also discovered N2 fixing stem nodules on *Casuarina sp.* caused by Frankia, an actinomycete.

Brefeld Introduced the practice of isolating soil fungi by "**Single Cell**" technique and cultivating / growing them on solid media. He used gelatin (first solidifying agent) in culture media as solidifying agent.

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Prokaryotic and Eukaryotic Microbes

Microorganisms can be broadly classified into 3 groups:

1) Bacteria
2) Archeabacteria
3) Eukaryotes

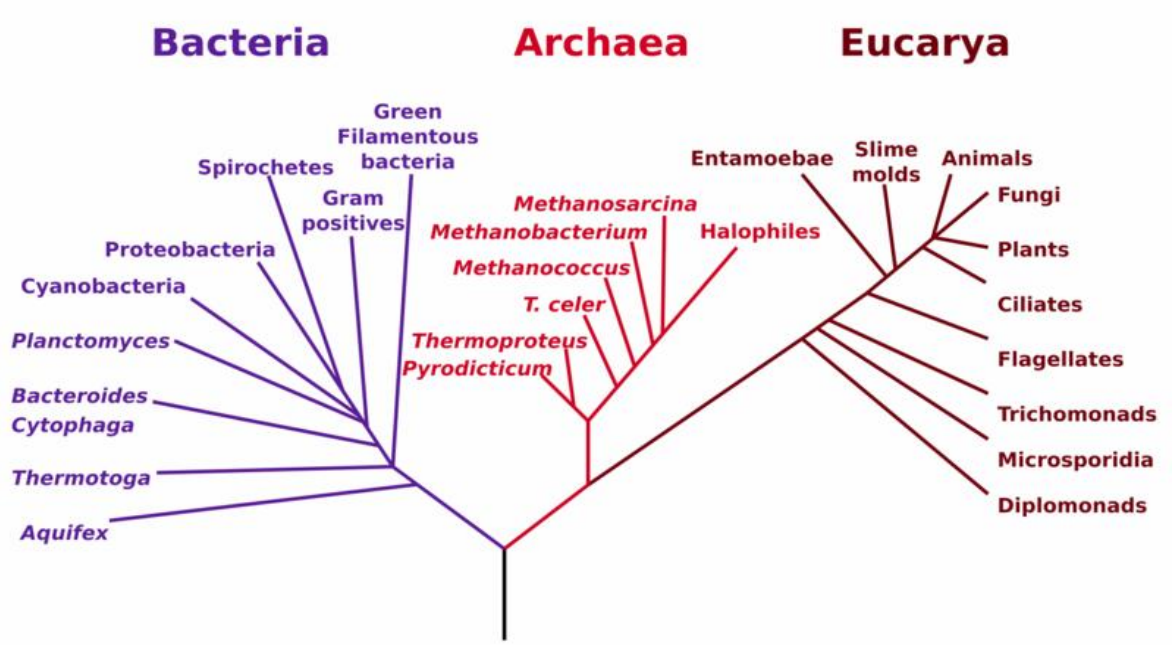

Fig.: Phylogenetic tree of life

- There are two basic types of cells, **eukaryotic and prokaryotic**. Prokaryotic cells are usually single, while eukaryotic cells are usually found in multicellular organisms.
- Prokaryotic cell is a single, common universal ancestor of all life. Prokaryotes were the only life-forms for billions of years. As a consequence of evolution in different environments the prokaryotes have evolved to become more genetically and physically diverse and adapted to different styles of life.
- Prokaryotes consist of two domains – Bacteria and Archaea.
- In contrast to most eukaryotes, prokaryotes reproduce asexually and reproduce their clones.

  While sexual reproduction in eukaryotes results in offspring with genetic material which is a mixture of the parents' genome. During reproduction, eukaryotes generate genetic variation by sexual reproduction whereas genetic variation mechanisms of prokaryotes are not tied to reproduction.

- Prokaryotes are generally smaller than eukaryotes. Prokaryotes have higher growth rates and shorter generation times. Diffusion limitation generally restricts the maximal size of prokaryotic cells. Because of the asexual reproduction and short generation time relative to larger organisms, prokaryotes pass the genome rapidly on to subsequent generations. Therefore, genetically changed genomes are also rapidly transferred. Therefore, prokaryotes swiftly adapt and colonize new niches and a wide range of habitats.
- Prokaryotes are one-celled and often live in clusters or colonies. Prokaryote species live among and interact with other species in communities and consortia. The microenvironment and the interactions hold important selection pressures which affect the evolution of the prokaryotes.

Prokaryotic Microorganisms

Prokaryotes are distinguished from eukaryotes on the basis of nuclear organization, specifically their lack of a nuclear membrane. Prokaryotes also lack most of the intracellular organelles and structures that are characteristic of eukaryotic cells (an important exception is the ribosomes, which are present in both prokaryotic and eukaryotic cells). Most of the functions performed by eukaryote organelles, such as mitochondria, chloroplasts, and the Golgi apparatus, are done by the prokaryotic plasma membrane.

Prokaryotic cells have three architectural regions:

(1) Appendages called flagella and pili—proteins attached to the cell surface;
(2) A cell envelope consisting of a capsule, a cell wall, and a plasma membrane; and
(3) A cytoplasmic region that contains the cell genome (DNA), ribosomes, and various sorts of inclusions.

Other features include:

- The plasma membrane (a phospholipid bilayer) separates the interior of the cell from its environment.
- Most prokaryotes have a **cell wall** (some exceptions are *Mycoplasma,* a bacterium, and *Thermoplasma,* an archaea). It consists of *peptidoglycan* in bacteria, and acts as an additional barrier against exterior forces. It also prevents the cell from "exploding" (cytolysis) from osmotic pressure against a hypotonic environment. A cell wall is also present in some eukaryotes like fungi, but has a different chemical composition.
- A prokaryotic chromosome is usually a circular molecule (an exception is that of the bacterium *Borrelia burgdorferi,* which causes Lyme disease). Even without a real *nucleus*, the DNA is condensed in a nucleoid. Prokaryotes can carry extrachromosomal DNA elements called plasmids, which are usually circular.

Eukaryotic Microorganisms

The eukaryotic cells are about 10 times the diameter of a typical prokaryote and can be as much as 1000 times greater in volume. The major difference between prokaryotes and eukaryotes is that eukaryotic cells contain membrane-bound compartments in which specific metabolic activities take place. Most important among these is the presence of a "well defined nucleus", having a membrane-delineated compartment that houses the eukaryotic cell's DNA. It is this nucleus that gives the eukaryote its name, which means "true nucleus."

- Eukaryotes are hypothesized to be more complex than prokaryotes. Prokaryotes have evolved a multitude of metabolic strategies and are found in a wide range of habitats, including conditions where most other organisms (Eukaryotes) fail to survive.

Other features include:

- The plasma membrane resembles that of prokaryotes in function, with minor differences in the set up. Cell walls may or may not be present.
- The eukaryotic DNA is organized in one or more linear molecules, called chromosomes, which are associated with histone proteins. All chromosomal DNA is stored in the *cell nucleus,* separated from the cytoplasm by a membrane. Some eukaryotic organelles also contain some DNA.
- Eukaryotes can move using cilia or flagella. The flagella are more complex than those of prokaryotes.

Table 1: Comparison of features of prokaroytic and eukaryotic cells

Character	**Prokaryotic cell**		**Eukaryotic cell**
Domain	**Bacteria**	**Archaea**	**Fungi, Algae, Protozoa**
Diversity	Prokaryotes are more diverse than eukaryotes		
Complexity	Prokaryotes are less complex than eukaryotes		
Genetic variation mechanisms	Asexual. Produce clones. May transfer genetic material horizontally.		Sexual recombination
Organisation	Uni-cellular		Uni-cellular or multicellular
Diameter (μM)	The smallest 0.2 ;;The largest >50		Typically between 2-200
Movement	Flagellum		Cytoskeleton
Cell division	Binary fission		Mitotic spindle
Electron acceptor	Oxygen or other compounds		Oxygen
Major structures	Nucleoid , cell wall, cytoplasmic membrane, ribosomes, inclusions		Organelles, nucleus & same basic elements of a prokaryote
Membrane-enclosed organelles	Absent		Present
Peptidoglycan in cell wall	Present	Absent	

Antibiotic sensitivity	Growth inhibited by streptomycin & chloramphenicol	Not inhibited by these antibiotics	
Membrane lipids	Unbranched hydrocarbons	Some branched hybrocarbons	Unbranched hybrocarbons
Species that survive above 65°C	Yes		No
Genetic material	Often only one, circular chromosome and small amounts of extrachromosomal DNA in plasmids		Several, linear chromosomes
Introns (non-coding parts of genes)	Absent	Present in some genes	Present
Typical number of gene copies	Haploid → Genotype reflects phenotype.		Diploid or multiploid → Recessive genes are not expressed.
Site of chromosomes	Cytoplasma		Nucleus
Site of RNA Synthesis	Cytoplasma		Nucleus
Site of protein synthesis	Cytoplasma		
RNA polymerase	One kind	Several kinds	
Initiator amino-acid for start of protein synthesis	Formyl-methionine	Methionine	

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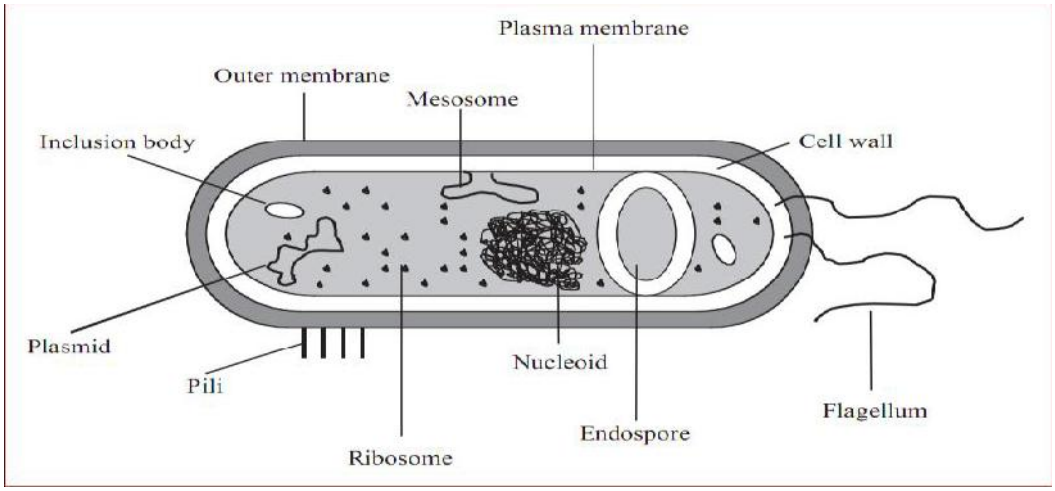
Bacteria: Cell structure

(Sing. Bacterium, Plu. Bacteria)

- Bacteria are prokaryotic, unicellular microorganisms.
- Size: 0.5 -2 um x 5-10 um.
- It can be grown on artificial media in laboratory, reproduce asexually by simple cell division i.e. Binary fission.
- They have rigid cell wall, cells are rod, spherical (cocci), bacilli (cylindrical rods), spiral and vibrios shape and some motile with flagella.
- They can be anaerobic, aerobic and facultative or obligate parasite.
- On the basis of chemical composition of cell wall the bacteria are divided in (1) Gram Positive and (2) Gram Negative.
- **Importance:** Some cause disease, some perform important role in natural cycling of elements which contributes to soil fertility, useful in industry for manufacture of valuable compounds, some spoil food and some make foods.

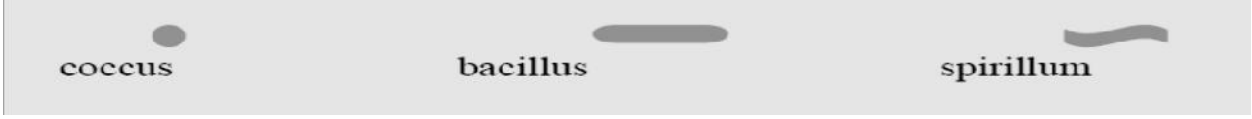


Structure of a typical bacterial cell

- surface area/ volume ratio is exceedingly high favoring unusually high rate of growth and metabolism of bacteria.
- No circulatory mechanism is needed to distribute the nutrients that are taken in, due to this high surface to volume ratio.

Shape & Arrangement of Bacteria:

- The shape of bacterial cell is governed by rigid cell wall. They may be spherical (Coccus – Cocci), straight rods (Bacillus – Bacilli), or rods that are helically curved (Spirillum – Spirilli) or they may be pleomorphic (exhibit a variety of shapes).



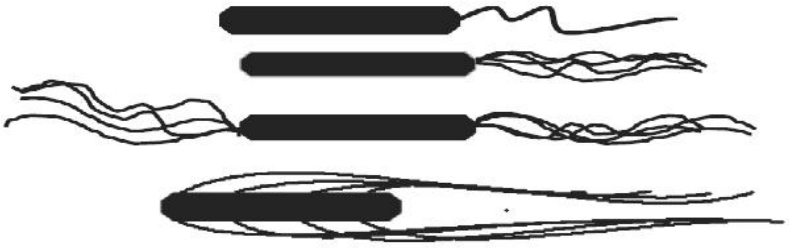
❖ **The Cocci are further grouped on the basis of arrangement of cells:**

- Diplococci, Streptococci, Tetracocci, and Staphylococci.
- Bacilli are mostly singular or in pairs (Diplococci).
- Some species may be Streptobacilli (Ex: *Bacillus subtilis)* or trichomes (Ex: *Beggiatoa)* or may have palisade arrangement *(Corynebacterium diphtheria).*
- Some other bacilli may form long, branched multinucleated filaments called hyphae, which collectively form mycelium *(*Ex: *Streptomyces)*

Structures external to cell wall

Flagella (flagellum) and motility: Bacterial flagella are hair like helical appendages that protrude through the cell wall and are responsible for swimming motility. It grows at the tip unlike hair, which grows at the bottom. The hook and filament are made up of protein known as flagellin. Flagellar arrangement may be

a). Monotrichous – a single polar flagellum
b). Lophotrichous – a cluster of polar flagella
c). Amphitrichous – flagella either single or clusters, at both cell poles and
d). Peritrichous –surrounded by lateral flagella.



Pili (Fimbriae):

They are hollow non-helical filamentous appendages that are thinner, shorter and more numerous than flagella. Do not function in motility. Different types of pili have different functions. F – pilus (Sex pilus) serves as the port of entry of genetic material during bacterial mating. Some pili play major role in human infection.

Capsule: Many bacteria synthesize organic exopolymers that form an envelope outside the cell wall. If this layer can be seen by light microscopy using special staining methods it is called a capsule. The material is called **"Slime**" if the layer is abundant and many cells are embedded in a common matrix. Most pathogenic bacteria produce either capsule or slime. The functions of the capsule are: (1) they may block attachment of bacteriophages (2) they may be antiphagocytic (3) they may provide protection against temporary drying by binding water molecules (4) they may promote attachment of bacteria to surfaces.

Sheaths: Sheath is a hollow tube formed in some species of bacteria to enclose chains or trichomes of bacterial cells. Sheath is commonly found in the species from fresh water and, marine environments.

Cell wall: In bacteria the cell wall is very rigid and gives the shape to the cell. Most of the bacteria retain their original cells even after subjected to very high pressure or severe physical conditions. It accounts for 10-40% of dry weight of the cell. Cell walls can be broken by sonic or ultrasonic treatment or by subjecting the cells to extremely high pressure and subsequent sudden release of pressure.

Structures internal to cell wall

- **Cytoplasmic membrane**: This is about 7.5 ηm thick and is immediately beneath the cell wall. This is primarily composed of phospholipids (20-30%) and proteins (60-70%). This membrane contains various enzymes involved in respiratory metabolism and in the synthesis of capsular and cell wall components. It is the site of generation of proton motive force, which drives ATP synthesis, certain nutrient transport systems and flagellar motility. Damage to this membrane may result in the death of the cell.
- **Protoplast and Sphaeroplast**: A protoplast is that portion of a bacterial cell consisting of the cytoplasmic membrane and the cell material bound by it.
- **Sphaeroplast** is a protoplast surrounded by the outer membrane of cell wall. In gram-negative bacteria only peptidoglycan layer can be removed but outer membrane is still intact surrounding the protoplast.
- **Mesosomes:** In many bacteria, especially Gram-positive bacteria, the cytoplasmic membrane appears to be infolded at more than one point. Such infoldings are called mesosomes. Mesosomes are thought to be involved in DNA replication, cell division and export of exocellular enzymes.
- **Cytoplasm**: The major cytoplasmic contents of bacterial cell include the nucleus, (without a membrane), ribosomes, proteins and other water soluble components and reserve materials. In most bacteria extra chromosomal DNA (Plasmid DNA) is also present.
- **Bacterial Chromosome**: The bacterial nucleus is not enclosed in a defined membranous structure. The nuclear material is generally confined to the center of the cell. It consists of single circular double stranded DNA molecule in which all the genes are linked. This nuclear material is generally designated as nucleoid.
- **Ribosomes:** Ribosomes are 70 S type consisting of 50 S and 30 S sub-units. Some ribosomes are free in the cytoplasm and some are attached to inner surface of the cytoplasmic membrane.
- **Volutin granules** (reserve source of phosphate), poly-β-hydroxybutyrate (PHB) and glycogen (both serving as source of carbon and energy) are some of the granules present in the cytoplasm of some bacteria. Gas vesicles are present in bacteria that grow in aquatic habitat.
- **Spores:** Spore is a metabolically dormant form, which under appropriate conditions can undergo germination and grow out to form a vegetative cell. Spores produced within the cell are called endospores and the spores produced external to cell are called exospores.
- **Endospores** are thick walled, highly refractile bodies that are produced (one per cell) *by Bacillus, Clostridium, Sporosarcina* and few other genera. They are generally formed at the end of the active growth or during stationary phase.

They are extremely resistant to desiccation, staining, disinfecting chemicals, radiation and heat.

- **Exospores** are formed external to the vegetative cell by budding at one end of the cell in the methane oxidizing genus *Methylosinus*. They are desiccation and heat resistant.
- **Conidiospores and Sporangiospores**: The bacteria, actinomycetes form branching hyphae. From the tips of these hyphae spores develop singly or in chains. If the spores are contained in an enclosing sac (sporangium), they are termed sporangiospores, if not they are called conidiospores. The spores can survive long periods of drying but they do not have high heat resistance.
- **Cysts:** Cysts are thick walled, desiccation resistant, dormant forms that develop by differentiation of vegetative cells. *Azotobacter* and some other genera produce cysts.

Differences in the cell wall of Gram positive and Gram negative eubacteria

CHARACTER	GRAM POSITIVE	GRAM NEGATIVE
1. Thickness	Thicker wall (20 – 25 ηm)	Thinner (10-15 ηm)
2. Layers	A single thick. layer	Two layers (a Peptidoglycan layer and outer membrane).
3. Peptidoglycan	Account for 50% dry weight of cell wall	Only about 10% of cell wall.
4. Other constituents	Polysaccharides and Techoic acids	Outer membrane is rich in phospholipids, proteins or lip polysaccharides. Peptidoglycan layer is linked to outer – membrane by Braun's lipoprotein.
5. Susceptibility to		
(a) Penicillin	More susceptible	Less susceptible
(b) Mechanical disintegration	Less susceptible	More susceptible

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CHEMOAUTOTROPHY

- Chemoautotrophs can grow in a mineral medium, taking carbon from C02 and energy from the oxidation of inorganic compounds. Some of these bacteria are capable of growing both Chemoorganotrophically and chemoautotrophically i.e. they are facultative autotrophs. Example of these types is *Alcaligenes eutrophus*. Other chemoautotrophic bacteria are obligate in nature e.g. *Thiobacillus, Nitrosomonas*.
- Reaction which yield energy in chemoautotrophs are the oxidation of H2, NH4+, NO_3^-, S and reduced-S compounds and Fe++. All these oxidations, except H_2 oxidation, couple electron transport to the cytochrome system and NAD+ reduction occurs by energy dependent reverse electron flow.
- The assimilation of CO2 in these organisms occurs through the reaction of the calvin cycle. When grown chemoautotrophically, cells contain high levels of the 2 enzymes of this pathway namely carboxy dismutase, phosphoribulokinase.
- Depending on the oxidisable organic substrate, the chemoautotrophic bacteria is classified as: Nitrifying bacteria, sulfur oxidizing bacteria, H2 oxidizing bacteria, Iron oxidizing bacteria and carbon monoxide bacteria.

Importance of chemoautotrophs:

- Chemoautotrophs play an important role in oxidation of reduced N and S compounds like NH_3 and H_2S to NO_3 and SO_4 respectively. Nitrates sulphates are the utilized forms of nutrients for higher plants.

PHOTOAUTOTROPHY

- Photoautotrophy refers to an autotrophic mode of metabolism in which organisms utilize light energy with the help of photosynthetic pigments and convert it to chemical energy in the form of ATP (photophosphorylation).
- These organisms synthesize organic compounds from CO_2, generally through Calvin - Benson cycle. The 2 processes i.e generation of ATP by photophosphorylation and CO_2 fixation together constitutes photosynthesis.
- Cyanobacteria/BGA do photosynthesis & release O_2 as by product. This type of photosynthesis is known as oxygenic. However, other bacteria that contain bacterio-chlorophyll and do photosynthesis but without O_2 evolution (Anoxygenic).

Photosynthesis includes two processes:

1. First process includes the reactions by which light energy is absorbed by the photosynthetic pigments and transformed into chemical bond energy. These reactions are photochemical in nature and are known as light reactions.
2. The second process include enzyme – catalyzed biochemical reactions involving CO2 fixation in which light has no direct role. These reactions are called dark reactions. 3. The products of light reactions are ATP and NADH2 or NADPH2. These products are used in the dark reaction for synthesis of sugar or other organic compounds from CO_2.

Classification of bacteria based on carbon, energy and electron sources

Carbon Sources	
Autotrophs	CO_2 sole or principal biosynthetic carbon source (section 10.3)
Heterotrophs	Reduced, preformed, organic molecules from other organisms (*chapters 9 and 10*)
Energy Sources	
Phototrophs	Light (section 9.12)
Chemotrophs	Oxidation of organic or inorganic compounds (*chapter 9*)
Electron Sources	
Lithotrophs	Reduced inorganic molecules (section 9.11)
Organotrophs	Organic molecules (chapter 9)

Bacterial growth

Growth -Growth is the orderly increase in all of the components of an organism.

In the laboratory, under favorable conditions, a growing bacterial population doubles at regular intervals. Growth of bacteria occurs by geometric progression: 1, 2, 4, 8, etc. or 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations). This is called **exponential growth** which is observed during **Log Phase**. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in Nature.

When a fresh medium is inoculated with a given number of cells, and the population growth is monitored over a period of time, plotting the data will yield a typical bacterial growth curve.

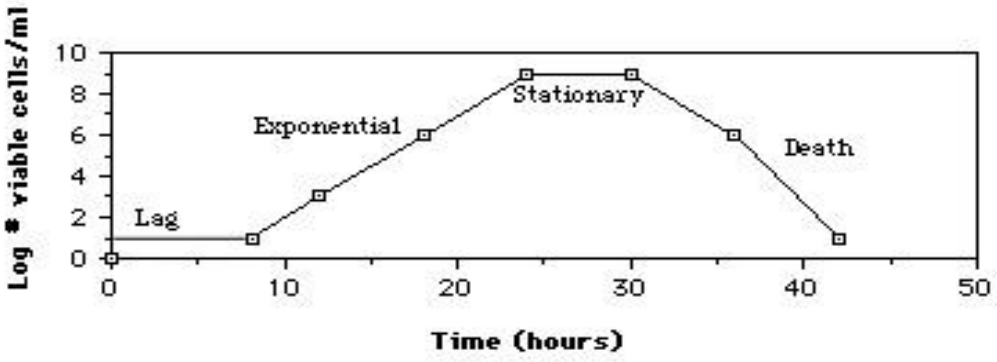

Fig. The typical bacterial growth curve.

When bacteria are grown in a closed system (also called a batch culture), like a test tube, the population of cells almost always exhibits these growth dynamics

1. **Lag Phase:** Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity.

The length of the lag phase is apparently dependent on: size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

2. **Exponential (log) Phase:** The exponential phase of growth is a pattern of balanced growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the

composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as **generation time**, also the **doubling time** of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time is done.

3. **Stationary Phase:** Exponential growth cannot be continued forever in a **batch culture** (e.g. a closed system such as a test tube or flask). Population growth is limited by one of three factors: 1. exhaustion of available nutrients; 2. accumulation of inhibitory metabolites or end products; 3. exhaustion of space, in this case called a lack of "biological space". In this stage some cells are dying while some other may be dividing. Bacteria that produce **secondary metabolites**, such as antibiotics, do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth). It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process.

4. **Death Phase:** If incubation continues after the population reaches stationary phase, a death phase follows, in which the viable cell population declines. (Note, if counting by turbidimetric measurements or microscopic counts, the death phase cannot be observed.). During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

GROWTH RATE- Growth rate is the change in cell number or mass per unit time. It is expressed as 'R' which is the reciprocal of generation time 'g'. It can be defined as the slope of the line when log of cells versus time is plotted (R = 1/g). Microbes generally respond linearly to a limiting nutrient concentration in the medium, which forms the principle for microbiological assays.

- **Generation Time- generation time** is the time interval required for the cells (or population) to divide.

G (generation time) = (time, in minutes or hours)/n(number of generations)

G = t/n

Factors affecting microbial growth

Sr. No	Factor	Type of organisms	Characteristics
1	Temperature	Psychrophile	"Cold-loving". Can grow at 0°C
		Mesophile	Best growth between 25 to 40°C. Optimum temperature commonly 37°C
		Thermophiles	Optimum growth between 50 to 60°C Many cannot grow below 45°C.
		Extreme Thermophiles (Hyperthermophiles)	Optimum growth at 80°C or higher
2	pH	Acidophiles	Grow at very low pH (0.1 to 5.4)
		Neutrophiles	Grow at pH 5.4 to 8.5
		Alkaliphiles	Grow at alkaline or high pH (7 to 12 or higher)
3	Osmotic pressure	Halophiles	Require 3.5 % salt concentration
		Extreme or Obligate Halophiles	Require 20 to 30% salt concentration
4	Oxygen	Aerobes	Require oxygen to live
		Anaerobes	Do not require oxygen and may even be killed by exposure
		Facultative aerobe	can live with or without oxygen
		Aero tolerant anaerobes	can tolerate oxygen and grow in its presence even though they cannot use it
		Microaerophile	can use oxygen only when it is present at levels reduced from that in air

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BACTERIAL GENETICS

- Genetics is the study of the inheritance (heredity) and the variability of the characteristics of an organism.
- **Genotype:** The genetic makeup of an individual. The information that codes for that organism's genetic characteristics. Collection of an individual's genes.
- **Phenotype:** Expressed properties of an individual. An individual's phenotype is a function of the genotype (and environment). Collection of an individual's proteins or gene products.

Gene expression

- The expression of gene involves three well recognized processes namely, **replication, transcription and translation**.
- DNA codes for RNA and proteins products. The flow of genetic information is DNA to RNA (via trancription) and RNA to protein (via translation).
- DNA only has 4 different nucleotides; while proteins have 20 different amino acids.

Central Dogma:  DNA ↔ RNA → Protein

DNA Replication: In molecular biology the production of a strand of DNA from the original strand is known as replication.

- Double stranded DNA on unwinding will be converted into two single strands.
- Each strand serves as a template for the synthesis of a new complementary chain, thus forming two new helices.

RNA Synthesis (Transcription)

- There are three types of RNA in bacterial cells:

1) mRNA: Messenger RNA, carries information for protein synthesis;

2) rRNA: Ribosomal RNA, forms part of ribosome and

3) tRNA: Transfer RNA, carries amino acids to growing protein during translation.

- The process in which a complementary single stranded mRNA is synthesized from one of the DNA strands is called **transcription**.
- The synthesis of polynucleotide chain of **mRNA** is catalyzed by the enzyme **RNA polymerase** and the activated ribonucleotides are the substrates for this enzyme.

Protein Synthesis (Translation)

- Translation is the next step in gene expression. It is the process in which the genetic information now present in the mRNA molecule directs protein synthesis.

- mRNA is read in codons or nucleotide triplets. The four bases, the number of sequences of triplets is 4^3 or 64 (4^n).
- These base triplets, each of which specifies a particular amino acid, constitute the genetic code.
- There are 64 possible codons for 20 amino acids.
- Here, AUG act as start codon, which codes for amino acid Methionine. While; UAA, UGA and UAG act as stop codons.
- Translation occurs on the ribosome, which is made up of two subunits, large (50s) and small (30s).
- tRNA molecules have an anticodon, which recognizes codons. They carry specific amino acids to the growing protein chain.

GENETIC RECOMBINATION

- Genetic Recombination is a process by which genetic elements from two separate sources are brought together in a single unit.
- Three kinds of genetic recombination in bacteria:

(1) **Transformation** is transfer of cell-free or naked DNA from one cell to another.

(2) **Conjugation** is transfer of genes between cells that are in physical contact with one another.

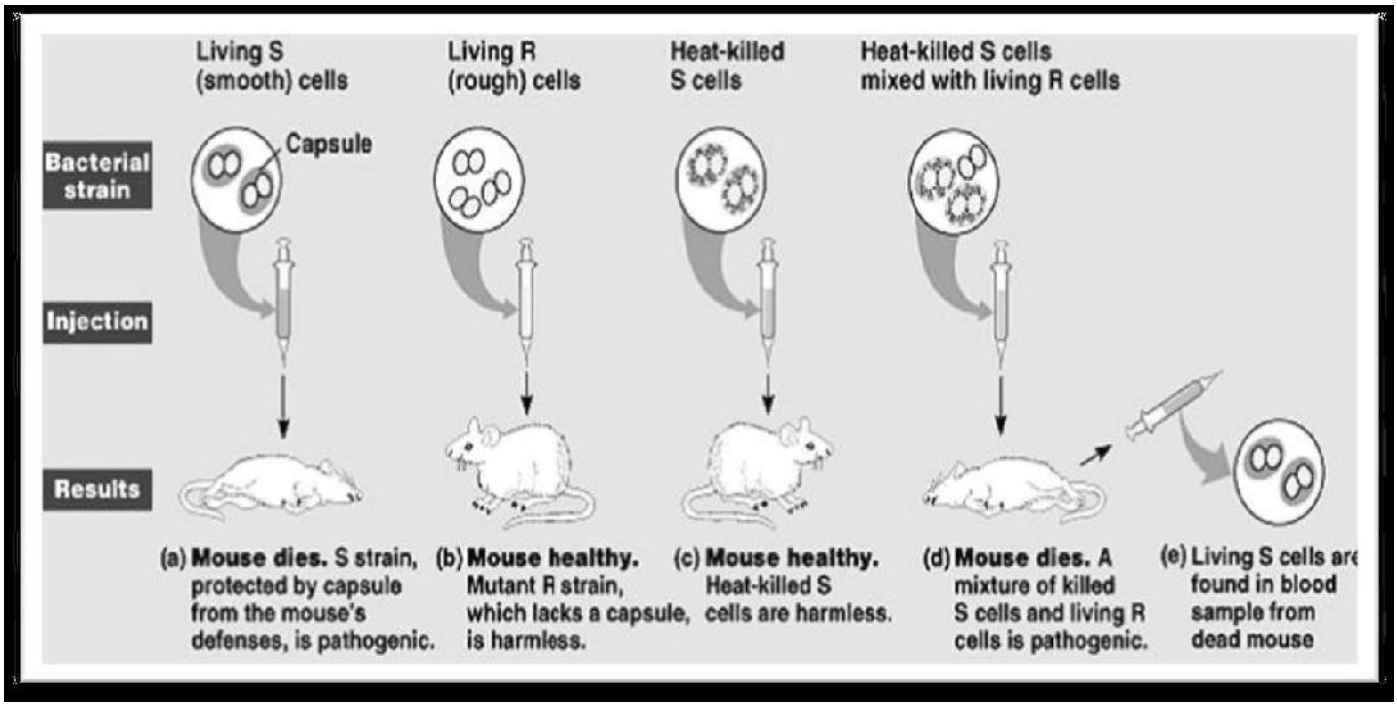
(3) **Transduction** is transfer of genes from one cell to another by a bacteriophage.

- In bacterial recombination, the cells do not fuse and usually only a portion of the chromosome from the donor cell (male) is transferred to the recipient cell (female).
- The recipient cell thus becomes a merozygote, a zygote that is partially diploid. Once merozygote transformation has occurred, recombination can take place.

Transformation:

- It is a process in which free DNA is taken up by a cell, resulting in a genotypic change in the recipient.
- It was the first mechanisms of bacterial genetic exchange to be discovered.
- In **1928, Frederick Griffith** made a series of experiments with laboratory mice and two strains of pneumonia causing bacterium, ***Diplococcus pneumoniae***.
- *This bacterium has two types of* strains. One type has **Smooth (S) capsulated cell, whereas another type has Rough (R)** non-capsulated cells.
- The disease is caused by smooth type of cells only, i.e. smooth-type cells are pathogenic (virulent), whereas rough type of cells are harmless on non-pathogenic (avirulent).

- Griffith found that injection of mice with an avirulent strain of ***Streptococcus pneumoniae*** *together with heat-killed cells of a virulent strain separately caused no disease.*
- On autopsy (examination of tissue of dead mice), these mice were found to contain live virulent cells of *S. pneumoniae.*
- However, when live, harmless (rough type) cells were injected in the body of mice, the animal remained healthy.
- The injection of dead, pathogenic (smooth-type) cells into the body of mice also did not cause any disease.
- These and subsequent experiments established that surviving cells were recombinant; they exhibited certain properties (including virulence) that were typical of the killed cells and others that were typical of the avirulent culture.
- Thus, a genetic exchange had occurred between the dead cells and the live ones.
- Genes are transferred from one bacterial cell to another in the form of naked DNA.
- Initial work done in 1928 by Frederick Griffith on two strains of *Streptococcus pneumoniae*.
- **Smooth strain:** Caused disease due to capsule.
- **Rough strain:** Did not cause disease.
- Experiments with heat killed smooth bacteria and live rough bacteria, demonstrated the presence of a transforming factor.
- The **"Transforming Principle"** was purified and indentified as DNA by **O.T. Avery, H. Macleoid and M. Mc Carty in 1944.**
- They defined DNA as the chemical substance responsible for heredity.
- Indeed, these experiments were the first to establish in any biological system that DNA is the macromolecule in which genetic information is encoded.
- The word Transformation then came to be used to describe genetic exchange among Prokaryotes that was mediated by DNA.
- In 1944, Avery and others demonstrated that transforming material was indeed DNA.
- This was important in establishing that genetic material was DNA.
- Thus, Transformation is the process whereby cell-free or naked DNA containing a limited amount of genetic information is transferred from one bacterial cell to another.

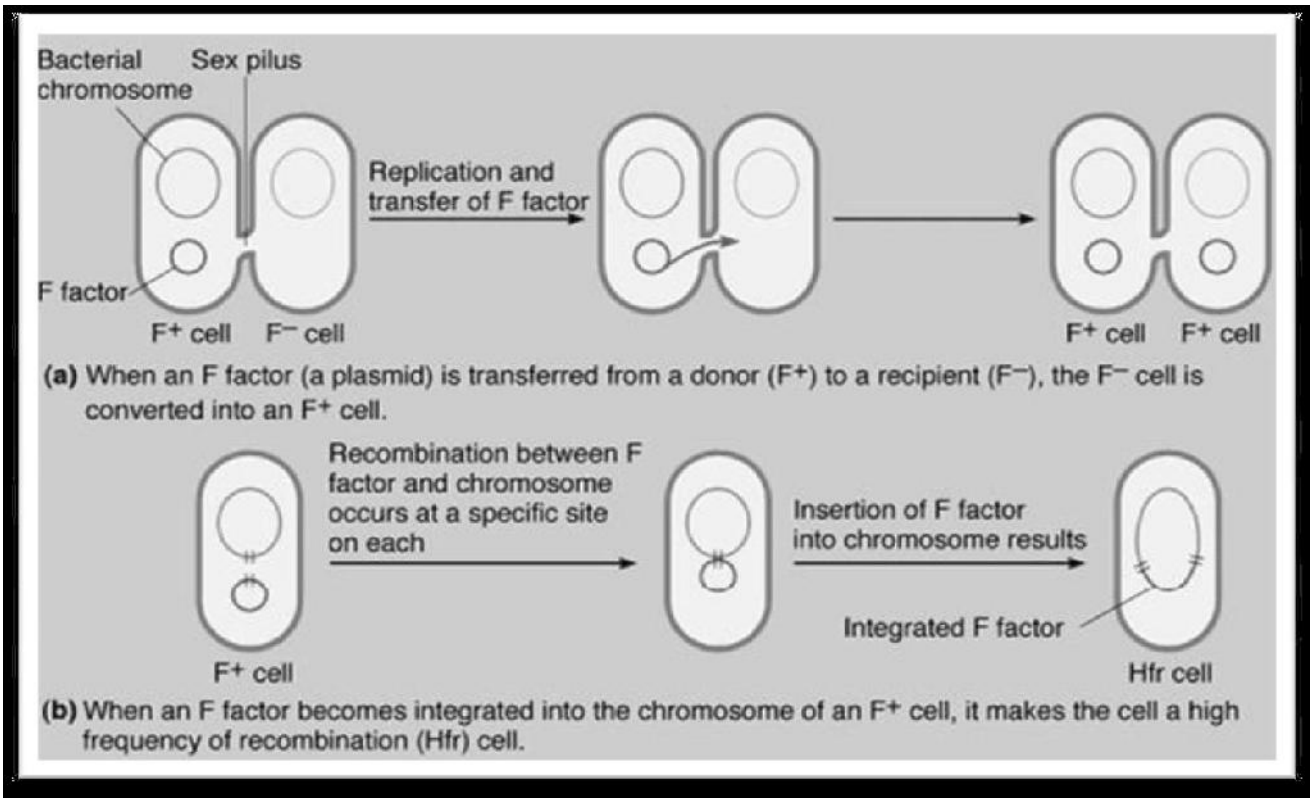


Conjugation

- Genetic recombination in which there is a transfer of DNA from a living **donor bacterium to a** recipient bacterium.
- Often involves a sex pilus.
- Conjugation in bacteria is a mechanism for gene transfer that requires cell-to-cell contact.
- In 1946, **J. Lederberg and E.L.Tatum** discovered a genetic exchange occurring between certain strains of *E.coli* that eventually proved to be different from transformation in respect of :

❖ exchange of genetic material in conjugation is dependent on direct contact between cells. However, in transformation, it occurred even if DNA was present in the medium.

❖ It is polarized i.e. certain strains designated as F+ (Fertility Plus) always acted as Donors (Males) and others designated as F- (Fertility minus) always acted as Recipients (Females).



- In Gram-negative bacteria, a sex pilus produced by the donor bacterium binds to the recipient. The sex pilus then retracts, bringing the two bacteria in contact.
- In Gram-positive bacteria, sticky surface molecules are produced which bring the two bacteria into contact.
- DNA is then transferred from the donor to the recipient.
- The process was termed conjugation.
- F+ strains contain a plasmid (termed the F plasmid or F factor) that carries all the genes that encode conjugative genetic transfer.
- Indeed, the F plasmid is not known to encode any additional function other than this one and its own replication.
- F- factors are double standard loops of DNA in the cytoplasm of F+ cells apart from the bacterial chromosome.
- When F+ and F- cells are mixed together, the F+ cells attach to the F- cells by means of sex pili. (F- factors contain genes coding for the synthesis of sex pili).
- During conjugation, the F factor is duplicated and the new copy is transferred across an intercellular bridge from the F+ to the F- cell.
- This converts the F- cell into an F+ cell.
- Once an F+, it is capable of transferring its F+ factor to another F- cell.
- F factor is a plasmid, separate from the bacterial chromosome. When an F factor is transferred, the bacterial chromosome of the F+ cell is not passed to the F- cell. Thus, in this event no recombinants are produced.

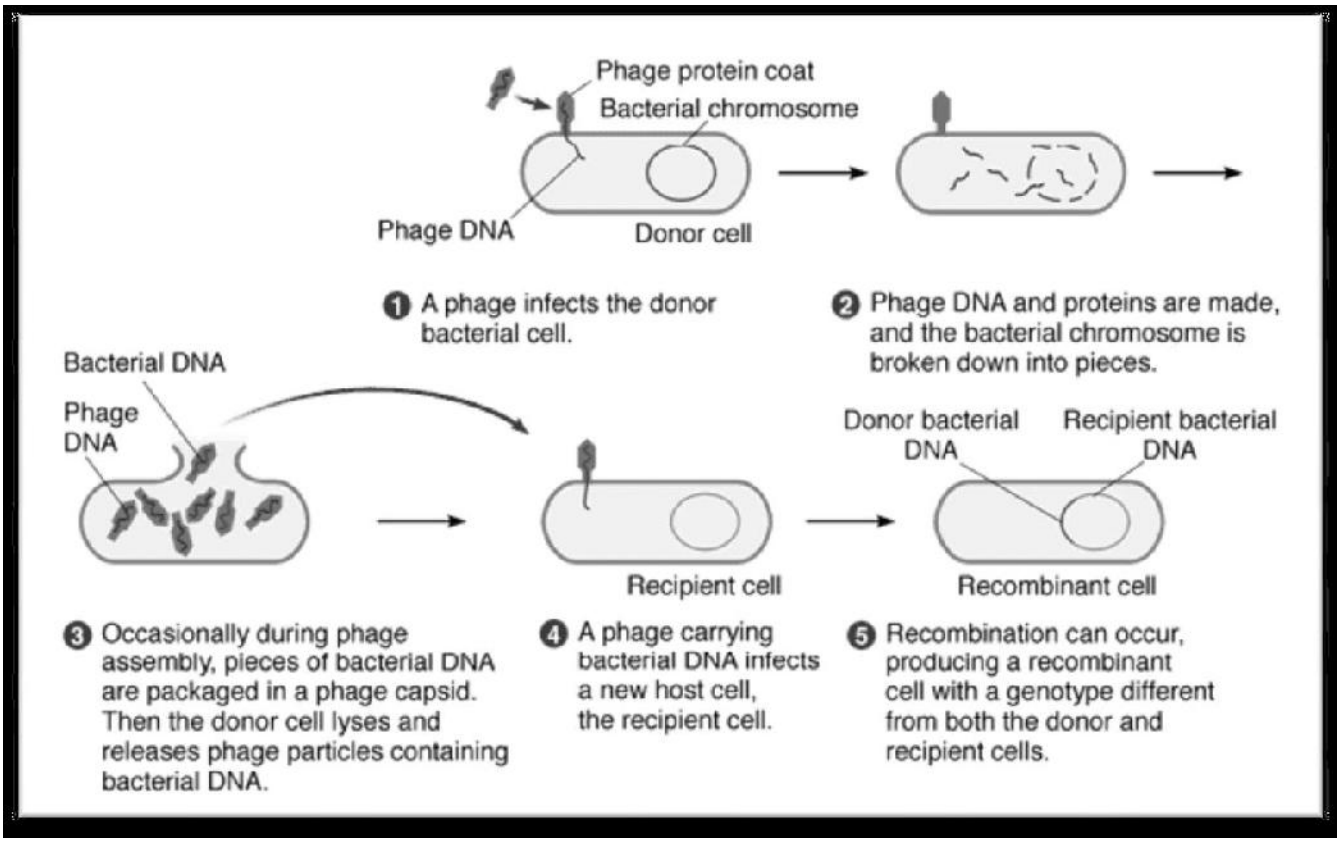
- Some male cells have the F factor integrated into their chromosome; they are called Hfr (High Frequency of Recombination) cells.
- During conjugation between an Hfr and F- cell, the chromosome of the Hfr cell replicates and the new copy of the chromosome is transferred to the recipient cell.
- Only Hfr cells can transfer their chromosomes.
- Replication of the Hfr chromosome being within the F factor and a small piece of the F factor leads the chromosomal genes into the F- cells.
- Most of the integrated F factor enters the recipient cell in the last, if at all.

Usually, the chromosome breaks before it is completely transferred.

Transduction

- Transduction is the transfer of fragments of DNA from one bacterium to another bacterium by a bacteriophage. **OR**
- This is a process in which bacterial DNA is transferred from one cell to another with the help of a virus.
- The process was discovered in **1952 by N. Zinder and J. Lederberg,** whose experiments were designed to demonstrate that transfer of genetic material between the strains of *Salmonella* (does not require cell-to-cell contact).
- Transduction may be **generalized or specialized.**
- Many genes for toxins are transferred by specialized transduction:
- *E. coli O157:H7: Shiga-like toxin.*
- *Corynebacterium diphtheriae: Diphtheria toxin.*
- *Streptococcus pyogenes: Erythrogenic toxin.*

- Recombination by this method requires a bacteriophage as a carrying agent (vector) to carry genes from one bacterium to another.
- In this case, the DNA is not passed naked, as in transformed or during cell-to-cell contact, as in conjugation.
- Rather, it is passed inside a bacterial virus (bacteriophage) from the donor to the recipient.
- Two types of transducing particles, and therefore, two types transduction exist.
- One of these is termed as **Generalized or Non-specialized Transduction,** because it mediates the exchange of any bacterial gene.
- The other is termed **Restricted or Specialized Transduction because** it mediates the exchange of only a limited number of specific genes.


Phage protein coat
Bacterial chromosome
Phage DNA
Donor cell
1 A phage infects the donor bacterial cell.
2 Phage DNA and proteins are made, and the bacterial chromosome is broken down into pieces.
Bacterial DNA
Phage DNA
Donor bacterial DNA
Recipient bacterial DNA
Recipient cell
Recombinant cell
3 Occasionally during phage assembly, pieces of bacterial DNA are packaged in a phage capsid. Then the donor cell lyses and releases phage particles containing bacterial DNA.
4 A phage carrying bacterial DNA infects a new host cell, the recipient cell.
5 Recombination can occur, producing a recombinant cell with a genotype different from both the donor and recipient cells.

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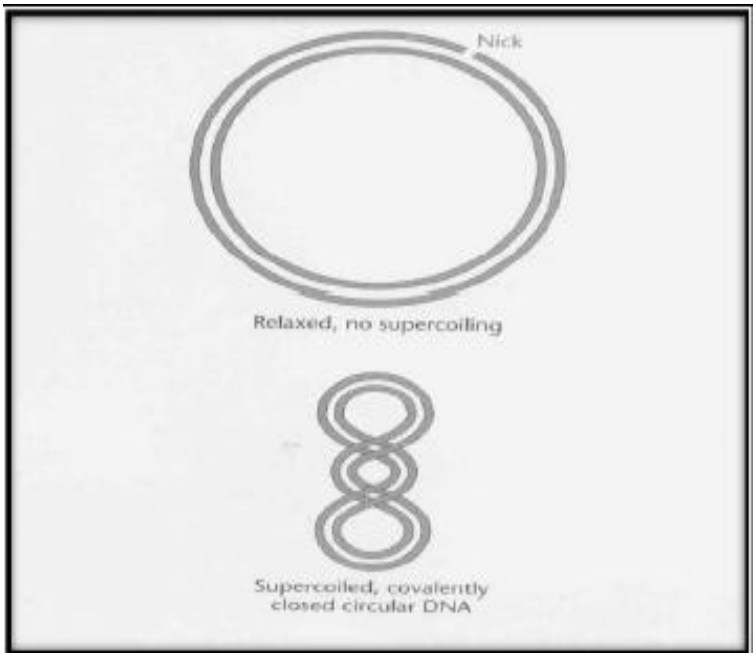
PLASMIDS

- It is an ―Extra-chromosomal piece of DNA that can replicate independently within a bacterial cell‖.
- Lederberg (1952) first to use the term Plasmids for extra-cellular and autonomous reproducing genetic structure.
- Morphologically they are circular, double stranded molecules of DNA that exist independently of chromosomal DNA in bacterial cell.
- They are capable of undergoing independent replication via **rolling circle model or theta model** of DNA replication.
- Plasmids are circular double stranded DNA molecules that appear as super-helical coil in morphology.
- Each super-helical turn usually consist of 400-600 base pairs.
- **pBR 322, pUC, Yest, Yeast episomal plasmids (YEps), Yeast integrating plasmids (YIps), Ti plasmids** are widely used as vector in genetic engineering.



Types of Plasmids

There are two types of plasmids according to their function.

1. Conjugative plasmids: It carries genes that promote the transfer of plasmids from host cell to recipient cell by conjugation.

- Fertility-F-plasmids which contain *tra-genes*. They are capable of conjugation.
- Ti plasmid of *Agrobacterium.*

2. Non- Conjugative plasmids: It carries genes that cannot promote the transfer of plasmids from host cell to recipient cell by conjugation.

- Resistance-(R) plasmids, which contain genes that can build a resistance against antibiotics or toxins.
- Col-plasmids, which contain genes that *code for colicines proteins that can kill* other bacteria.
- Degradative plasmids, which enable the digestion of unusual substances, e.g., toluene or salicylic acid.
- Virulence plasmids, which turn the bacterium into a pathogen.

Important Characteristics of naturally occurring plasmids

- They can replicate independently of the main chromosome.
- They are species specific to one or few species of bacteria.
- They can undergo reversible integration into bacterial chromosome.
- A few plasmid can pickup and transfer chromosomal gene.
- They usually contain up to 40 genes.
- They do not occur free in nature.

Episoms

- The plasmids that remains attached to the bacterial chromosome and can integrate into or out of the main chromosome, designated as ―episomes.
- Often it is not possible to differentiate between plasmids and episomes and the two terms are mostly synonymously used.

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Transposons

- A transposable element (transposon) is a DNA sequence that can change its position within a genome, sometimes creating or reversing mutations and altering the cell's genetic identity and genome size. Transposition often results in duplication of the transposable element (TE). Barbara McClintock's discovery of these jumping genes earned her a Nobel Prize in 1983.
- Transposons in bacteria usually carry an additional gene for functions other than transposition, often for antibiotic resistance. In bacteria, transposons can jump from chromosomal DNA to plasmid DNA and back, allowing for the transfer and permanent addition of genes such as those encoding antibiotic resistance (multi-antibiotic resistant bacterial strains can be generated in this way). Bacterial transposons of this type belong to the Tn family. When the transposable elements lack additional genes, they are known as insertion sequences.

General characteristics of TE

- They were found to be DNA sequences that code for enzymes, which bring about the insertion of an identical copy of themselves into a new DNA site.
- Transposition events involve both recombination and replication processes which frequently generate two daughter copies of the original transposable elements.
- One copy remains at the parent site and another appears at the target site.
- A transposable element is not a replicon. Thus, It cannot replicate apart from the host chromosome.

Transposable elements in Bacteria

There are three main types: (1) insertion sequences or IS elements, (2) composite transposons, and (3) Tn3 elements.

(1) IS Elements: IS elements are compactly organised. Typically, they consist of fewer than 2500 nucleotide pairs and contain only genes whose product is involved in promoting or regulating transposition.

(2) Composite Transposons: Composite transposons, which are bacterial cut-and-paste transposons denoted by the symbol Tn, are created when two IS elements insert near each other.

(3) Tn3 Elements: The elements in this group of transposons are larger than the IS elements and usually contain genes that are not necessary for transposition.

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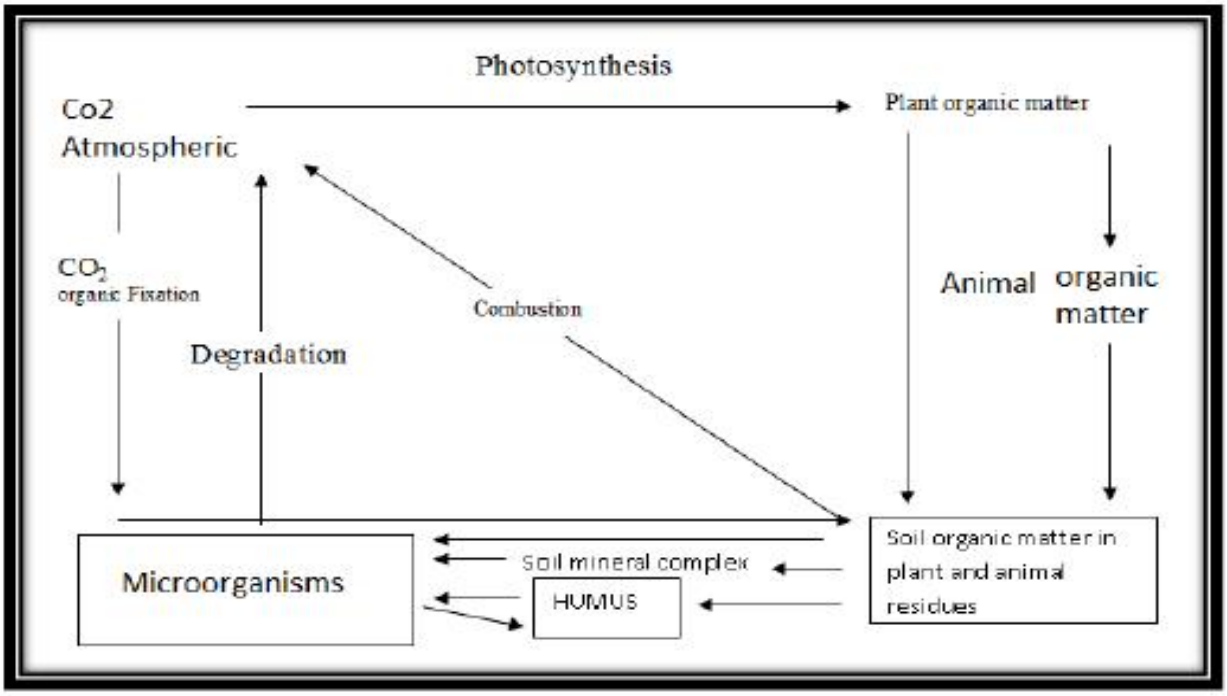
Role of microbes in soil fertility and crop production: Carbon, Nitrogen, Phosphorus and sulphur cycles.

1. CARBON CYCLE



- Carbon is the basic constituent of all organic compounds.
- The ultimate source of organic carbon compound in nature is the carbon dioxide present in the atmosphere and in dissolved state in the water on the earth.
- Green plants, certain bacteria and algae use CO_2 through photosynthesis in the presence of sunlight, to form carbohydrates, simple fats and polysaccharides in plants which are utilized and digested by animals.
- Flesh eating animals (carnivores) feed on herbivores and the carbon compounds are again digested and converted into the other forms.
- Once the atmospheric carbon is fixed by photoautotrophs, it becomes non-available for the generation of new plant life.
- Therefore, it is essential for the carbonaceous materials to be decomposed and returned the atmosphere, otherwise the stock of carbon in nature would be exhausted.
- The return of carbon is brought about through the decomposition organic carbon in nature.
- Some carbon is also released to the atmosphere as CO_2 in respiration and combustion of both plants and animals.
- The soil microorganisms play an important role in completing the cycle.

- They convert organic matter into body substances, liberate CO_2 and water, increase and concentrate the nitrogen content, and bring down the ratio between carbon and nitrogen in the soil. This improves soil fertility.
- The released CO_2 goes back to complete the cycle and this ensures that there is no major lock-up in organic tissues on earth.

Degradation of organic carbon compounds

- Organic carbon compounds added to the soil are degraded by microorganisms and the CO_2 generated in the process is released into the atmosphere.
- The process involves the metabolic activities of many kinds of microorganisms such as fungi and bacteria.
- These organisms consume organic materials for the synthesis of their cell constituents and release CO_2 through respiration.
- The most abundant organic material in plants is cellulose. It is readily attacked by many species of bacteria and fungi.
- The initial enzymatic attack is by cellulose which splits cellulose to cellobiose. In turn, the Cellobiose is split to glucose and is metabolized by many microorganisms, complete oxidation yields CO_2 and H_2O.
- The process can be summarized as follows:

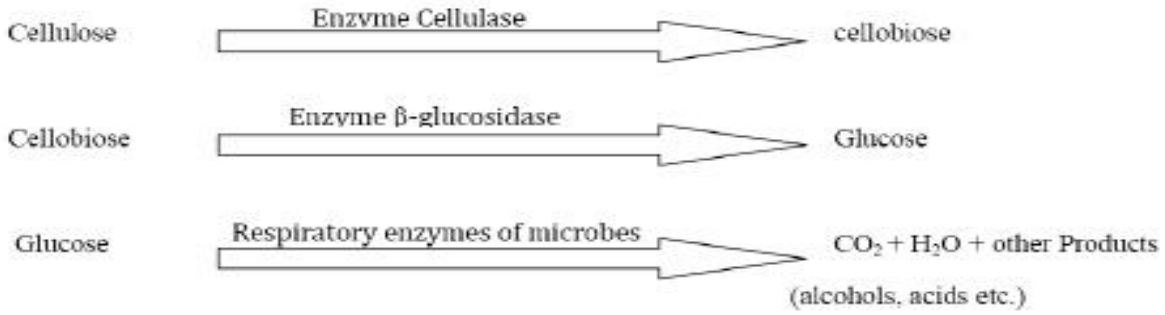


Fig.: Degradation of cellulose

Similar degradation pathways occur for other major substances such as protein, hemicellulose, lignin, pectin etc. All of these transformations may occur in soil.

Role of microorganisms in carbon cycle

1. Microorganisms degrading cellulose
   - Bacteria: Species of *Cellulomonas, Cytophaga, Bacillus, Clostridium, Pseudomonas, Streptomyces* etc.
   - Fungi: Species of *Trichoderma, Chaetomonium, Aspergillus, Penicillium* etc.

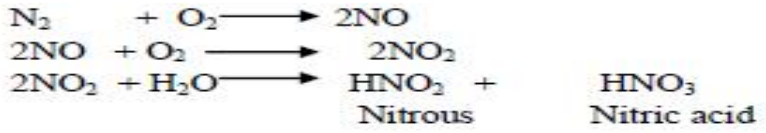
2. Microorganisms degrading hemicellulose
   - Bacteria: Species of *Bacillus, Pseudomonas, Cytophaga, Streptomyces, Actinomyces* etc.

- Fungi: Species of *Chaetomonium, Aspergillus, Penicillium* etc.

3. Microorganisms degrading lignin
   - Bacteria: Species of *Pseudomonas, Micrococcus, Flavobacterium, Arthobacter,*
   - Fungi: Species of *Polyporus, Poria, Trametes, Mycena, Clavaria, Aspergillus, Phanerochaete*

NITROGEN CYCLE

- In atmosphere, 78% nitrogen is present.
- Less then 0.1% of this N is fixed.
- Through rainwater trace N is dissolved and added in soil. In process of lightning $N_2 + O_2$ combine to form nitrous and nitric acids.



- Nitrogen demand of plant is very high, so nitrogenous fertilizers are required to be added to soil.
- Due to symbiotic N-fixation 10-500 kg N/ha/year is fixed in soil.
- Due to non-symbiotic process N-fixed in soil ranges between 50-150 kg/year/ha.

Steps of Nitrogen Cycle:

1. Proteolysis /decomposition of protein
2. Ammonification
3. Nitrification
4. Denitrification
5. Nitrogen fixation

1) Proteolysis/Decomposition/ Hydrolysis of Proteins

- In the first stage, proteinase break down the large molecules of proteins into smaller units of polypeptides.
- In the 2nd stages, polypeptides are broken down further into tripeptides and dipeptide and then in to free amino acid by the enzyme peptidases.
- It involves decomposition of protein into amino acids by microbes (*Clostridium histolytiacum, C. sporogenes, Proteus, Pseudomonas, Bacillus).*
- Protein is made up of amino acids and linked together by peptide bond.
- Protein content of plant residue like paddy straw is < 1% while legume contains about 20% protein.
- Animal residues and organic manures contain large amount of protein.

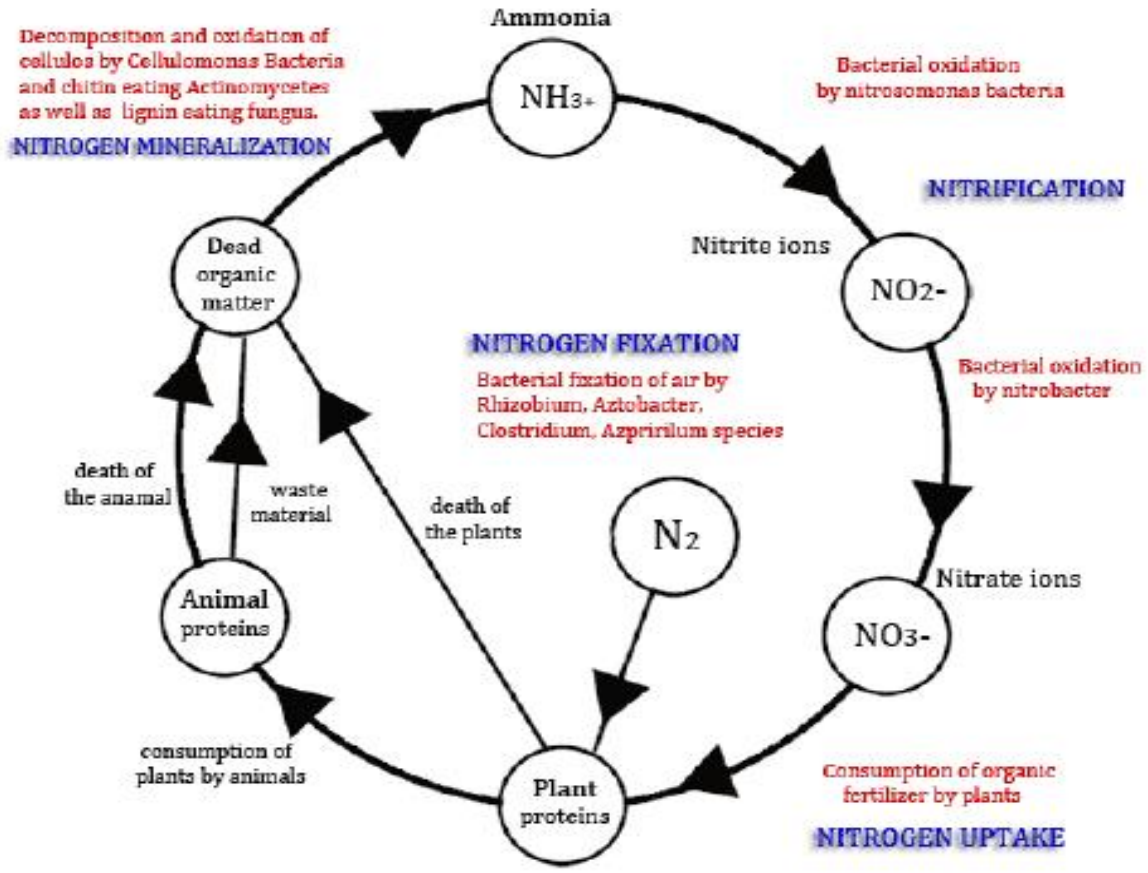


Fig.: N-Cycle

i) Large molecules of proteins → (break down by proteinase enzyme) → Small units of polypeptides contain two-more amino acids

ii) polypeptides → (breaking) → Tripeptides & dipeptides → (Enzyme peptidases) → free amino acid

Fig.: Proteolysis

The proteins are broken down by microorganisms with the help of proteolytic enzyme.

Protein → (Proteinases by fungi) → Polypeptides → (Peptidases by bacteria) → Amino acids

Amino acids are broken down by oxidative deamination by aerobic microorganisms.

Amino acids utilized by:

- Soil Microorganisms
- Plants with the help of mycorrhiza.
- Converted into ammonia (NH3) gas. This process is called Ammonification

2) Ammonification

- It is process by which Amino acids are converted into ammonia with the help of microorganisms.
- Bacteria are relatively more active than other organisms.
- Spore-forming bacteria *Pseudomonads, Actinomycetes* and fungi seem to readily attack amino acids in soil.
- However, in acid soils, fungi are more important agents of ammonification than bacteria.
- Amino acids are broken down by oxidative deamination by aerobic micro organisms.

$$\underset{\text{Alanine}}{CH_3.CHNH_2.COOH} + 1/2\ O_2 \xrightarrow{\text{alamine deaminase}} \underset{\text{Pyruvic acid}}{CH_3\ COCOOH} + \underset{\text{Ammonia}}{NH_3}$$

- Bacteria breakdown complex nitrogenous substances to obtain energy, and in this process NH_3 is released.

Many organisms utilize urea to liberate ammonia

$$NH_2CONH_2 + 2H_2O \longrightarrow \underset{\text{Ammonium carbonate}}{(NH_4)_2\ CO_3} \longrightarrow 2NH_3 + CO_2 + H_2O$$

- Microorganisms like *Bacillus, Proteus, Micrococcus, Sarcina, Aerobacter,* etc are known to quickly convert urea to ammonium carbonate and then to ammonia.

$$\underset{\text{Urea}}{} \xrightarrow{\text{Urease}} \underset{\text{ammonium carbonate}}{} \xrightarrow{\text{Urease}} \underset{\text{ammonia}}{}$$

3). Nitrification

- Microorganisms convert ammonia to nitrate, the process is called nitrification.
- It occurs in two steps, each step preformed by a different group of bacteria.

I) Oxidation of ammonia to nitrite by Oxidizing bacteria. → *Nitrosomonas*

$$2NH_3 + 3O_2 \longrightarrow 2HNO_2 + 2H_2O$$

2) Oxidation of nitrite to nitrate by oxidizing bacteria. → *Nitrobacter*

$$HNO_2 + {}^1/_2\ O_2 \longrightarrow HNO_3$$

- Decomposition of organic matter in soil results in increased concentration of nitrate in soil.
- Nitrification was discovered to be a biological process by Schloesing and A Muntz in 1877.
- This was further confirmed by R. Warrington 1886, who showed that special bacteria are involved in this process.

- S. Winogradsky (1888-91) isolated & brought out important characteristics of bacteria. Which are capable to oxidize ammonia to obtain energy, just like photosynthesis in plants.
- Instead of light as a source of energy, chemical oxidation may provide energy to microorganisms.
- Organisms responsible for conversion of NH_3 to NO_2

1. Ammonium oxidation - (NH_3 to NO_2)

Nitrosomonas Spp.; Nitrosovibrio tenuis; N. europaea; N. briensis and Nitrosococcus nitrosus.

2. Nitrite oxidation : Convert nitrite to nitrate (NO_2 to NO_3)

Nitrobacter winograsdsky and Nitrospira gracilis

4. Denitrification

- This is reverse process of nitrification *i.e.* nitrate is reduced to nitrites and then to nitrogen gas and ammonia.
- This process is harmful at the view point of agriculture because it reduces soil fertility.
- Some aerobic organisms, *Pseudomonas denitrificans,* also reduce nitrate under certain conditions.
- The presence of denitrifying bacteria in the soil is sufficient.
- The enzyme required is nitrate reductase or nitratase. This enzyme is found in bacteria viz. *E. coli, P. aeruginosa, Micrococcus denitrificans.*
- Denitrification may result in the formation of **gaseous nitrogen.**

$$\underset{\text{Nitrate}}{2NO_3} \longrightarrow \underset{\text{Nitrite}}{2NO_2} \longrightarrow \underset{\text{Nitric oxide}}{2NO} \longrightarrow \underset{\text{Nitrous oxide}}{N2O} \longrightarrow \underset{\text{Nitrogen}}{N_2}$$

  - This is an undesirable process as it results in loss of nitrogen from the soil and hence a decline in nutrients for plant growth.

5. Nitrogen fixation

- In this biological process, **atmospheric nitrogen** utilized by nitrogen fixing bacteria with the help of nitrogenase enzyme and converts to **ammonia**, readily utilizable form of nitrogen by plants.
- Symbiotic, non symbiotic and associative symbiotic nitrogen fixing bacteria plays an important role in this process.

- Symbiotic nitrogen fixation: Carried out by ***Rhizobium*** with leguminous plants.
- Non-symbiotic nitrogen fixation: by ***Azotobacter*** directly into soil.
- Associative nitrogen fixation: by ***Azospirillum*** with roots of grass.

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BIOLOGICAL NITROGEN FIXATION

A number of microorganisms are able to use molecular nitrogen in the atmosphere as their source of nitrogen". **The conversion of molecular nitrogen into ammonia is known as nitrogen fixation**

- Nitrogen fixation is generally divided into two types: **Symbiotic and non-symbiotic nitrogen Fixation.**
- Two group of microorganisms :

(1) Symbiotic: Microorganisms those living in roots of plants

(2) Non Symbiotic : Microorganisms those living freely and independently in the soil

- **Symbiotic N_2 fixation :**

• Nodulation & Symbiotic nitrogen fixation is carried out by *Rhizobium* group of bacteria in association with a particular group of leguminous plants. On the basis of the specificity of symbiotic interaction and morphological differences in the bacterium, seven different species of *Rhizobium* have been identified, which is referred to as **cross-inoculation groups**.

Cross inoculation groups of *Rhizobium*

Sr. No.	**Cross inoculation group**	***Rhizobium* spp.**	**Host it can nodulate**
1	Alfalfa group	*R. meliloti*	Alfalfa and melilotus
2	Clover group	*R. trifoli*	Trifolium
3	Pea group	*R. leguminosarum*	Pisum, Victa, Lathyras
4	Bean group	*R. phaseoli*	Phaseolus (Bean)
5	Lupine group	*R. lupine*	Luines
6	Soybean group	*R. japonicum*	Glycine
7	Cowpea	*Rhizobium* spp.	Vigna, Arachis, Cajanus, Dolichos

- Approximate quantities of N2 fixed by different symbiotic bacteria range from 10 to 500 kg/ha/year.
- Certain *Rhizobium* form stem nodules in legumes like *Sesbania rostrata as observed* by Dreyfus *et. al, in 1985*. It has capacity to fix nitrogen as high as 150 kg/ha in 52 days. It is named recently as *Azorhizobium caulinodans.*

Non-Symbiotic Nitrogen Fixation :

- Nitrogenase, the key enzyme in nitrogen fixation, has been isolated from several freeliving nitrogen fixing microorganisms *viz. Clostridium, Azotobacter, Rhodospirillum, Anabaena.*

- It has been estimated that the amount of N_2 fixed by the non-symbiotic process

Associative N2 Fixation :

- *Azospirillum* is associated with the roots of grasses and is capable of fixing atmospheric nitrogen.
- The blue-green algae *Anabaena azollae* are symbiotically associated with the water fern *Azolla. Azolla* is used as green compost for rice cultivation *A. azollae* is capable to fix atmospheric nitrogen. ranges between 20 and 50 lbs/acre/year.

The essential reactants in the bacterial nitrogen fixation process are :

1. Components I & II
2. A strong reducing agent,
3. ATP molecules,
4. A regulating system for NH_3 production and utilization,
5. A system that protects the N_2 fixing system from inhibition by O_2.

The overall biochemical reaction for nitrogen fixation can be expressed as:

$$N_2 + 6e^- + 12\ ATP + 12\ H2O \xrightarrow{\text{Nitrogenase}} 2NH4^+ + 12ADP + 12Pi + 4H^+.$$

The enzyme nitrogenase is very sensitive to oxygen and get deactivated in presence of oxygen, so each of the nitrogen fixing organisms have evolved specialized mechanisms to protect their nitrogenase from oxygen deactivation

Name of the organism	Mechanism to prevent deactivation of nitrogenase
Azotobacter	High respiration rate
Azospirillum	Microaerophilic nature
Acetobacter	Endophytic colonization
Rhizobium	Leghaemoglobin

PHOSPHORUS CYCLE

Phosphorus is never found in the free state in nature, because contact with air causes combination with oxygen. It is found abundantly as calcium, phosphate in many minerals.

- P_2O_5 (phosphate) constitute nearly 0.1% of earth crust.
- Rock phosphate are present in parent rocks. It occur in soil in inorganic & organic forms.In cultivated soil, they are present in about 1100 kg/ha but most of them are not available to plants.
- Only about 1% of the total soil phosphorus is in available form.

- Therefore when phosphates are applied as fertilizers, they are utilized more by the plants than the soil phosphates.

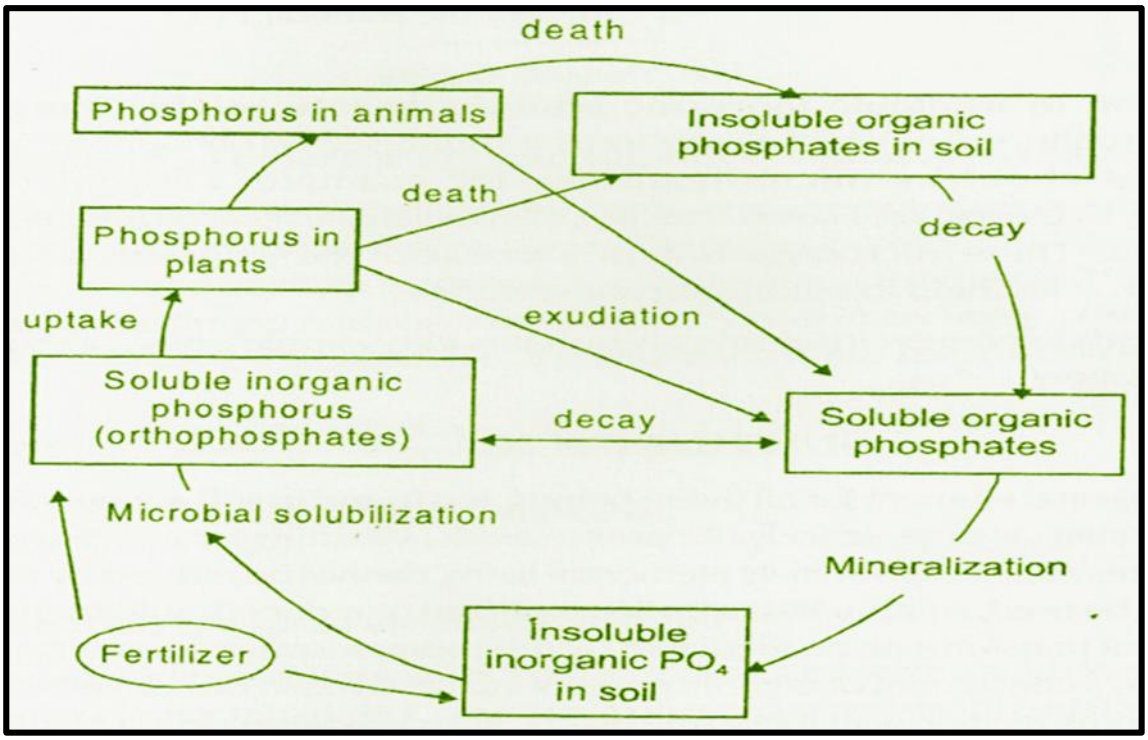


Fig.: P-CYCLE

- The residue of man, animals, plants, birds etc contain several phosphates. When they reach the soil, they are acted upon by several microorganisms, break-down the phosphorus containing compounds with the liberation of mineral elements like Ca, Fe, Na and this process is known as Mineralization.
- On the death of the bacteria, the phosphate is made available for plants.
- Inoculation of organic compost with Phosphate Solubilizing *Bacillus* (PSB) and nitrogen fixing *Azotobacter chrococcum* help in improving the quality of manure by reducing the C:N ratio from 15 to 12 and substantially improving the available phosphate.
- The activity of microorganisms in phosphate solubilization influenced by various soil factors viz. pH, moisture & aeration.

(1) Both organic and inorganic phosphorus occur in soil.

- The inorganic forms of phosphorus are compounds of Ca, Fe, and Al.
- The organic phosphorus containing compounds are derived from plants and microorganisms and are composed of nucleic acids, phospholipids and phytin.
- Organic matter derived from dead and decaying plant debris is rich in inorganic sources of phosphorus.

(2) The deficiency of phosphorus may occur in crop plants growing on soils containing adequate phosphates.

- This may be partly due to the fact that plants are able to absorb phosphorus only in an available form.
- Soil phosphates are rendered available either by plant roots or by soil microorganisms through secretion of organic acids.
- Therefore, phosphate dissolving soil microorganisms play some role in correcting phosphorus deficiency of crop plants.
- They may also release soluble inorganic phosphates (H_2PO_4) into soil through decomposition or phosphate rich organic compounds.
- On the other hand, certain microorganisms, through assimilation, may immobilize available phosphates in their cellular material.
- Such immobilization process in soil may also contribute to phosphorus deficiency to crop plants.

(3) Solubilization of phosphates by plant roots and microorganisms is depended on soil pH.

- In neutral or alkaline soils having a high content of calcium, precipitation of calcium phosphates takes place.
- Microorganisms and plants roots readily dissolve such phosphates and render them easily available to plants.
- On the contrary, acid soils are generally poor in calcium ions there fore, phosphates are precipitated in the form of ferric or aluminium compounds which are not so easily amenable to solubilization by plant roots or by soil microorganisms.
- If such conditions prevail in acid soils, deficiency of phosphorus in plant may also occur.

P-deficiency in soil can be corrected by inoculating seed or soil with phosphate dissolving microorganisms along with phosphatic fertilizers.

(4) Labeled phosphates (^{32}P) test : it is used to test the phosphate solubilizing property of soil microorganisms and also to find out the extent of phosphorus uptake by the plant.

- Such experiments have been done in sterilized and unsterilized soils using tri-calcium and rock phosphates, apatite and bone meal.
- The results seed to be inclusive since several contradictory report have been made on this aspect.
- Many fungi (*Aspergillus, Penicillium)* and bacteria *(Bacillus and Pseudomonas)* are potential solubilizers of bound phosphates (PO_4 unavailable form).
- Phosphate dissolving bacteria and fungi are known to reduce the pH of the substrate by secretion of a number of organic acids such as formic, acetic, propionic, lactic, gluconic, fumaric and succinic acids.
- These acids (Hydroxy acids) may form chelates with cations such as Ca and Fe, and results in effective solubilization of phosphates.
- The application of sulphur coupled with *Thiobacilli (Phosphomin biofertilizer*) has also the potential rendering alkali soil fit for cultivation of crops.

- The formation of H_2SO_4 in soil following additions of elemental sulfur augments nutrient mobilization by increasing the level of soluble phosphates of potassium, calcium, manganese, aluminum and magnesium.
- The solubility of phosphorus is called the mobilization of phosphoric acid.
- Mobilization of inorganic phosphorus is brought about by many microorganisms.
- Species of *Pseudomonas, Mycobacterium, Micrococcus, Flavobacterium, Penicillium, Sclerotium, Aspergillus* and others are active in the conversion.

SULPHUR CYCLE

Sulphur is present in different forms :

- Trace in air
- In industrial area, atmosphere contains high amount of sulfur, due to burning of coal.
- Sulfur in air, reaches the soil through rainwater.
- Sulfur and H_2S abundantly coming out from volcanoes.
- Sulfur is present in some springs.

Plants utilize Sulfur in the dissolved from as sulfate.

Cruciferous plants require up to 40 kg S/ha

Cereals requires < 10 kg S/ha.

The bacteria capable of oxidizing in organic sulphur compounds may be aerobic or anaerobic. *Thiobacillus, Thiorix, Thioploca, Beggiatoa*

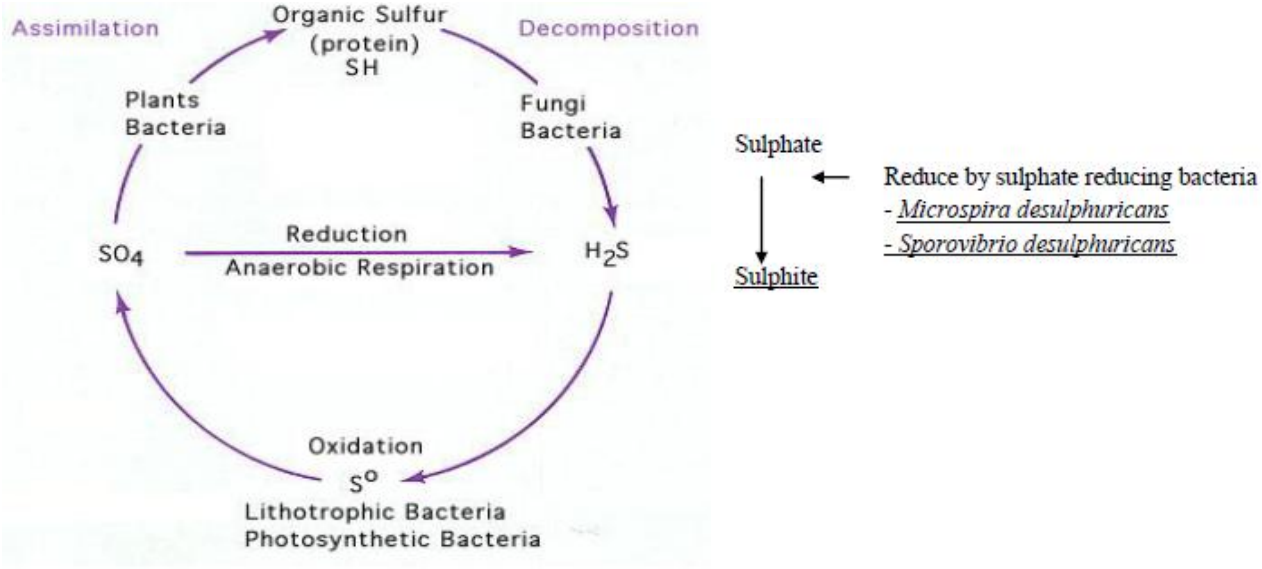


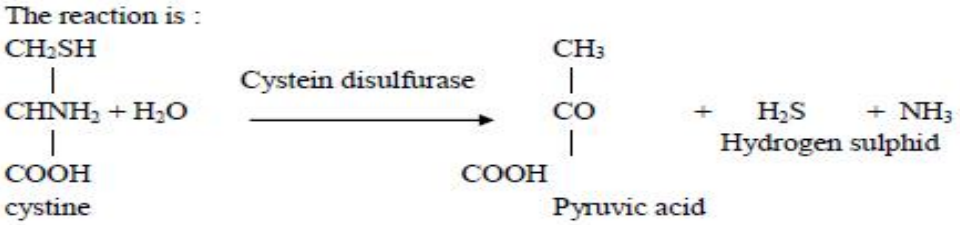
Fig.: S-CYCLE

Some of the Biochemical changes by micro-organisms involved in this cycle are as follows:

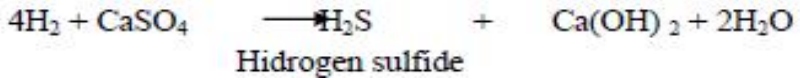
1. Elemental sulfur can not be utilized by plants or animal. Bacteria, *Thiobacillus thiooxidans is capable of oxidizing sulphur to sulphates :*

The reaction is : $2S + 2H_2O + 3O_2 \quad 2H_2SO_4$

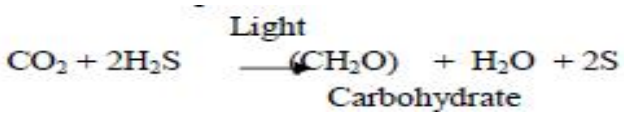
2. Sulfate is assimilated by plants and is incorporated into sulfur-containing amino acids and then into proteins. Degradation of protein (Proteolysis) librate amino acids, some of which contain sulfur is released from the amino acids by enzymatic activity of many bacteria.



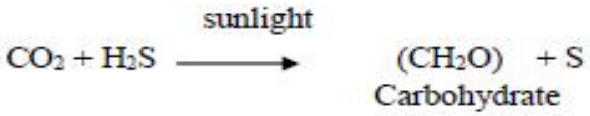
3. Sulfates may also be reduced to H_2S by soil microorganisms. The bacterium involved is *Disulfotomaculum.*



4. H_2S resulting from sulfate reduction and amino acid decomposition is oxidized to elemental sulfur. This reaction is characteristics of some pigmented sulfur bacteria :



The H_2S may be photosynthetically utilized and elemental sulfur may be released.



- The sulfates and sulphuric acid, when dissolved in water, are made available for plant growth.
- The plants utilize sulfates to form various amino acids, hormones, growth factors, etc. They are either eaten away by animals or returned to soil as organic waste.
- The plant parts eaten by the animals are also in some form or other returned to the soil.
- When various complex organic sulfur compounds reach the soil, they are attacked by the soil organisms and the cycle of events continues.

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Azolla

Azolla is a genus of aquatic ferns in the family Salviniaceae. Azolla is a tiny water fern common in ponds, ditches and rice fields. It has been used as a bio-fertiliser for rice in all major rice growing countries including India, Thailand, Korea, Philippines, Brazil and West Africa. The nitrogen accumulated in the Azolla is made available to the rice crop when the fern decomposes. The alga inhabits some of the cells on the underside of the Azolla frond and fixes atmospheric nitrogen. They form a symbiotic relationship with the cyanobacterium/BGA *Anabaena azollae*, which fixes atmospheric nitrogen, giving the plant access to the essential nutrient.

It is dependent on the fern for photosynthates which supply the energy for nitrogen fixation. In addition to nitrogen, the decomposed Azolla also provides K, P, Zn and Fe to the crop. It also controls aquatic weeds which would otherwise compete with the crop for nutrients.

Azolla is a highly productive plant. It doubles its biomass in 3–10 days, depending on conditions, and yield can reach 8–10 tonnes fresh matter/ha in Asian rice fields.

The species of Azolla are: A. *filiculoides, A. Mexicana, A. caroliniana*, *Azolla microphylla*, *Azolla nilotica*, *Azolla pinnata*

Why use Azolla in rice?

Azolla in association with blue-green alga anabaena can fix atmospheric Nitrogen (N) into ammonia which can be utilized by rice plant when it is incorporated into soil. Azolla contains from 2–5% N, 0.3–6.0% Potassium (K) (dry weight).

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Blue Green Algae (BGA)/Cyanobacteria

- BGA is also known as **Cyanophyta**, is a phylum of bacteria that obtain their energy through photosynthesis,[4] and are the only photosynthetic prokaryotes able to produce oxygen. The name "cyanobacteria" comes from the color of the bacteria. Cyanobacteria (which are prokaryotes) used to be called "blue-green algae". They have been renamed 'cyanobacteria' in order to avoid the term "algae", which are eukaryotes.
- Like other prokaryotes, cyanobacteria have no membrane-sheathed organelles. Photosynthesis is performed in distinctive folds in the outer membrane of the cell (unlike green plants which use chloroplasts for this purpose).
- By producing and releasing oxygen (as a byproduct of photosynthesis), cyanobacteria are thought to have converted the early oxygen-poor, reducing atmosphere, into an oxidizing one.
- Cyanobacteria are a group of photosynthetic, nitrogen fixing bacteria that live in a wide variety of moist soils and water either freely or in a symbiotic relationship with plants or lichen-forming fungi.[
- They range from unicellular to filamentous and include colonial species. Colonies may form filaments, sheets, or even hollow spheres. Some filamentous species can differentiate into several different cell types: **vegetative cells** -- the normal, photosynthetic cells that are formed under favorable growing conditions; **akinetes** -- climate-resistant spores that may form when environmental conditions become harsh; and thick-walled **heterocysts** -- which contain the enzyme nitrogenase, vital for nitrogen fixation.

Nitrogen fixation

Cyanobacteria can fix atmospheric nitrogen in anaerobic conditions by means of specialized cells called heterocysts. Heterocysts may also form under the appropriate environmental conditions (anoxic) when fixed nitrogen is scarce. Heterocyst-forming species are specialized for nitrogen fixation and are able to fix nitrogen gas into ammonia (NH_3) and which in turn may convert to nitrates ($NO{-}3$), which can be absorbed by plants and converted to protein and nucleic acids (atmospheric nitrogen is not bioavailable to plants, except for those having endosymbiotic nitrogen-fixing bacteria, especially the Fabaceae family, among others).

Free-living cyanobacteria are present in the water of rice paddies.Cyanobacteria such as*Anabaena* (a symbiont of the aquatic fern *Azolla*), can provide rice plantations with biofertilizer.

Carbon fixation

Cyanobacteria use the energy of sunlight to drive photosynthesis, a process where the energy of light is used to synthesize organic compounds from carbon dioxide. Because they are aquatic organisms, they typically employ several strategies which are collectively known as a "carbon concentrating mechanism" to aid in the acquisition of inorganic carbon (CO_2 or bicarbonate). Among the more specific strategies is the widespread prevalence of the bacterial microcompartments known as **carboxysomes.** These icosahedral structures are believed to tether the CO_2-

fixing enzyme, RuBisCO, to the interior of the shell, as well as the enzyme carbonic anhydrase, using the paradigm of metabolic channeling to enhance the local CO_2 concentrations and thus increase the efficiency of the RuBisCO enzyme.

Heterocysts

These are specialized nitrogen-fixing cells formed during nitrogen starvation by some filamentous cyanobacteria, such as *Nostoc*, *Cylindrospermum*, and *Anabaena* . They fix nitrogen from dinitrogen (N_2) in the air using the enzyme nitrogenase, in order to provide the cells in the filament with nitrogen for biosynthesis. Nitrogenase is inactivated by oxygen, so the heterocyst must create a microanaerobic environment. The heterocysts possess a unique structure and physiology .

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Mycorrhiza

- Some non-pathogenic fungi help in plant growth by forming associations with the host plant roots called mycorrhizae (myca- fungi, rhiza -root). Some examples of such fungi are *Trichoderma, Gigaspora, Glomus*, etc.
- One group of mycorrhizae forms a sheath around the fine lateral roots and replaces the root hairs by dichotomous branching of the fungal hyphae. They are called **ectomycorrhizae** because they do not traverse intracellularly. The ectomycorrhizae help the plant by Solubilizing nutrients near the plant roots and making it easy for the plants to feed. They also prevent the roots from being attacked by nematodes (by entangling them).
- Another group called the **endomycorrhizae** penetrate the roots and establish symbiotic relation with the plants.

The fungi help the roots in obtaining inorganic nutrients while obtaining essential organic nutrients from the host.

There is yet another group called **ect-endomycorrhiza or vesiculararbuscular mycorrhiza (VAM fungi)** wherein they are partly outside the host roots and partly intracellular.

Vesicular Arbuscular Mycorrhiza (VAM)

VAM is the symbiotic association between plant roots and soil fungus.

Suitable for: Turmeric, Banana, Rubber, Coffee, Tea, Pepper, Cardamom, Cocoa, Fruit trees, Tree seedlings and species etc.

Advantage of VAM:

(a) VAM is highly versatile and colonizes 85% of the plant families. It penetrates the roots, forms arbuscules and vesicles in the cortical cells of the roots and hyphae and spores in the soil.
(b) VAM plays a great role in inducing plant growth.
(c) The mychorrhiza penetrates the roots, mobilizes & supplies phosphorus and other micronutrients such as zinc, Manganese, iron, copper, Cobalt, Molybdenum etc. from the surrounding area to the plant.
(d) Increases the plant vigor by inducing drought resistance of young seedlings
(e) Mycorrhizae increase the resistance to root borne or soil borne pathogens and Nematodes.
(f) Enhanced colonization of introduced population of beneficial soil organisms like *Azotobacter, Azospirillum, Rhizobium* and Phosphate Solubilizing Bacteria around mycorrhizal roots thereby, exerting synergistic effects on plant growth.

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RHIZOSPHERE

- The **rhizosphere** is the narrow region of soil that is directly influenced by root secretions and associated soil microorganisms. The rhizosphere contains many Bacteria and microorganisms that feed on sloughed-off plant cells, termed *rhizodeposition*, and the proteins and sugars released by roots. Much of the nutrient cycling and disease suppression needed by plants occurs immediately adjacent to roots due to root exudants and communities of microorganisms.
- Term "Rhizosphere" was introduced for the first time by the German scientis**t Lorenz Hiltner (1904)** to denote that region of soil which is subjected to the influence of plant roots.
- The rhizosphere differs from the bulk soil because of the activities of plant roots & their effect on soil organisms. A major characteristic of the rhizosphere is the release of organic compounds into the soil by plant roots. These compounds called **root exudates** makes the environment different in rhizosphere and bulk soil. The exudates increase the availability of nutrients in the rhizosphere & also provide a carbon source for heterotrophic microorganisms. The exudates cause the no. of microbes to be far greater in the rhizosphere than in the bulk soil. The population of organisms in the rhizosphere can be 500 times higher than in bulk soil.
- Organisms in the rhizosphere can affect the plant roots by altering the movement of carbon compounds from roots to shoots. Many microorganisms are beneficial and are called Plant growth promoting rhizobacteria (PGPR). Various root microbes association can increase nutrient uptake by plants in nutrient poor environment such as symbiosis (eg. Mycorrhizal or Rhizobia) & specific association (Associative N_2 fixing bacteria–*Azospirillum).* Some microorganisms produce hormones that stimulate plant growth and some microorganisms are antagonistic to plant pathogens. However, some soil microorganisms are pathogenic & attack living plant roots.

R:S ratio: H. Katznelson (1946) - the ratio between the microbial population in the rhizosphere (R) and in the non-rhizospheric 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.

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 is always ≥1.

Rhizoplane:- The rhizoplane is the surface of the plant roots in the soil. The rhizoplane is the site of the water & nutrient uptake & the release of exudates in to the soil. As roots grow they cast dead cells & navigate around the soil particles making the rhizoplane highly irregular, blurring the dividing line between the root surface & soil.

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PHYLLOSPHERE

- The above-ground parts of plants are normally colonized by a variety of bacteria, yeasts, and fungi. While a few microbial species can be isolated from within plant tissues, many more are recovered from the surfaces of healthy plants. The aerial habitat colonized by these microbes is termed the phyllosphere, and the inhabitants are called epiphytes.
- Bacteria are by far the most numerous colonists of leaves, often being found in numbers averaging 10^6 to 10^7 cells/cm^2 (up to 10^8 cells/g) of leaf.
- **Ruinen** (1956), Dutch microbiologist coined the term "**Phyllosphere**" to denote the region of leaf influenced by microorganisms.
- The leaf surface microbes may perform an effective function in controlling the spread of air borne microbes inciting plant disease. Resistance to disease causing microbes has also been attributed to fungistatic compounds secreted by leaves such as malic acid etc.
- Phyllosphere bacteria are often pigmented due to direct solor radiation. Any change in phyllosphere, affects plant growth which in turn affects the physiological activity of root system. Such changes in the root results in an altered pH & spectrum of chemical exudation causing a change in rhizosphere microflora.
- Thus there is a link between phyllosphere microflora and rhizosphere microflora.
- There is a continuous diffusion of plant metabolites from leaves which support the microbial growth & in turn these microbes protect the plant from pathogens.
- The microbial ecology of the phyllosphere has been viewed mainly through the biology of gram-negative bacteria such as *Pseudomonas syringae* and *Erwinia (Pantoea) spp.*, two of the most ubiquitous bacterial participants of phyllosphere communities.

Factors that may influence the microhabitat experienced by bacteria on leaves:

- First, the leaf itself is surrounded by a very thin laminar layer in which moisture emitted through stomata may be sequestered, thereby alleviating the water stress to which epiphytes are exposed.
- Second, some cells in a leaf bacterial population, particularly in plant-pathogenic populations, may not reside in exposed sites on the leaf surface but instead may at least locally invade the interior of the leaf, avoiding the stresses on the exterior of the leaf by residing in substomatal chambers or other interior locations. Thus, while some phytopathogens may have the option of avoiding stresses, most other epiphytes apparently must tolerate them in some way.

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Microbes in Human Welfare

Microorganism in silage production

Silage is fermented, high-moisture stored fodder which can be fed to cattle, sheep and other such ruminants. It is fermented and stored in a process called *ensilage*, *ensiling* or *silaging*, and is usually made from grass crops, including maize, sorghum or other cereals, using the entire green plant.

The crops suitable for ensilage are the ordinary grasses, clovers, alfalfa, vetches, oats, rye and maize; various weeds may also be stored in silos. Silage must be made from plant material with a suitable moisture content: about 50% to 60% depending on the means of storage, the degree of compression, and the amount of water that will be lost in storage, but not exceeding 75%. **Ryegrasses** have high sugars and respond to nitrogen fertiliser better than any other grass species. These two qualities have made ryegrass the most popular grass for silage making for the last sixty years.

Silage is made either by placing cut green vegetation in a silo or pit, by piling it in a large heap and compressing it down so as to leave as little oxygen as possible and then covering it with a plastic sheet, or by wrapping large round bales tightly in plastic film. After harvesting, crops are shredded to pieces about 0.5 in (1.3 cm) long. The material is spread in uniform layers over the floor of the silo, and closely packed. When the silo is filled or the stack built, a layer of straw or some other dry porous substance may be spread over the surface. In the silo the pressure of the material, when chaffed, excludes air. Extra pressure may also be applied by weights in order to prevent excessive heating.

Fermentation

The main process that we associate with ensiling is the fermentation of sugars by lactic acid bacteria. Once the crop is anaerobic, lactic acid bacteria grow rapidly and quickly become, in most cases, the dominant microorganisms on the crop. They ferment sugars mainly to lactic acid, acetic acid, ethanol and carbon dioxide. The ratio of products depends upon the species. Assuming a mix of hetero- and homofermentative lactic acid bacteria, lactic acid will be the dominant product on a molar basis followed in descending order by carbon dioxide, acetic acid, and ethanol. The acids lower crop pH to between 4.0 and 5.0 in alfalfa and grass silages and below 4.0 in corn silage.

Both the low pH and the acids are beneficial in preserving the crop. The principal bacterial competitors of the lactic acid bacteria under anaerobic conditions (enterobacteria, clostridia and bacilli) are all inhibited by achieving a sufficiently low pH. Once pH drops below 4.5 to 5.0, enterobacteria and bacilli will be inhibited, and enterobacterial populations will usually drop below detectable levels within a few days after the pH is below 5.0. Fermentation by the lactic acid bacteria usually takes silage pH to a level that inhibits these bacteria. So often we forget about these two groups unless we have a high acetic acid silage that is feeding poorly.

Silage undergoes anaerobic fermentation, which starts about 48 hours after the silo is filled, and converts sugars to acids. Fermentation is essentially complete after about two weeks.

- Before anaerobic fermentation starts, there is an aerobic phase in which the trapped oxygen is consumed. How closely the fodder is packed determines the nature of the resulting silage by regulating the chemical reactions that occur in the stack. When closely packed, the supply of oxygen is limited, and the attendant acid fermentation brings about decomposition of the carbohydrates present into acetic, butyric and lactic acids. This product is named sour silage.
- The role of microorganism in silage making is directly related to the production of the following acids - lactic acid, acetic acid, propionic acid and butyric acid. **Lactic acid** is the most important of the four. If formed rapidly and sufficient quantity preserves the ensiled plant material therefore no decay.
- **The lactic acid bacteria** covers a number of genus (*Lactobacillus*, *Pediococcus*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Leuconostoc*) that are found in silage. They all produce lactic acid as their principal product from fermenting sugars, but other products, particularly acetic acid, ethanol and carbon dioxide, are common. **Lactobacilli** occur in large amounts in green plants. Their development in the ensiled material depends on the presence of large quantities of sugar in the plant material. Crop plants like maize that have large carbohydrate content are easier to ensile than legume plants with low carbohydrate content because of the presence of high levels of lactobacilli.
- In the past, the fermentation was conducted by indigenous microorganisms, but, today, some bulk silage is inoculated with specific microorganisms to speed fermentation or improve the resulting silage. Silage inoculants contain one or more strains of lactic acid bacteria, and the most common is *Lactobacillus plantarum*. Other bacteria used include *Lactobacillus buchneri*, *Enterococcus faecium* and *Pediococcus* species.

Undesirable microbes in silage production

- Yeasts are perhaps the most significant aerobic microorganisms on the crop relative to silage quality. Some yeasts can grow anaerobically, fermenting sugars to ethanol.
- Molds are the filamentous fungi present on the crop. These microorganisms are strictly aerobic.
- Acetic acid bacteria are aerobic bacteria that are capable of growing at low pH. They grow on ethanol, producing acetic acid. However, once ethanol has been exhausted, they can grow on acetic acid, producing carbon dioxide and water. This will raise pH and permit other aerobic microorganisms to grow.
- Enterobacteria are also the principal competitors of the lactic acid bacteria for the sugars in the crop.
- Clostridia are obligate anaerobes, and their effects on silage quality usually occur long after the lactic acid bacteria have stopped actively growing in the silo.

Biofertilisers

Bio-Fertilisers: Bio-fertilisers are preparations containing active or latent cells of efficient strains of certain microbes that can utilise the atmospheric nitrogen to increase the nitrogen content of soil, and can dissolve the insoluble phosphate of

the soil to release the phosphorus it contains in the soluble form for increasing crop yield. Bio-fertilizers are of the following types:

- Bacterial
- Fungal
- Algal
- Aquatic fern

How Bio-fertilizers Work ?

- There is an abundance of biopolymers like proteins, fats, fibers and other carbohydrates in natural soils.
- Bacteria in soil digest these large biopolymers to respective smaller monomers.
- Proteins are digested to amino acids, carbohydrates and fiber to sugars and fats/lipids to fatty acids by the soil bacteria.
- Plants can easily absorb these small molecules or monomers.
- Additionally, the soil-bacteria help the plant-roots to absorb Major and minor nutrients present in the soils.
- The soil-bacteria also release bio-chemicals which accelerate the plant growth.

Advantages of Bio-fertilizers in general

- Add nutrients (Nitrogen) to the soil / make them (Phosphorous) available to the cop.
- Secrete certain growth promoting substances.
- Under certain conditions they exhibit anti-fungal activities and thereby protect the plants from pathogenic fungi.
- Harmless and Eco-friendly low cost agro-input supplementary to Chemical Fertilizers.
- Improve soil structure (porosity) and water holding capacity.
- Enhance seed germination.
- Increase soil fertility, Fertilizer Use Efficiency and ultimately the yield by 15-20 % in general

(1) Bacterial Biofertiliser

- Here, live cells of bacteria are used as fertilisers.
- They are the principal nitrogen fixers in the soil and may be either **free-living or symbiotic** (within the nodules of legume roots, leaf nodules of certain plants and stem nodules of *Sesbania grandiflora) or associative* Symbiotic.
- Some bacterial genera can solubilize phosphorus from the bound form (*Bacillus, Pseudomonas sp.).*

- There are certain other bacterial members which during their metabolic activity, make available certain essential trace elements like manganese, calcium and zinc in the soil for plant absorption.
- Some bacteria like *Agrobacterium sp. and Pseudomonas fluorescence* supply growth hormones like Indole Acetic Acid (IAA) to the plants.
- Certain groups of bacteria like the *Pseudomonas fluorescence living in association with the* rhizosphere of most of crop plants (rhizobacteria promoting plant growth) supply all the essential nutrients required for the growth of the crop and in addition, protects the plant roots from the attack by soil-borne pathogens (saprophytic suppression).
- The nitrogen fixing ability of the bacteria can be studied under the following heads:

(A) Nitrogen fixers

- *Rhizobium, Azorhizobium, Synorhizobium and Bradyrhizobium* are **symbiotic nitrogen fixers** and are found in association with legume plants.
- These bacteria results into formation of nodules in the legume plants, where nitrogen fixation takes place.
- The most important obligate aerobic nitrogen fixers are *Azotobacter, Beijerinckia, Derxia, Achromobacter, Mycobacterium and Bacillus. Facultative anaerobic bacteria belong to the genera Aerobacter,*
- *Klebsiella and Pseudomonas (i.e., they can survive both in the presence and absence of* oxygen). (**Facultative anaerobe**)
- **Anaerobic nitrogen- fixing** bacteria are represented by the genera *Clostridium, Chlorobium, Chromatium, Rhodospirillum, Rhodomicrobium, Rhodopseudomonas, Desulphovibrio and Methanobacterium.*
- *Apart from fixing nitrogen, Azotobacter chroococcum has the ability to synthesise and secrete B-vitamins, growth hormones and* antifungal antibiotics into its environment.
- *Azotobacter* has one drawback*; its nitrogen fixing* ability is regulated by the presence of nitrogenous compounds in its environment.
- *Azospirillum* show associative symbiosis with cereals, sugarcane and some grasses.

(B) Phosphatic Bio-fertilisers/ Phosphate Solubilizing Bacteria (PSB)

- The soil is inhabited by a group of bacteria termed as phospho bacteria which have the capacity of releasing bound phosphates in the soil and thus making it available for the plants.
- They act in different ways. Some bacteria secrete organic acids such as lactic acid, acetic acid and citric acid which solubilize the bound phosphate to forms which are available to plants.
- Others produce sulphuric acid by oxidation of sulphur which acts like the organic acids in making phosphate available.
- Carbonic acid formed by the action of carbon dioxide released by bacteria during respiration and water acts in a similar way.

- Some organisms give off hydrogen sulphide which reacts with the iron salt, ferrous phosphate, to form ferrous sulphide and thus releases the phosphate.
- Microbes are also responsible for decay of dead animal and plant litter in the soil during which humic and fulvic acids are formed which bind the metal ions such as Fe, AI, Mn and Ca and release the phosphate ions for the plants.
- The most common varieties of phosphobacteria are *Pseudomonas species and Bacillus megaterium.*
- Like *Rhizobium,* they are used as seed inoculants.
- Most of the cultivable soil being alkaline in nature contains less available phosphorus.
- Due to higher concentration of Calcium, whenever phosphatic fertilizers are applied in such soil, the large quantity of it gets fixed as Tri-Calcium Phosphate as it is water insoluble and hence becomes unavailable to the crop.
- Certain soil microorganisms have inherent capacity to dissolve part of the fixed phosphorus and make it available to the crop by secreting certain organic acids.
- Phosphate Solubilizing Bacteria are useful for all the crops i.e. Cereals, Cash crops, leguminous crops, horticultural crops, vegetables etc.

(2) Cyanobacteria (BGA) as Bio-fertilisers

- Another group of free-living nitrogen fixers are the cyanobacteria commonly called the bluegreen algae (BGA).
- More than a hundred species of BGA can fix nitrogen.
- Nitrogen fixation takes place in specialized cells called the **heterocysts** (large, thick walled and metabolically inactive cells) which depend on vegetative cells for energy to fix nitrogen while the fixed nitrogen is utilised by the vegetative cells for growth and development.
- BGA are very common in the rice fields (the micro-aerophilic condition and alkalinity are conducive to the algal population).
- If no chemical fertilisers are added, inoculation of the algae can result in 10-14 % increase in crop yields.
- Unlike Azotobacter, the BGA are not inhibited by the presence of chemical fertilisers.

3) Aquatic Fern (Azolla) as a Bio-fertiliser

- **Azolla is a tiny water fern** common in ponds, ditches and rice fields.
- It has been used as a bio-fertiliser for **rice** in all major rice growing countries including India, Thailand, Korea, Philippines, Brazil and West Africa.
- The nitrogen accumulated in the Azolla is made available to the rice crop when the fern decomposes.

- The nitrogen fixing work is accomplished by the symbiotic relationship between the fern and a BGA, ***Anabaena azollae.***
- The alga inhabits some of the cells on the underside of the Azolla frond and fixes atmospheric nitrogen.
- It is dependent on the fern for photosynthates which supply the energy for nitrogen fixation.
- In addition to nitrogen, the decomposed Azolla also provides K, P, Zn and Fe to the crop.
- It also **controls aquatic weeds** which would otherwise compete with the crop for nutrients.

(4) Actinomycetes as Biofertiliser

- ***Frankia*** is an actinomycete and forms nitrogen fixing nodules in trees and shrubs.
- The organism invades the cells of a developed lateral root and causes it to fuse into a nodule.
- Entry into the host changes the structure of the microbe.
- Scientists are hopeful that some day they may be able to make fruit trees like apple, pear, plum, raspberry, etc. by **fixing nitrogen** through the involvement of Frankia.

(5) Fungi as Bio-fertiliser

- Some non-pathogenic fungi help in plant growth by forming associations with the host plant roots called **mycorrhizae** (myca- fungi, rhiza -root).
- Some examples of such fungi are *Trichoderma, Gigaspora, Glomus, etc.*
- One group of mycorrhizae forms a sheath around the fine lateral roots and replaces the root hairs by dichotomous branching of the fungal hyphae. They are called **ectomycorrhizae** because they do not traverse intracellularly.
- The ectomycorrhizae help the plant by Solubilizing nutrients near the plant roots and making it easy for the plants to feed.
- They also prevent the roots from being attacked by nematodes (by entangling them).
- Another group called the **endomycorrhizae** penetrate the roots and establish symbiotic relation with the plants.
- The fungi help the roots in obtaining inorganic nutrients while obtaining essential organic nutrients from the host.
- There is yet another group called **ect-endomycorrhiza or vesiculararbuscular mycorrhiza (VAM fungi)** wherein they are partly outside the host roots and partly intracellular.

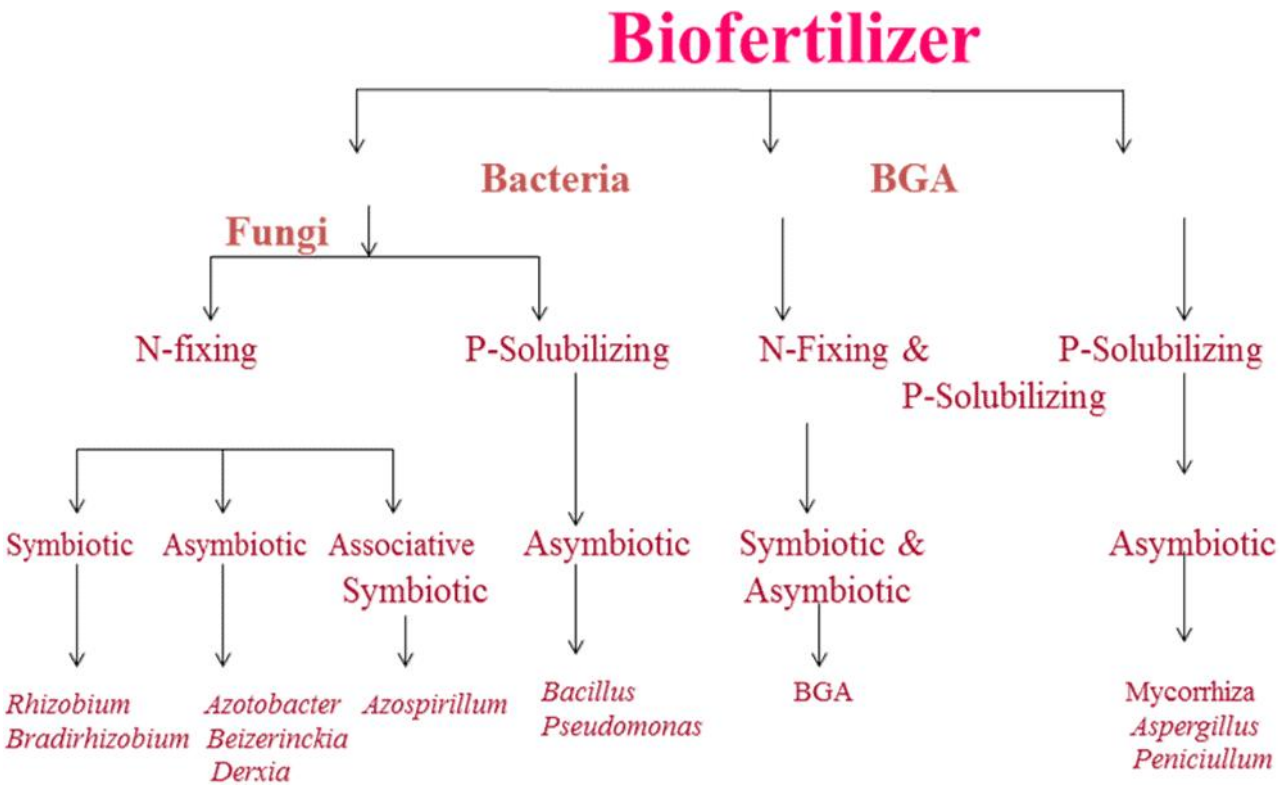
Vesicular Arbuscular Mycorrhiza (VAM)

- VAM - Vesicular Arbuscular mycorrhiza - is the symbiotic association between plant roots and soil fungus.
- VAM plays a great role in inducing plant growth.

- Mycorrhizae increase the resistance to root borne or soil borne pathogens and Nematodes.
- Enhanced colonization of introduced population of beneficial soil organisms like *Azotobacter, Azospirillum, Rhizobium* and Phosphate Solubilizing Bacteria around mycorrhizal roots thereby, exerting synergistic effects on plant growth.
- **VAM is suitable for:** Turmeric, Banana, Rubber, Coffee, Tea, Pepper, Cardamom, Cocoa, Fruit trees, Tree seedlings etc.

Advantages:

- VAM is highly versatile and colonizes 85% of the plant families.
- It penetrates the roots, forms arbuscules and vesicles in the cortical cells of the roots and hyphae and spores in the soil.
- The mychorrhiza penetrates the roots, mobilizes & supplies phosphorus and other micronutrients to the plants.
- Solubilize phosphate and transports micronutrients such as zinc, Manganese, iron, copper, Cobalt, Molybdenum etc from the surrounding area to the plant.
- Increases the plant vigor by inducing **drought resistance** of young seedlings.
- VAM protects the plants from the fungal pathogens



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Biopesticides

The United States Environmental Protection Agency (EPA) defines **biopesticides** as, "certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals." Based on the active ingredient, biopesticides are categorized as microbial pesticides, plant-incorporated-protectants, and biochemical pesticides.

Biological control: The term **"biological control"** and its abbreviated synonym **—biocontrol** have been used in different fields of biology. It has been used to describe the use of live predatory insects, entomopathogenic nematodes, or microbial pathogens to suppress populations of different pests, insects and disease causing microorganisms.

- In general, biopesticides are less toxic, more target specific, and/or decompose faster following application compared to conventional pesticides.
- All of these features contribute to the idea that application of biopesticides can result in less pollution compared to some of the conventional chemical pesticides.
- **One disadvantage** is that the user of biopesticides needs to have a greater understanding about the pest control needs of his or her crop.
- This is because most biopesticides have a more limited target range than chemical pesticides.

Biocontrol Organism	**Target Disease**
Bactericide	
Bacteriophages of *Xanthomonas* and *Pseudomonas syringae*	Bacterial spot in pepper and tomatoes and bacterial speck in tomatoes
Pseudomonas syringae	Ice inducing bacteria and biological decay
Pantoea agglomerans	Fireblight (*Erwinia amylovora)*
Fungicides	
Biocontrol Organism	**Target Disease**
Streptomyces lydicus	**Soilborne pathogens:** *Pythium, Rhizoctonia, Phytophthora, Fusarium, Verticillium* and other root decay fungi. **Foliar pathogens:** *Podosphaera, Botrytis, Schlerotinia, Monilinia, Alternaria, Peronospora and* other foliar fungi.
Bacillus pumilus	Rust, powdery mildew, cercospora, and brown spot
Coniothyrium minitans	*Sclerotinia minor, Sclerotinia sclerotiorum*
Bacillus subtilis	*Rhizoctonia, Fusarium, Alternaria, Aspergillus*, and others that attack the root systems of plants
Trichoderma harzianum	*Fusarium, Pythium*, and *Rhizoctonia*
Bacillus subtilis	Bacterial spot, powdery mildew, rust, gray mold, leaf blight, scab, and others fungi.

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Microbial insecticides

Microbial insecticides are comprised of microscopic living organisms (viruses, bacteria, fungi, protozoa, or nematodes) or the toxins produced by these organisms. They are formulated to be applied as conventional insecticidal sprays, dusts, liquid drenches, liquid concentrates, wettable powders, or granules. Each product's specific properties determine the ways in which it can be used most effectively.

Microbial insecticides are a new form of pesticide that work by infecting selected insect populations. Though this sounds potentially dangerous, many argue that it is actually quite safe, since the application of microbial insecticides is specific to the species one is trying to kill. Microbial insecticides usually have no effect on animal populations, unless diminishing a certain bug in the area interrupts the food chain. Each type usually works against only one type of insect.

(1) Bacteria

Following bacterial species are used as biocontrol:

1. ***Bacillus thuringiensis var. kurstaki*** kill the caterpillar stage of a wide array of butterflies and moths.
2. In contrast, ***Bacillus popillae*** (milky spore disease) kills Japanese beetle larvae but is not effective against the closely related annual white grubs (masked chafers in the genus *Cyclocephala)* that commonly infest lawns.

Common pests that are controlled with *B.thuringiensis var. kurstaki (Bt):*

European corn borer in corn, Indian meal moth in stored grain, Cabbage looper, Diamond back moth, Tomato/tobacco hornworm, Gypsy moth, Spruce budworm, Tent caterpillars, Fall webworm, Mimosa webworm, Bagworms, Spring and fall cankerworm.

(2) Fungi

- Fungi are important natural control agents that limit insect populations.
- Most of the entomopathogenic fungal species spread by means of asexual spores called conidia.
- Although, conidia of different fungi vary greatly in ability to survive adverse environmental conditions, desiccation and ultraviolet radiation are important causes of mortality in many species.
- Where viable conidia reach a susceptible host, free water or very high humidity is usually required for germination.
- Unlike bacterial spores or virus particles, fungal conidia can germinate on the insect cuticle and produce specialized structures that allow the fungus to penetrate the cuticle and enter the insect's body.
- Fungi do not have to be ingested to cause infections.

- In most instances, as fungal infections progress, infected insects are killed by fungal toxins, not by the chronic effects of parasitism.
- Many important fungal pathogens attack eggs, immatures, and adults of a variety of insect species.
- Others are more specific to immature stages.

Following Fungi has insecticidal properties:

- *Beauveria bassiana:* This soil fungus has a broad host range that includes many beetles and fire ants. It infects both larvae and adults of many species.
- *Nomuraea rileyi:* In soybeans, may cause dramatic reductions in populations of foliage-feeding caterpillars.
- *Vericillium lecanii:* This fungus has been used in greenhouses to control aphids and whiteflies.
- *Lagenidium giganteum:* This aquatic fungus is highly infectious to larvae of several mosquito genera.
- *Hirsutella thompsonii:* It is a pathogen of the citrus rust mite.

(3) Viuses

- The larvae of many insect species are vulnerable to devastating epidemics of viral diseases.
- The viruses that cause these outbreaks are very specific, usually acting against only a single insect genus or even a single species.
- Specificity of the virus-host relationship makes viruses an ideal candidate for use in the control of specific pest population.
- There are two major groups of insect viruses:

  (i) Polyhedrosis virus: where many virus particles are embedded in each protein crystal.

  When they occur in the host cell nucleus, they are called **nuclear polyhedrosis virus (NPV**), and when they occur in the host cytoplasm they are called **cytoplasmic polyhedrosis virus (CPV**).

  (ii) Granulosis virus: where only one virus is contained in each protein crystal.
- GV either develop in the nucleus or cytoplasm of the host cells (NGV or CGV).
- **NPV and CGV** come under the group of **Baculoviruses** and they are the most extensively studied insect viruses.
- Infection is caused by ingestion of contaminated food containing NPV / GV followed by cell invasion beginning in the midgut.
- GV when sprayed on leave, enter the larvae feeding on the foliage.
- The viruses multiply fast in the larvae and make them lethargic.
- Affected larvae become sluggish and stop feeding and die.

For example, **NPV** controls the pest ***Helicoverpa armigera***.

(4) Protozoa

- Species in the genera ***Nosema and Vairimorpha*** seem to offer the greatest potential for use as insecticides.
- ***Nosema locustae*** has been used to reduce grasshopper populations in rangeland areas, and adequate control has been achieved when treatments were applied to large areas while hoppers were still young.

Disadvantages of Microbial Insecticides/biopesticides:

- A particular biopesticides/microbial insecticide is toxic to only a specific species or group of insects. Therefore, one biopesticides may control only a portion of the pests present in a field, garden, or lawn.
- Heat, desiccation (drying out), or exposure to ultraviolet radiation reduces the effectiveness of several types of microbial insecticides
- Special formulation and storage procedures are necessary for some microbial pesticides.
- Because several microbial insecticides are pest-specific, the potential market for these products may be limited.

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Biogas

Biogas is a term used to represent a mixture of different gases (varied composition) produced as a result of action of anaerobic microorganisms on domestic and agricultural wastes. Depending on where it is produced, biogas can also be called swamp, marsh, landfill or digester gas.

Composition:

The composition of biogas varies depending upon the origin of the anaerobic digestion process. Landfill gas typically has methane concentrations around 50%. Advanced waste treatment technologies can produce biogas with 55-75% CH_4.

Typical composition of biogas:	
Matter	**%**
Methane, CH_4	50-75
Carbon dioxide, CO_2	25-50
Nitrogen, N_2	0-10
Hydrogen, H_2	0-1
Hydrogen sulphide, H_2S	0-3
Oxygen, O_2	0-2

- Biogas plants also provide highly enriched organic fertilizer.
- In 1961, a **Gobar Gas Research Station** was also established at **Ajitmal (district Etawah in U.P.**), which has designed a variety of gas plants suited to Indian conditions and supplying electricity at a cheap rate.
- The digesters in 'gas plants' are almost entirely burried underground, with a fixed dome that serves as a gas holder.

Substrates:

- The substrate usually employed for biogas generation is a waste product of industrial, agricultural, animal husbandry, or domestic and municipal origin.
- Therefore, the waste would contain a variable proportion of non, biodegradable matter in form of plastics, inorganic materials, lignin, etc. Lignin is virtually non-degradable under anaerobic conditions.

Factors Affecting Biogas Production:

Biogas yield is measured as m3 gas/kg volatile solids.

- Type of waste,
- Temperature during digester operation,
- The retention time (the period of time a given sample of waste/substrate stays in the digester/fermenter before it flows out) and
- The presence of inhibitors.

FOUR groups of bacteria are involved in Biogas Production:

(1) Hydrolytic and Fermentative Bacteria-

- This group includes both obligate and facultative anaerobes, and may occur upto 10^8-10^9 cells/ml of sewage sludge digesters.
- They remove the small amounts of O_2 present and create anaerobic conditions.
- These bacteria hydrolyze and ferment the organic materials, e.g., cellulose, starch, proteins, sugars, lipids, etc., and produce organic acids, CO2 and H2.
- But usually only 50% of the polysaccharides present in the waste may be digested.

(2) Syntrophic H2 Producing Bacteria:

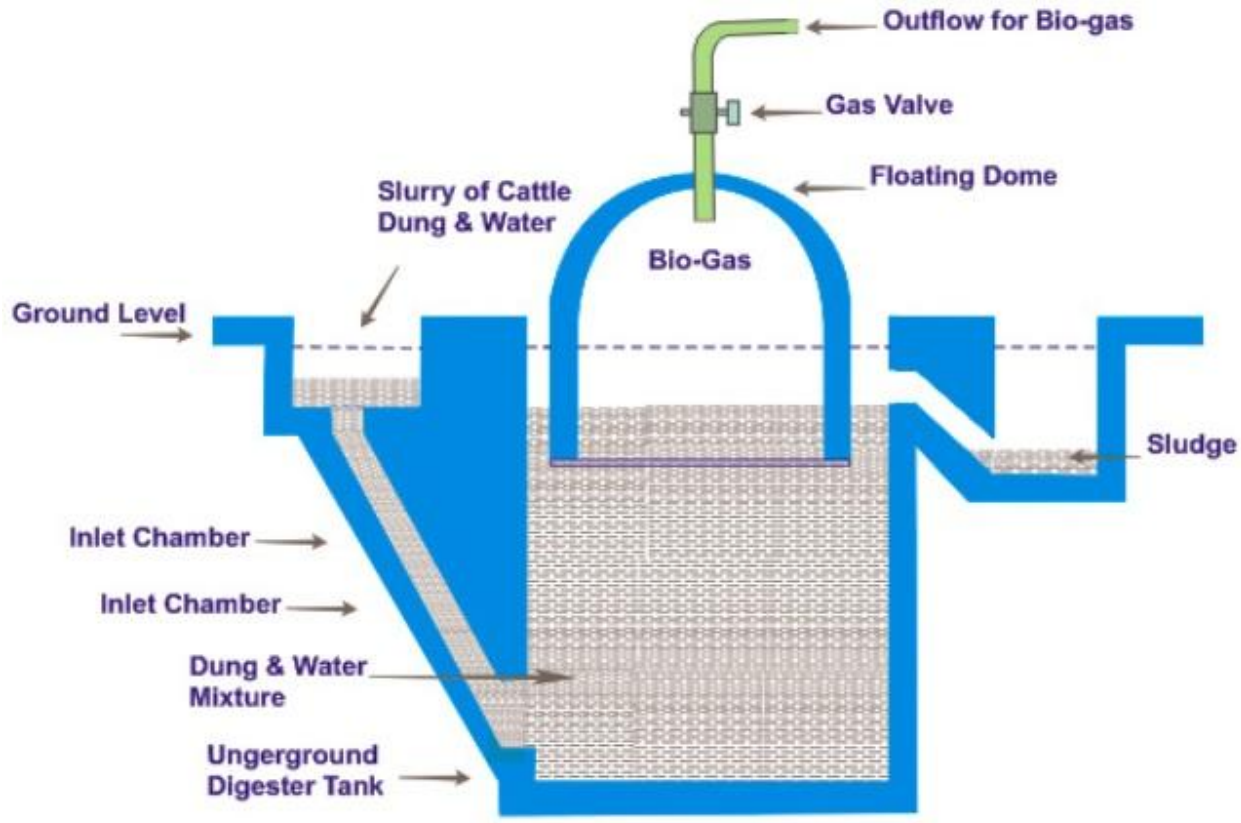
- This group is also called obligate H2 producing or obligate proton reducing bacteria since they oxidise NADH by reducing H+ to H2 and thereby produce hydrogen.
- These bacteria breakdown organic acids having greater than two carbon atoms in their chain to produce acetate, CO2 and H2.
- However, they are able to grow freely and produce H2 only under low H2 partial pressure, which is maintained by methanogens.
- Sewage sludge digesters have about 4 x 10^6 cells/ml of this group. Examples of these bacteria are *Syntrophomonas wolfei, and S. wolinii.*

(3) Methanogenic Bacteria:

- This group of bacteria converts acetate, and CO2 + H2 into methane.
- Thus methanogens remove the H2 produced by obligate H2 producing bacteria, thereby lowering the H2 partial pressure and enabling the latter to continue producing H2.
- Methanogenic bacteria are the strictest possible anaerobes known.
- They may occur up to 10^6-10^8 cell/ml of the slurry in digesters.
- These belong to the new kingdom called Archaebacteria and oxidise H2 by reducing CO2 to obtain energy.
- Examples of methanogenic bacteria are *Methanosarcina barkeri, Methanobacterium omelianskii, etc.*
- 4H2 + CO2 CH4 + 2H2O (ΔG^o = -139 kJ/mol)

(4) Acetogenic Bacteria:

- These bacteria oxidise H2 by reducing CO2 to acetic acid, which is then used up by methanogens to generate methane, CO2 and H2.
- Thus acetogenic bacteria also remove H2 and enable the obligate H2 producing bacteria to continue their function.



Floating Dome type Bio-gas Plant

Advantages of Biogas:

1. The technology is cheaper and much simpler than those for other biofuels, and it is ideal for small scale application.
2. Recovery of the product (methane) is spontaneous as the gas automatically separates from the substrates.
3. Dilute waste materials (2-10% solids) can be used as substrate.
4. Organic pollutants are removed from the environment and used to generate useful biogas; this helps clean up the environment.
5. Aseptic conditions are not needed for operation.
6. Any biodegradable matter can be used as substrate.
7. Biogas is suitable for heating boilers, firing brick and cement kilns, and for running suitably modified internal combustion engines.
8. There is reduced risk of explosion as compared to pure methane.
9. Anaerobic digestion inactivates pathogens and parasites, and is quite effective in reducing
10. the incidence of water borne diseases.

Disadvantages of Biogas:

1. The product (biogas) value is rather low; this makes it an unattractive commercial activity.

2. The biogas yields are lower due to the dilute nature of substrates.
3. The process is not very attractive economically (as compared to other biofuels) on a large industrial scale.
4. Recombinant DNA technology and even strain improvement techniques can not be used to enhance the efficiency of the process.
5. The only improvement in the process, can be brought about by optimising the environmental conditions of the anaerobic digestion.
6. Biogas contains some gases as impurities, which are corrosive to the metal paris of internal combustion engines.

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BIODEGRADATION

- The United States Environmental Protection Agency defines biodegradation as, —A process by which microbial organisms transform or alter (through metabolic or enzymatic action) the structure of chemicals introduced into the environment. In this process organic (carbon-based) material is changed through chemical processes from complex molecules into simpler molecules.
- An example of this is a banana peel being reduced from cellulose to water, carbon dioxide gas, and humus in a compost pile.
- Biodegradation is a waste management and recycling system that degrades everything from yard waste to crude oil. This process helps to keep our planet clean and healthy.

The Biodegradation Process

- Biodegradation is the process by which organic substances are broken down into smaller compounds using the enzymes produced by living microbial organisms.
- The microbial organisms transform the substance through metabolic or enzymatic processes.
- Although biodegradation processes vary greatly, the final product of the degradation is most often carbon dioxide and/or methane.
- Biodegradable matter is generally organic material such as plant and animal matter and other substance originating from living organisms, or artificial materials that are similar enough to plant and animal matter to be put to use by microbes.
- Some microorganisms have naturally occurring catabolic processes that can degrade a huge range of compounds including hydrocarbons (e.g. oil), polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), pharmaceutical substances, radionuclides and metals.

Organic materials can be degraded aerobically, with oxygen, or anaerobically, without oxygen.

Bioremediation: It can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. Bioremediation may be employed to attack specific soil contaminants, such as degradation of chlorinated hydrocarbons by bacteria. An example of a more general approach is the cleanup of oil spills by the addition of nitrate and/or sulfate fertilisers to facilitate the decomposition of crude oil by indigenous or exogenous bacteria

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THANK YOU

AGRIL. MICRO 6.2

BIOPESTICIDES AND BIOFERTILIZERS

(2+1)

COURSE CONTENT

PART I: BIOPESTICIDES

1. HISTORY AND CONCEPT OF BIOPESTICIDES

What is Biopesticide?

Bio means involving life or living organisms. Pesticide includes substance or mixture of substances intended for preventing, destroying or controlling any pest. Biopesticide refers introduction of any living organism such as microorganism, parasitoids, predators, botanicals, biochemical etc that controls pests by biological non-toxic means e.g. *Bacillus thuringiensis*, *Beauveria,* NPV, EPN, *Trichograma,* Hormones etc. All the living organisms or it's by products, which are used and exploited for the control of pest/insect, are called biopesticides.

How biopesticides work?

Biologicals are used to control pests, pathogens and weeds by a variety of means. Microbial biocontrols may include a pathogen or parasite that infects the target. Alternatively, they might act as competitors or inducers of plant host resistance. Biochemical biocontrols can also act through a variety of mechanisms. Some act by inhibiting the growth, feeding, development or reproduction of a pest or pathogen. Still other biocontrols may be used to form a barrier on the host, so as to act as a feeding or infection inhibitor.

History of Biopesticides

Plant extracts were likely the earliest agricultural biocontrols, as history records that nicotine was used to control plum beetles as early as the 17th century. Experiments involving biological controls for insect pests in agriculture date back as far as 1835, when Agostine Bassi demonstrated that white muscadine fungus (*Beauveria bassiana*) could be used to cause an infectious disease in silkworm. Experiments with mineral oils as plant protectants were also reported in the 19th century. During the rapid institutional expansion of agricultural research during the early 20th century, an ever-growing number of studies and proposal for biocontrols were developed.

The first, and still most, widely used biocontrols included spores of the bacteria *Bacillus thuringiensis* (Bt). In 1901, Bt was isolated from a diseased silkworm by Japanese biologist Shigetane Ishiwata. Ernst Berliner in Thuringen, Germany, then rediscovered it ten years later in a diseased caterpillar of flour moth. The Bt pathogen was classified in 1911 as type species *Bacillus thuringiensis* and remains the most widely used biocontrols to this day.

In the early 1920s, the French began to use Bt as a biological insecticide. The first commercially available Bt product, Sporeine, appeared in France in 1938. In the US in the 1950s, widespread use of biocontrols began to take hold as a host of research on Bt efficacy was published.

In the latter half of the 20th century, research and development continued at a low level because of the widespread adoption of cheaper but more toxic synthetic chemical insecticides. During this time, new products were developed and applied; especially in niche markets where petroleum based chemicals were not registered, not effective, or not economical. For example, in 1956, the Pacific Yeast Product Company developed an industrial process known as submerged fermentation, which allowed production of Bt on a large scale. In 1973, *Heliothis* NPV was granted exemption from tolerance and the first viral insecticide, Elcar received a label in 1975.

In 1977, *Bacillus thuringiensis* var. *israelensis* (toxic to flies) was discovered, and in 1983 the strain *tenebrionis* (toxic to beetles) was found. In 1979, the U.S. EPA registered the first insect pheromone for use in mass trapping of Japanese beetles. In the 1990s, researchers began testing kaolin clay as an insect repellent in organic fruit orchards. It was made commercially available, particularly for use in organic systems, in 1999.

Biological development for the control of plant diseases has undergone a similar transformation. During the early 20th century, studies of soil microbiology and ecology had led to the identification of many different microorganisms that act as antagonists or hyperparasites of pathogens and insect pests. A number of these were shown to be useful in field scale inoculations, but few were developed commercially because of the rapid adoption of chemical pesticides during that time period. Commercial success stories from the 1980s and 1990s include products containing *Agrobacterium radiobacter* for the prevention of crown gall on woody crops and *Pseudomonas fluorescens* for the prevention of fireblight in orchards where the streptomycin had been overused and resistant pathogen populations were abundant. In the greenhouse and potting mix industry, products containing a variety of microbes that suppressed soilborne pathogens were introduced into the market.

As the costs of overusing such synthetic chemicals became apparent, there was resurgence in academic and industrial research related to biocontrols development. And with the rapid expansion of organic agriculture during the past decade, adoption rates have rapidly increased. Because of this, development of new and useful biocontrols has continued to increase rapidly since the mid 1990s. In fact, more than 100 biocontrols active ingredients have been registered with the U.S. EPA Biologicals division since 1995. Many of these have been introduced Biologicals division since 1995. Many of these have been introduced commercially in a variety of products. Many of the active ingredients currently approved for use in the U.S.A. can be found in publicly available databases.

Some examples of biocontrols developed in more recent years

***Agrobacterium radiobacter* Strain K84**

Agrobacterium radiobacter Strain K84 is a naturally occurring bacterium found in many soils and in plant root zones. This biocontrols is used in the greenhouse and nursery environment to control crown gall, an important plant disease.

Bacillus spp.

Bacillus licheniformis, B. pumilus, and *B. subtilis* are naturally occurring soil bacteria with fungicidal properties that together have become one of the fastest growing biocontrols in today's market. Successes include uses as seed treatments or dressings, foliar application and soil-applied control of diseases in a variety of crops.

Paecilomyces lilacinus

Paecilomyces lilacinus is used to control nematodes that attack plant roots in field crops including many vegetables, fruit, turf, and ornamental crops.

***Trichoderma* spp**.

Trichoderma spp. is another biocontrols technology developed in the 1990 s that has been widely commercialized in recent years. *Trichoderma*is a genus of fungi that helps to control plant disease by stimulating plant host defenses and growth, and, under certain conditions, parasitizing harmful fungi within the plant root zone.

Azadirachtin

Azadirachtin is an insect growth regulator derived from neem tree seeds. Known to affect some 200 species of insects, *azadirachtin* disrupts insect feeding and inhibits its ability to molt as it changes from the pupa to adult stage.

Beauveria bassiana

Beauveria bassiana is a naturally occurring soil fungus that grows as white mold. This insect pathogen can be used to control a wide range of target pests, which become infected and develop white muscadine disease, killing the pest within a matter of days.

***Cydia pomonella** granulo virus* (CpGV)

CpGV is a natural pathogen of the codling moth, a major pest of tree fruits such as apples and pears. Developed through research begun in the 1980's, commercial use of CpGV in both organic and conventional systems has gained in popularity over the last ten years as codling moth has displayed resistance to many traditional insecticides.

Dysphania ambrosioides

An extract of the plant *Dysphania ambrosioides (*syn. *Chenopodium ambrosioides)* is used to control a number of sucking insect pests such as aphids, leafhoppers, whiteflies, and mites in citrus, grapes, tree nuts, and vegetables. This product breaks down the pest's exoskeleton, adversely affects its respiratory system, and interrupts its ability to navigate (find food).

2. IMPORTANCE, SCOPE AND POTENTIAL OF BIOPESTICIDE

During recent years the concept of integrated pest management is arise but still farmers have continued to rely heavily on chemical insecticides for pest control. The intensive and indiscriminate use of insecticides for crop protection has created problems such as resistance of insects to insecticides, resurgence and toxic residue in / on the crop plants and agro-ecosystem pollution etc and also have adverse effects on the non target organisms such as pollinators, parasitoids, predators and wild animals.

In order to overcome these problems, biological control is one of the safe approaches for pest management. Insect control through an eco-friendly manner is no longer a dream because many biological, botanical and natural pesticides are promoted successfully in the world market by now. Insect pathogens are effectively demonstrated for pest control on different crops, since the Second World War.

The chemical insecticides are valuable for control of insects, but due to their continuous and over usage during last few decades have posed several serious problems.

- Development of resistance in pests.
- Resurgence of minor pests.
- Toxicity in the environment, Accumulation of pesticides residues.
- Biological magnification of pesticide residues through the food chain.
- General environmental pollution

In view of the several disadvantages associated with the unscientific use of pesticides in agriculture, there is an urgent need for minimizing the use of chemical pesticides in the management of insect pests through eco-friendly pest management tactics such as biological control or biopesticide. Biopesticide aims at suppressing the pest in environmentally safe manner without affecting other non target organisms. It provides the most effective, environmentally sound and socially acceptable methods of managing diseases, pests and weeds".

Biopesticides:

In nature every ecosystem exists in a balance. Growth and multiplication of each organism depends on the food chain, its predators, parasites, etc. In biological control system, these interrelations are exploited. The natural enemy of a pest, disease or weed is selected; its biology is studied for mass multiplication and utilizes the same to check the target pest. They are also specific in their action and perish once their feed (i.e. the pest) is exhausted. Thus they are based on natural principles; do not leave any residue, safe and economical. Among the alternatives, biological control of pests is one of the important means for checking pest problems in almost all agro-ecological situations.

Bio pesticides are living organisms, which can intervene the life cycle of insect pests in such a way that the crop damage is minimized. The agents employed as biopesticides, include parasites, predators and **disease causing fungi, bacteria and viruses**, which are the natural enemies of pests.

Utilization of naturally occurring parasites, predators and pathogens for pest control is a classical biological control. On the other hand, these bio agents can be conserved, preserved and multiplied under Laboratory condition for field release. Once these bio-agents are introduced in the field to build their population considerably, they are capable of bringing down the targeted pest' population below **economic threshold level (ETL)**. However, the crux lies in their mass production and application at the appropriate time.

Advantages of biopesticides over chemical pesticides:

Biopesticides are preferred over chemical pesticides for the following reasons:

- No harmful residues.
- Target specific and safe to beneficial organisms like pollinators, predators, parasites etc.
- Growth of natural enemies of pests is not affected, thus reducing the pesticide application.
- Environmental friendly.
- Cost effective for long term use.
- Important component of IPM as 1st line and 2nd line of defense, chemicals being the last resort.

Difference between chemical pesticide and biopesticide

Factors	**Synthetic Pesticides**	**Bio-pesticides**
Cost effectiveness	Cheap but increased spraying cost	Costlier but reduced number of applications
Persistence and residual effect	High	Low
Knockdown effect	Immediate	Delayed
Handling and Bulkiness	Easy but danger and Hazardous	Bulky : Carrier based Easy : Liquid formulation
Pest resurgence	More	Less
Effect on Beneficial flora	More harmful	Not harmful
Target specificity	Mostly broad spectrum	Mostly host specific
Nature of control	Curative	Preventive
Shelf life	More	Comparatively Less

The market share of bio-pesticide is only 2% as compared to synthetic pesticide

Last three decade has witnessed a tremendous breakthrough in this aspect, especially on standardization of production techniques of ***Trichoderma, Gliocladium, Paecilomyces, Pseudomonas, NPV and Bacillus*** spp. to use them against many insect pests and diseases. The popularity of biopesticides has increased in recent years, as extensive and systematic research has greatly enhanced their effectiveness. Also, techniques for the mass production, storage, transport and application of biopesticides have been improved in recent years.

Commercial production of biopesticides:

Though there are more than about 300 biopesticide production units existing in the country, as on today, they are able to meet the demand of only less than 10% of cropped area. There exists a wide gap, which can only be bridged by setting up of more and more units for production of biopesticides. This requires large-scale investment and private participation. Some of the local small-scale industries have already started production and marketing of *Beauveria bassiana, Metarhizium anisopliae, Trichoderma spp., Paecilomyces lilacinus, and* NPV. There is a scope to enhance production and use of biological control agents in the days to come as the demand is on the increase every year.

Few microbial pesticides marketed in India:

Sr. No.	Name of Agent/s	Common Product Names
1	*Bacillus thuringiensis*	Delfin, Halt, Dipel, Biolap etc.
2	Nuclear polyhedrosis virus (NPV)	*Ha*NPV, *Sl*NPV
3	Muscardine fungi, *Beauveria bassiana* and *Metarhizium anisopliae*	Basina, Biosoft, *Metarhizium, Beauveria* spores
4	*Trichoderma viride* *T. harzianum*	Tricho-XP, Mnitor WP, Monitor S, Trichomycil, T-2 Trichoderma spore powder and granules, etc.
5	*Pseudomonas fluorescence*	*P. fluorescence* wattable powder
6	*Paecilomyces lilacinus*	Yorker, Tcicho X-P (Combi product)
7	Entomopathogenic nematodes	Green commando, Soil commando, *Steinernema* sp.

(More than 50 products are now available in India out of which majorities are produced by small-scale regional producers and few products are also from State Agricultural Universities, but majority of them are not registered products OR having provisional registrations)

Characteristics for development of a biopesticide:

- **Efficacy:** A highly effective biocontrol strain or other material must be obtained or produced**.** Such strains must not only have appropriate mechanisms for biocontrol, but it should also (a) be able to compete and persist in the environment in which it must operate, and (b) ideally, be able to colonize and proliferate after application. But better to use existing natural strains, which should be sufficiently effective.
- **Cost:** Inexpensive or less expensive production and formulation of the biocontrol agent or other material must be developed. The production process must result in biomass with excellent shelf life even under adverse storage conditions.
- **Application:** Delivery and application methods that permit the full expression of the biocontrol agent. Delivery systems must ensure that biocontrol agents will grow well and achieve their purpose. Delivery and application processes must be developed on a crop by crop and application by application basis. No general solutions exist, and so biocontrol systems must be developed for each crop. An effective method of use is to use the biocontrol

agent in conjunction with chemical pesticides. The chemicals provide good short-term protection and the biocontrol provides long-term protection. As a consequence, yields frequently are increased over use of the chemical alone.

- **Market Potential:** Considering the negative effects of indiscriminate case of pesticides, importance for organic farming and promotion of sustainable farming practices it is estimated that there will be further scope for new units, particularly in the states of Maharashtra, Gujarat, Rajasthan, Madya Pradesh, Tamil Nadu, AP, UP, West Bengal and Karnataka, where crops such as sugarcane, pulses, cereals and vegetable crops are grown in large scale. At present, in some states, state government is purchasing the product from the private parties and selling it to the individual farmers at a subsidized rate.

Future prospects for biopesticide research, production and use in India:

Due to chemical pesticide problems in India, there is an urgent need to promote environmental friendly biopesticides in the country. Moreover recent Government policies also favor production and use of biopesticide.

- More potential industrial fermentation technique for production of biopesticides needs to be developed to fulfill the ever increasing demand in the market.
- Large-scale field demonstrations at farmer's fields are required to increase awareness and adoption of biopesticides for pest management.
- Use of effective biopesticides should be accelerated in integrated management and economics needs to be worked out.
- Interest to be focused on developing new methods of biological control. In particular, identification of proteins and the genes encoding them from various microbial agents and newer microbes.

3. DEFINITIONS AND CLASSIFICATION OF BIOPESTICIDES

The term biopesticides defines compounds that are used to manage agricultural pests by means of specific biological effects rather than as broader chemical pesticides. It refers to products containing biocontrol agents – i.e., natural organisms or substances derived from natural materials (such as animals, plants, bacteria, or certain minerals), including their genes or metabolites, for controlling pests. According to the FAO definition, biopesticides include those biocontrol agents that are passive agents, in contrast to biocontrol agents that actively seek out the pest, such as parasitoids, predators, and many species of entomopathogenic nematodes. Thus biopesticides cover a wide spectrum of potential products that can be classified in major four groups as:

1. **Microbial pesticides**
2. **Botanical pesticides**
3. **Biochemical/Semiochemical pesticides**
4. **Plant incorporated Protectants (PIPS).**

Classification of biopesticides:

1. Microbial pesticides and other entomopathogens:

Pesticides that contain microorganisms, like bacteria, fungi, or virus, which attack specific pest species, or entomopathogenic nematodes as active ingredients. Although most of these agents attack insect species (called entomopathogens; products referred to as bioinsecticides), there are also microorganisms (i.e., fungi) that control weeds (bioherbicides). They suppress pest by producing a toxin specific to the pest, causing a disease or preventing establishment of other microorganisms through competition or other modes of action.

Microbial insecticides are a new form of pesticide that works by infecting selected insect populations. Though this sounds potentially dangerous, but it is actually safe, since the application of microbial insecticides is specific to the species one is trying to kill. Microbial insecticides have no effect on animal populations, unless diminishing a certain bug in the area interrupts the food chain. Each type usually works against only one type of insect.

Types of microbial pesticides:

There are five main categories of microbial pesticides based on the active ingredient used as bellow.

1) Bacteria based: Bacterial biopesticides are probably the most widely used and cheaper than the other methods of pest bioregulation. Insects can be infected with many species of bacteria but those belonging to the genus *Bacillus* are most widely used as pesticides.

***Bacillus thuringiensisI* (Bt)**:

- Discovered in Japan in early 20th century and first become a commercial product in France in 1938.
- Control lepidopterous pests like American bollworm in cotton and stem borers in rice.
- Bt has developed many molecular mechanisms to produce pesticidal toxins i.e. Cry and VIP; most of toxins are coded for by several *cry* genes.

- Since its discovery in 1901 as a microbial insecticide, Bt has been widely used to control insect pests important in agriculture, forestry and medicine.
- When ingested by pest larvae, Bt releases toxins which damage the mid gut of the pest, eventually killing it.
- Main sources for the production of Bt preparations are the strains of the subspecies kurstaki, galeriae, dendrolimus for agriculture use.

***Bacillus popilliae*:**

- A naturally occurring bacterium that is used for control of white grub.
- Cause 'milky spore disease' in the larvae of the grub or beetle and establish a resident population capable of causing mortality over several seasons if soil conditions are appropriate.
- *Bacillus popilliae* was the first insect pathogen to be registered in the U.S. as microbial control agent.

2) Fungi based: Fungi often act as important natural control agents that limit insect populations. Most of the species that cause insect diseases spread by means of asexual spores called conidia. Where viable conidia reach a susceptible host, free water or very high humidity is usually required for germination. Unlike bacterial spores or virus particles, fungal conidia can germinate on the insect cuticle and produce specialized structures that allow the fungus to penetrate the cuticle and enter the insect's body. In most instances, as fungal infections progress, infected insects are killed by fungal toxins, not by the chronic effects of parasitism. Many important fungal pathogens attack eggs, immatures, and adults of a variety of insect species.

***Beauveria bassiana*:**

- This common soil fungus has a broad host range that includes many beetles and fire ants. It infects both larvae and adults of many species.
- Habitat: Foliage and soil
- Insect Host: White flies, beetles & caterpillars (including *Helicoverpa* sp.)

Metarhizium anisopliae:

- This common soil fungus has a broad host range that includes many beetles and other soft body insects.
- Habitat: Foliage and soil
- Insect host: Frog hoppers, beetles

Vericillium lecanii:

- This fungus has been used in greenhouses to control aphids and whiteflies.
- Habitat: Glasshouse foliage
- Insect host: Aphids, whiteflies & scales.

Nomuraea rileyi:

- Naturally occurring epidemic infections of *Nomuraea rileyi* cause dramatic reductions in populations of soybean foliage-feeding caterpillars.

Hirsutella thompsonii:

- It is a pathogen of the citrus rust mite.

3) Virus based:

- The larvae of many insect species are vulnerable to devastating epidemics of viral diseases.
- The viruses that cause these outbreaks are very specific, usually acting against only a single insect genus or even a single species.
- Specificity of the virus-host relationship makes viruses an ideal candidate for use in the control of specific pest population.
- The most important group of viruses used for biocontrol belongs to the highly host-specific family of *Baculoviridae*, which are pathogenic for invertebrates.
- Baculoviruses have been found in over 700 species of invertebrates, mainly Lepidoptera and are considered an effective and selective means for biological insect control.
- Based on the morphology of their occlusion bodies (OBs), they can be distinguished between nucleopolyhedroviruses (NPVs) and granuloviruses (GVs).
- The OBs are composed of a crystalline protein matrix, mainly consisting of a single protein, the so-called 'polyhedrin' in NPVs and 'granulin' in GVs.

A. Polyhedrosis virus

- Polyhedrosis virus where many virus particles are embedded in each protein crystal.
- When they occur in the host cell nucleus, they are called Nuclear Polyhedrosis Virus (NPV).
- When they occur in the host cytoplasm they are called Cytoplasmic Polyhedrosis Virus (CPV).

B. Granuloisis virus

- Granulosis virus where only one virus is contained in each protein crystal.
- They either develop in the nucleus or cytoplasm of the host cells (NGV or CGV).

- The viral DNA replicates in the nuclei of the host cells and then spreads throughout the body of the larvae, turning it into a "virus factory."
- The infected insect stops feeding within a few days, dies and disintegrate.
- *Ha*NPV (Helicoverpa NPV): It is highly effective on *H. armigera*, pest of cotton, gram, pea, pigeon pea, tomato, cabbage, ground nut, millets and oilseeds.
- *Sl*NPV (Spodoptera NPV): It is highly effective against *S. litura* caterpillar, pest of cotton, gram, pigeon pea, cabbage, tomato, chillies & oilseeds.

4) Nematode based:

- Entomopathogenic nematodes (EPNs) are insect-specific parasites in the genera *Steinernema* (Steinernematidae) and *Heterorhabditis* (Heterorhabditidae) that are obligatory associated with symbiotic bacteria (*Xenorhabdus* spp. and *Photorhabdus* spp., respectively) which are responsible for rapidly killing host insects.
- After entering a host insect, the infective juvenile stage (IJ) of EPNs releases its symbiotic bacteria. In addition to killing the host, the bacteria digest host tissues, and produce antibiotics to protect the host cadaver from saprophytes and scavengers.

- After two to three reproductive cycles, when host nutrients are depleted, infective juveniles are produced and begin leaving the host insect. This stage is capable of immediately infecting a new host or may persist for months in the absence of a host.
- The entomogenous nematodes *Steinernema feltiae, S. riobravis, S. carpocapsae* and *Heterorhabditis heliothidis* are the species most commonly used in insecticidal preparations.
- Within each of these species, different strains exhibit differences in their abilities to infect and kill specific insects.
- In general, however, these nematodes infect a wide range of insects.
- On a worldwide basis, laboratory or field applications have been effective against over 400 pest species, including numerous beetles, fly larvae, and caterpillars.

5) Protozoa based:

- Protozoa are single-celled eukaryotic organisms that exist in both water and soil.
- The protozan *Nosema locustae* is known to be a natural biocontrol agent of many grasshopper species.
- Nosema infects at least 90 species of grasshoppers.
- It is non-toxic to humans and other mammals.
- It infects and weakens young grasshoppers and adversely affects female grasshoppers' ability to reproduce.

2. Botanical pesticides:

- These are naturally occurring plant material that may be crude preparation of the plant parts ground to produce a dust or powder or liquid that can be used in full strength or dilute form in a carrier such as clay, talc or diatomaceous earth.
- Several plant based insecticides as nicotinoids, natural pyrethrins, rotenoids, neem products etc are used.
- They are generally acting in one of two ways 1) Contact poison and 2) Stomach poison.
- There are about 25,000 plant species evaluated among which, 2500 found useful for pest management and around 1005 exhibited insecticidal activity including 384 anti-feedants, 297 repellents, 27 attractants and 31 possess growth inhibiting properties.
- Neem tops the list of 2,500 plant species that are reported to have pesticidal properties and is regarded as the most reliable source of eco-friendly biopesticidal property.
- "Azadirachtin" affects the reproductive and digestive process of pest.
- Neem products (Neem Seed Kernel Extract: NSKE) are effective against more than 350 species of arthropods, 12 species of nematodes, 15 species of fungi, three viruses, two species of snails and one crustacean species.
- Majority of formulations contain 300 to 1,500 ppm azadirechtin.
- Recent formulations contain 10,000 to 50,000 ppm.
- *Chrysanthemum* (*Tanacetum*), Nicotiana, Derris are other members.

Examples:

Name	Plant part used	Active ingredients
Neem (*Azadirachta indica*)	Oil and leaves	Azadirachtin
Castor (*Ricinus communis*)	Leaves and oil	Ricin
Onion (*Allium cepa*)	Bulb	Oleic acid, α asarone, β asarone
Custard apple (*Annona squamosa*)	Leaves and bark	Annonin, squamocin

Some of the plant product registered as biopesticides

Plant product used as biopesticides	Target Pests
Limonene and Linalool	Fleas, aphids and mites, also kill fire ants, several types of flies, paper wasps and house crickets
Neem	A variety of sucking and chewing insect
Pyrethrin	Ants, aphids, roaches, fleas, flies, and ticks
Rotenone	Leaf-feeding insects, such as aphids, certain beetles (asparagus beetle, bean leaf beetle, Colorado potato beetle, cucumber beetle, flea beetle, strawberry leaf beetle, and others) and caterpillars, as well as fleas and lice on animals
Ryania	Caterpillars (European corn borer, corn earworm, and others) and thrips
Sabadilla	Squash bugs, harlequin bugs, thrips, caterpillars, leaf hoppers, and stink bugs

3. Biochemical/Semiochemical pesticides:

The chemical messages that trigger various behavioral responses are collectively referred as semiochemicals. Pesticides based on these naturally occurring substances that control pests by non-toxic mechanisms, in contrast to chemical pesticides that contain synthetic molecules that directly kill the pest. Biochemical pesticides include substances that interfere with growth or mating, such as insect growth regulators, or substances that repel or attract pests, such as pheromones.

Pheromones:

Biochemical pesticides include substances, such as insect sex pheromones, which interfere with mating, as well as various scented plant extracts that attract insect pests to traps. Man-made pheromones are used to disrupt insect mating by creating confusion during the search for mates or can be used to attract male insects to traps. Pheromones are often used to detect or monitor insect populations or in some cases, to control them.

Pheromones are chemicals emitted by living organisms used to send messages to individuals usually of the opposite sex of the same species. Pheromones of hundreds of insect species have been chemically elucidated, including the sex pheromone. When used in

combination with traps, sex pheromones can be used to determine what insect pests are present in a crop and what plant protection measures or further actions might be necessary to assure minimal crop damage. If the synthetic attractant is exceptionally effective and the population level is very low, some control can be achieved with pheromone traps or with the "attract and kill" technique.

Insect Growth Regulators:

An insect growth regulator (IGR) is a substance (chemical) that inhibits the life cycle of an insect. Two main groups of insect growth regulators (IGRs) being used in a commercial scale are the juvenile hormone analogues (JHAs) and the chitin synthesis inhibitors (CSIs).

JHA:

- Five different formulations of methoprene are available commercially for the control of mosquitoes, flies, beetle ants and fleas.
- Another, JHA, fenoxycarb, used for control of several fruit pests.
- Pyriproxyfen effective against sucking pests.
- Diofenolan for control of scale insects and lepidopteran pests.

CSI:

- Acyl urea group used for control of foliage feeders and tissue borers.
- Diflubenzuron used against several pests of cotton.

4. Plant-Incorporated Protectants (PIPs):

These include pesticidal substances that are produced in genetically modified plants/organisms (GMO) (i.e., through the genetic material that has been incorporated into the plant). One approach, to reduce destruction of crops by phytophagous pests, is to genetically modify plants to express genes encoding insecticidal toxins. The adoption of genetically modified (GM) crops has increased dramatically in the last years. Genetically modified (GM) plants possess a gene or genes that have been transferred from a different species.

The production of transgenic plants that express insecticidal δ-endotoxins derived from the soil bacterium *Bacillus thuringiensis* (Bt plants) were first commercialized in the US in 1996. The expression of these toxins confers protection against insect. The lethality of Bt endotoxins is highly dependent upon the alkaline environment of the insect gut, a feature that assures these toxins are not active in vertebrates, especially in humans. These proteins have been commercially produced, targeting the major pests of cotton, tobacco, tomato, potato, corn, maize and rice, notably allowing greater coverage by reaching locations on plants which are inaccessible to foliar sprays.

There are numerous strains of Bt, each with different Cry proteins and more than 375 Cry proteins have been identified. Most Bt maize hybrids express the Cry1Ab protein, and a few express the Cry1Ac or the Cry9C protein, all of which are targeted against the European corn borer (*Ostrinia nubilalis* Hubner) (Lepidoptera), a major pest of maize in North America and Europe. Some recent maize hybrids express the Cry3Bb1 protein, which is targeted against the corn rootworm complex (*Diabrotica* spp.).

Cotton expressing the Cry1Ac protein is targeted against the cotton bollworm (*Helicoverpa*), which is a major pest of cotton; potato expressing the Cry3A or Cry3C is targeted against the Colorado potato beetle (*Leptinotarsa decemlineata* Say) (Coleoptera), which is a major pest of potato; and Cry4 proteins are targeted against some Diptera, such as certain flies (e.g., *Lycoriella castanescens* Lengersdorf) and mosquitoes (e.g., *Culex pipiens* L.).

4. VIRULENCE, PATHOGENICITY AND SYMPTOMS OF ENTOMOPATHOGENIC PATHOGENS AND NEMATODES

1. Entomopathogenic bacteria

1) *Bacillus thuringiensis*

The most commonly used microbial agents to control noxious insects world over is *Bacillus thuringiensis.* The efficacy of Bt is quite impressive than other biopesticide and easily available in market. Amongst all insect pathogenic microbes, Bt has received prime attention and the product based on this bacterium is widely demonstrated among all the biological insecticides.

Bt is a gram positive, aerobic, sporulating bacterium that synthesizes crystalline proteins during later stage of growth as secondary metabolites which is highly toxic to agriculturally important pest especially caterpillars belonging to lepidopterans at very low concentration. The outstanding feature of Bt is the production of protenaceous delta (δ) endotoxin (crystal) that accounts up to 30 % of total protein content of the bacterium.

The crystal contains insecticidal proteins that may vary in type, quantity as well as toxicity against different groups of insects depending on the bacterial strain and place of isolation. Bt δ endotoxin (cry proteins) has acquired acceptability as eco-friendly biopesticides because of its specificity towards insects having alkaline gut and by enlarge safer to higher animals. Bt is under extensive use for control of insects in agriculture, horticulture, forestry and also for mosquito control over past three decades.

Mode of action*:*

The crystal protein of Bt is effective to insects having specific gut pH (alkaline) and /or the specific gut membrane structures required for binding of the toxin. The protein toxin binds to specific receptors present in the midgut epithelial membrane and disrupts membrane's ion exchange potential and osmotic equilibrium causing cell lyses and finally this damage leads to gut paralysis and ultimately cause death of the susceptible host. Infected insect stop feeding and die due to the combined effect like starvation and tissue damage. Bt spores have also capacity to cause disease (septicemia) in the insect by invading in heamocoel system.

The mode of action of *Bacillus thuringiensis* is summarized in the following sequential stages:

1. Ingestion of sporulated *Bacillus thuringiensis* by an insect larva.
2. Solubilization of the crystalline ICP in the midgut at high pH (alkaline, >9.0).
3. Activation of the ICP by gut proteases enzymes.
4. Binding of the activated ICP to specific receptors in the midgut cell membrane.
5. Insertion of the toxin in the cell membrane and formation of pores and channels in the gut cell membrane, followed by destruction of the epithelial cells.
6. Subsequent *Bacillus thuringiensis* spore germination. Septicemia may enhance mortality.

Symptoms:

Larvae infected by Bt become inactive, stop feeding and have watery excretion. The head capsule may appear to be larger compared to body size. The larva becomes flaccid and dies, usually within days. A typical putrefied smell comes out from dead cadavers. The body content

turns brownish-black as they decompose, while in case of other bacteria it may turn red or yellow.

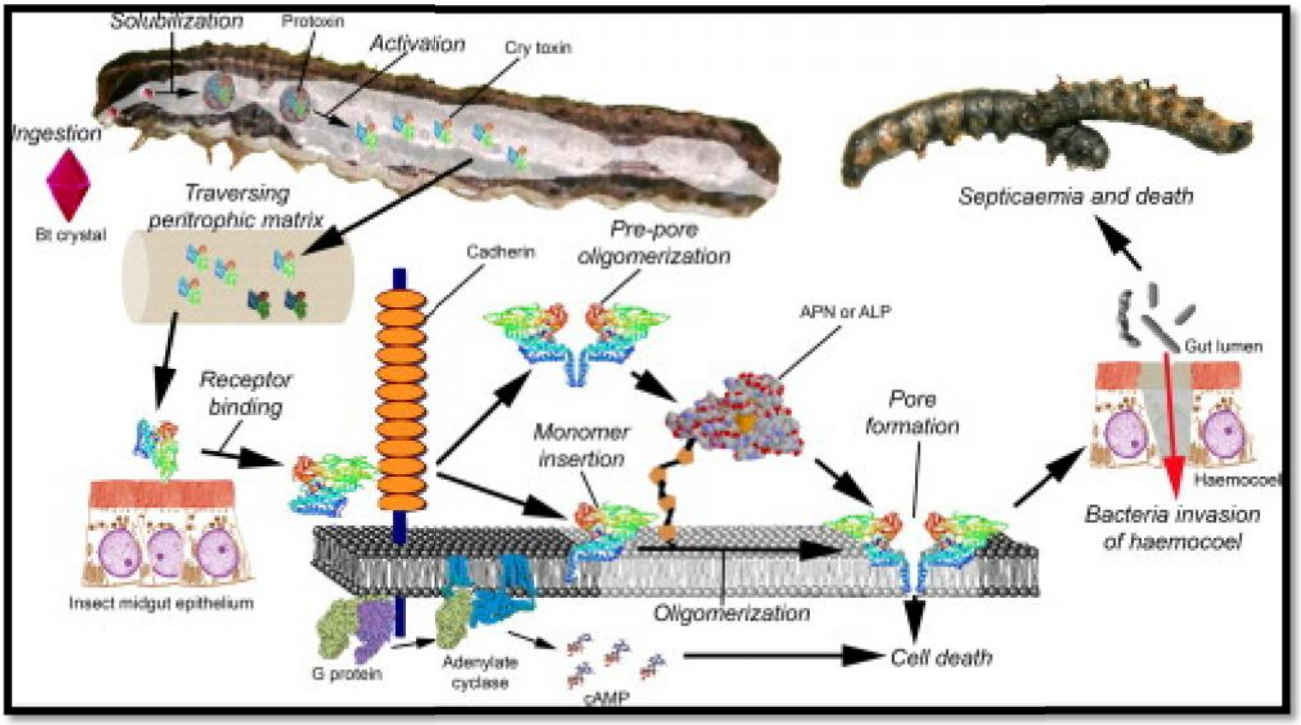


Figure: Mode of Action of *B. thuringiensis* on caterpillars.

Bt was first found to affect a range of lepidopteran insects, the major agricultural pests. Subsequently, discovery of newer strains expanded its host range. The host range of Bt is constantly widening with the large amount of commercial interest and at present, extensive screening programmes are in progress world over. Although most of the Bt strains are toxic to lepidoptera, few strains are known to be toxic against Coleopterans (root–grubs), Dipterans larvae especially mosquitoes, lice, mites and phytonematodes too (Table).

Table: Host range of *B. thuringiensis*

Order	**Susceptible families**	
Insecta:	Most of the lepidopteran families susceptible	
Lepidopterans	Sphingidae	- Hawk moths
	Pieridae	- Cabbage worms
	Lymantridae	- Tussock moths
	Tortricidae	- Leaf roller moth
	Noctuidae	- Cutworms
Dipterans	Culcidae	- Mosquitoes
	Simuliidae	- Black flies
	Anisopodidae	- Gnats
	Chironomidae	- Nudges
	Psychodiae	- Moth flies
	Tipulidae	- Crane flies

Coleopteran	Cyrysomelidae	- Leaf beetles
Phthiraptera	Philopteridae	- Bird lice
	Treichodectidae	- Mammalian lice
Arachnida: **Acari**	Dermanyssidae	- Animal mites
	Tetranichidae	- Plant mites
Nematoda: **Strongylida**	Trichostrongylidae	- Animal parasitic
	Tylenchidae	- Plant parasitic

2) *Bacillus popilliae*

Bacillus popilliae is a highly fastidious bacterium that is the primary etiological agent of the so called milky diseases of scarab larvae. These insects are the immature stages of beetles, such as the Japanese beetle *Popillia japonica*, that are important grass and plant pests belonging to the coleopteran family *Scarabaeidae*.

The term "milky disease" is derived from the opaque white color that characterizes diseased larvae and results from the accumulation of sporulating bacteria in larval hemolymph (blood). The disease is initiated when grubs feeding on the roots of grasses or other plants ingest the bacterial spores. The spores germinate in the midgut and vegetative cells invade the midgut epithelium, where they grow and reproduce, changing in form as they progress toward invasion of the hemocoel (body cavity). After passing through the basement membrane of the midgut, the bacteria colonize the blood over a period of several weeks and sporulate, reaching populations of 10^8cells/ml. For larvae that ingest a sufficient number of spores early in development, the disease is fatal. Dead larvae in essence become bag of spores that serve as a source of infection for up to 30 years.

Despite decades of research, suitable media for the growth and mass production of *P. popilliae in vitro* have not been developed. Thus, the technical material (i.e., spores) used in commercial formulations is produced in living, field-collected scarab larvae.

2. Entomopathogenic fungi

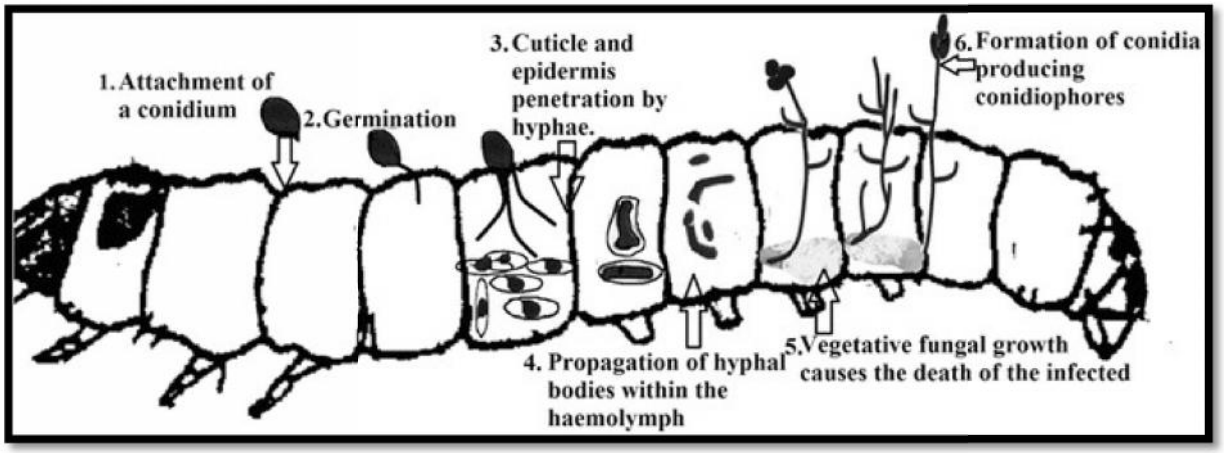
Agostino Bassi in 1835, first time formulated the germ theory by the use of white muscardine fungus on the silkworm that was then named in his honor as Beauveria *bassiana* and gave the idea of using insect infecting fungi for the control of insect pest management.

Mode of action:

The development of fungal infections in terrestrial insect is largely influenced by environmental conditions. High humidity is vital for germination of spores and transmission of the pathogen from one insect to another. Unlike bacteria and virus, which must be consumed, toxicity from entomopathogenic fungi most often occurs from contact of the fungal conidia with the host cuticle. With most entomopathogenic fungi, disease development involves following steps which are popularly known as 'Mycosis':

1. Attachment of the infective units like spore or conidia or zoospores to the insect epicuticle.
2. Germination of the infection unit on the cuticle.

3. Penetration of the cuticle, either directly by germ tube or by infection pegs from appresoria.
4. Multiplication of the hyphal body in the haemocoel.
5. Death of the host: Growth of the mycelia phase with invasion of virtually all host organs and penetration of the hyphae from the interior through the cuticle to the exterior of the insect.
6. Production of infective units on the exterior of the insect.



Salient features of mycosis:

I. **Spore germination:** the adhesion of spores into the insect's integument may be a passive mechanism and involve mucilaginous material. Spore germination largely depends on the environmental humidity and temperature. Optimum temperature for development, pathogenicity and survival of the fungi merely falls between 20 to 30˚C. Very high humidity (~ 80 %) is required for spore germination. the germination spore form a germ tube that serves as a penetration hyphae and an appressorium may also be produced.

II. **Penetration:** the mode of penetration is determined by the characteristics of the integument, its thickness, sclerotization and the presence of antifungal and nutritional substances. The larvae are more susceptible immediately after moulting before the cuticle is fully hardened. the process of penetration through the cuticle by hyphae germinating from spores involves chemical and physical forces. The enzymes detected on germ tubes are proteases, aminopeptidases, lipase, esterase and chitinase. The germ tube produces appressorium, when the integument is hard. Fungi can infect insect through spiracles and other body openings.

III. **Replication:** The fungi produce hyphal bodies and may avoid the immune system of the insect by (i) Developing protoplasts that are not recognized by the haemocysts, (ii) forming hyphal bodies that multiply and disperse rapidly and (iii) by producing mycotoxins. After death of the insect, the fungus grows saprophytically in the haemocoel to form a mycelia mass that forms a hard or firm sclerotium. The reproductive spores are produced within the sclerotium or sporengiophore or conidiophores.

Mycotoxins: Symptoms such as partial or general paralysis, sluggishness, and decreased irritability in mycosed insects are consistent with the action of neuromuscular toxins. *B.*

bassiana and *M. anisopliae* produced significant amounts of toxic compounds within their hosts. For example, the toxins Beauvericin, Bassianolide, Isarolides and Beauverolides have been isolated from *B. bassiana* infected hosts, toxins Destruxins (DTXs) and Cytochalasins have been isolated from *M. anisopliae* infected hosts.

IV. **Spore dispersal:** The spores of many fungi are forcibly discharged and carried by wind. Certain fungi from conidia that are enclosed in mucilaginous slime. Such conidia may attach to a passing insect or other invertebrates for dispersal.

Symptoms of mycosis:

1. Loss of appetite, irritability and paralyses.
2. Discoloured patches on integuments and increased acidity in blood.
3. The body hardens and covered by dense white mycelial mat or growth.
4. Mummified larvae/insect adheres to leaves, stem and fruiting body with upright position on its prolegs at the time of death.
5. Death occurs within 4-7 days depending on host insects and environmental conditions.

Host range:

1. ***Beauveria bassiana***: They are used particularly to control sucking pests and caterpillars infesting crop plants. These fungi are used to control the caterpillars of yellow stem borer and leaf folder of rice, white grub of groundnut, sugarcane pyrilla, coconut rhinoceros beetle, caterpillars of pulses, tomato and cotton, diamond back moth, leaf eating caterpillars of tobacco and sunflower etc.
2. ***Metarrhizium anisopliae*:** This fungus is used to control mainly coconut rhinoceros beetle, groundnut cut worm, rice brown plant hopper, diamond back moth and early shoot borer, top shoot borer and internode borer of sugarcane.
3. ***Lecanicelium lecanii***: This fungus is mainly used to control whiteflies, aphids, thrips, brown plant hopper, scale insects, mealy bugs and other sucking insect pests of crop plants.
4. ***Nomuraea rileyi*:** It is used to control pod borers, cut worms, cabbage borers etc.
5. ***Hirsutella thompsonii*:** These fungi are used to control different hoppers and bug pests, whiteflies, red mites etc.

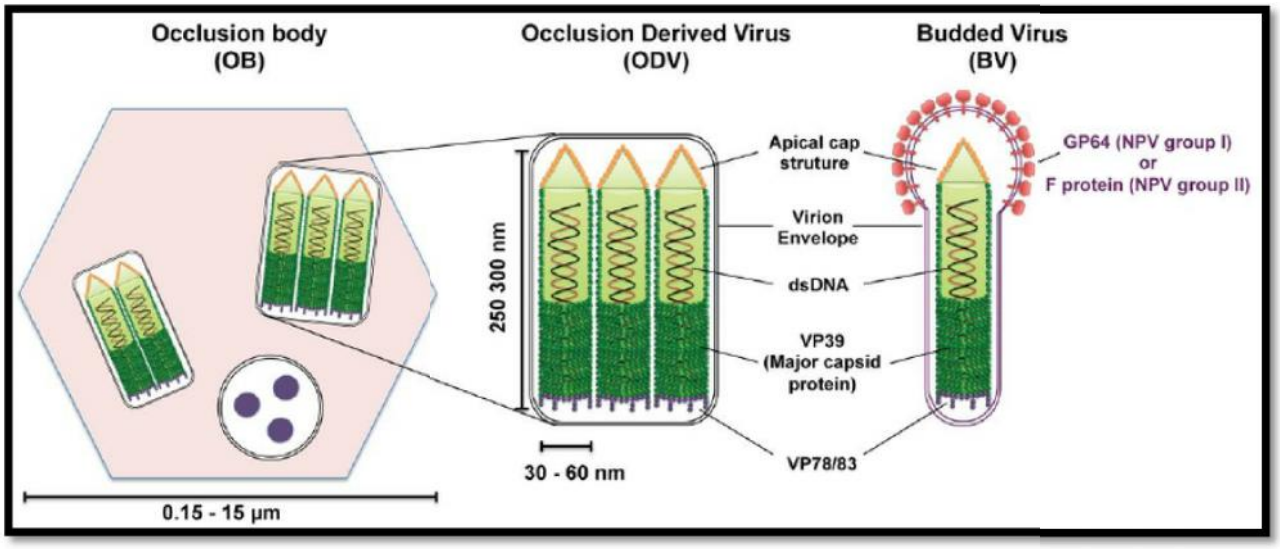
3. Entomopathogenic virus

Viruses in the family Baculoviridae, a diverse family of more than 700 viruses, are the best known of all the insect viruses. This is because they are very common in some of our most important insect pests, disease symptoms are easily recognized and they have the most potential for development as microbial insecticides. The major reason for interest in baculoviruses is their environmental safety. They are often, genus or species specific and safe to non target organisms.

Mode of action / Life cycle of baculoviruses

The baculovirus life cycle involves two distinct forms of virus. Occlusion derived virus (ODV) is present in a protein matrix (polyhedrin or granulin) and is responsible for the primary infection of the host while the budded virus (BV) is released from the infected host cells later during the secondary infection.

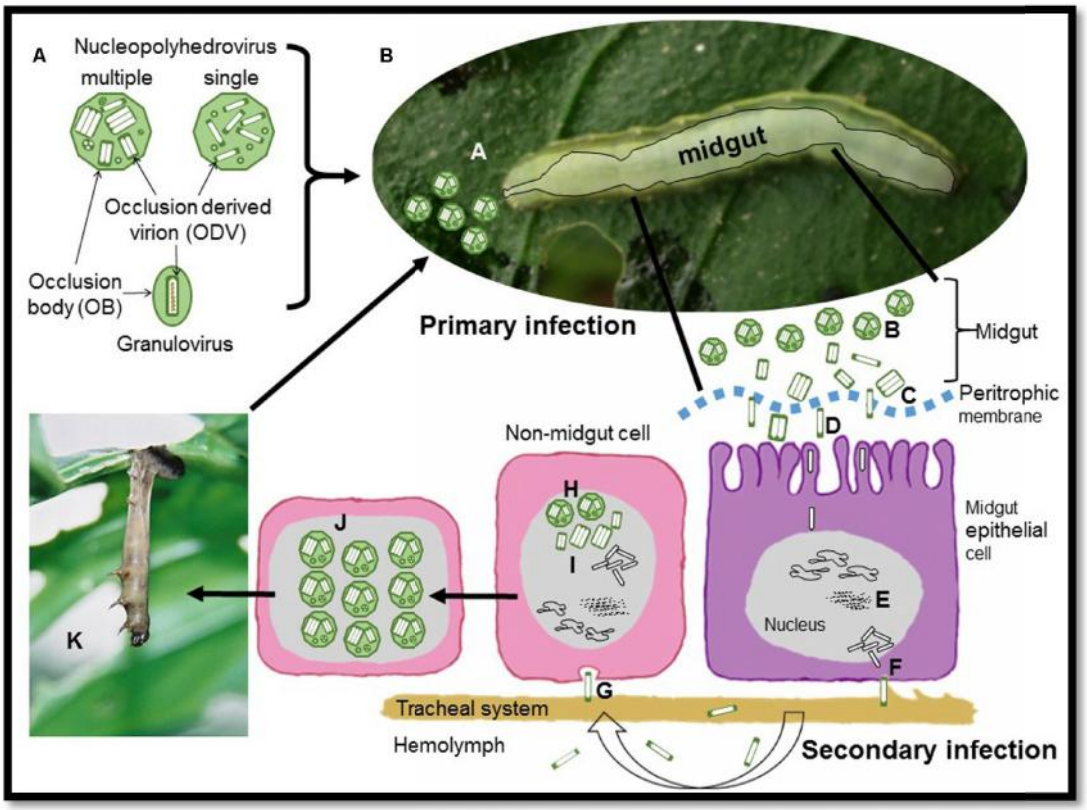
Typically, the initial infection occurs when a susceptible host insect feeds on plants that are contaminated with the occluded form of the virus. The protein matrix dissolves in the alkaline environment of the host midgut (stomach), releasing ODV that then fuse to the columnar epithelial cell membrane of the host intestine and are taken into the cell in endosomes. Nucleocapsids escape from the endosomes and are transported to nucleus. Viral transcription and replication occur in the cell nucleus and new BV particles are budded out from the lateral side to spread the infection systemically. During budding, BV acquires a loosely fitting host cell membrane with expressed and displayed viral glycoproteins.



Baculovirus infection can be divided to three distinct phases: Early (0–6 h post-infection), Late (6–24 h p.i.) and Very late phase (18–24 to 72 h p.i.).

While BV is produced in the late phase, the ODV form is produced in the very late phase acquiring the envelope from host cell nucleus and embedded in the matrix of occlusion body protein. These occlusion bodies are released when cells lyse to further spread baculovirus infection to next host. The extensive lysis of cells frequently causes the host insect to literally disintegrate, thus the reason for the historic name "wilting disease." The complete ODV-polyhedrin particles are resistant to heat and light inactivation, whereas the naked BV virion is more sensitive to environment.

When infecting a caterpillar, the advanced stages of infection cause the host to feed without resting, exhibit negative geotropism and then to climb to the higher parts of trees, including exposed places they would normally avoid due to the risk of predators. This is an advantage for the virus if (when the host dissolves) it can drip down onto leaves which will be consumed by new hosts.



Symptoms:

- Discoloration (brown and yellow)
- Stress
- Decomposition (liquefication)
- Lethargy (slow movement to no movement at all; refusal to eat)
- Hanging body on to the top branches of the plant.

Host range: Larvae of major lepidopteran insect including codling moth, *Spodoptera litura*, *Helicoverpa armigera*, *H. zea*, *H. virescens*, *Autographa californica* and many more.

4. Entomopathogenic nematodes

Entomopathogenic nematodes occur naturally in soil environments and locate their host in response to carbon dioxide, vibration and other chemical cues. They infect many different types of insects living in the soil like the larval forms of moths, butterflies, flies and beetles as well as adult forms of beetles, grasshoppers and crickets. Species in two families, Heterorhabditidae and Steinernematidae have been effectively used as biological insecticides in pest management programs.

Mode of action / Life cycle of EPN

The infective juvenile stage (IJ) is the only free living stage of entomopathogenic nematodes. The juvenile stage penetrates the host insect via the spiracles, mouth, anus, or in some species through intersegmental membranes of the cuticle and then enters into the hemocoel. Both *Heterorhabditis* and *Steinernema* are mutualistically associated with bacteria of

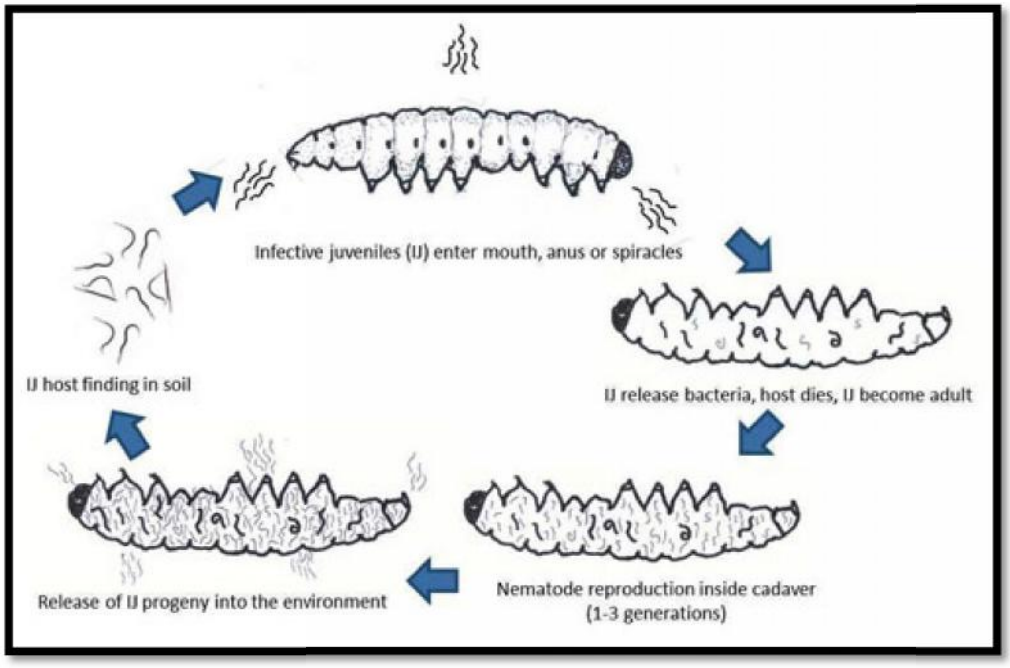
the genera *Photorhabdus* and *Xenorhabdus*, respectively. The juvenile stage release symbiotic bacteria from their intestines into the hemocoel. The bacteria multiply in the insect hemolymph and the infected host usually dies within 24 to 48 hours. After the death of the host, nematodes continue to feed on the host tissue, mature and reproduce. The progeny nematodes develop through four juvenile stages to the adult. Depending on the available resources one or more generations may occur within the host cadaver and a large number of infective juveniles are eventually released into environment to infect other hosts and continue their life cycle.

Symptoms

The insect cadaver becomes red if the insects are killed by heterorhabditids and brown or tan if killed by steinernematids. The color of the host body is indicative of the pigments produced by the monoculture of mutualistic bacteria growing in the hosts.

Searching Behavior

Entomopathogenic nematodes use two search strategies: ambushers or cruisers. Ambushers such as *St. carpocapsae* have an energy conserving approach and lie-in-wait to attack mobile insects (nictitating) in the upper soil. Cruisers like *St. glaseri* and *H. bacteriophora* are highly active and generally subterranean, moving significant distances using volatile signals and other methods to find their host underground. Therefore, they are effective against less mobile pests such as white grubs. Some nematode species such as *St. feltiae* and *St. riobrave* use an intermediate foraging strategy (combination of ambush and cruiser type) to find their host.



Host range

The major pests that are killed using these nematodes include white grubs, black vine weevil, turf grass pests, mole crickets, weevils and cutworms.

5. MASS PRODUCTION TECHNOLOGIES OF BIOPESTICIDES

5.1 Scope for Production of Biopesticides

Though there are about 140 bio-pesticide production units existing in the country as on today, they are able to meet the demand of only less than 1% of cropped area. There exists a wide gap, which can only be bridged by setting up of more and more units for production of Bio-pesticides. There is a scope to enhance production and use of biological control agents in the days to come as the demand is on the increase every year. This requires large scale investment and private participation

To achieve optimum results, bio-pesticide facilities are to be set up in areas which have appropriate climatic conditions. Because temperature control is less costly in locations where there are no extreme conditions. Besides the climatic conditions, the proximity of the location to the market is also important. However, care must be taken that the production facilities are set up at least a quarter of a mile away from farming areas, so as to prevent the contamination of production facilities by insecticides from the farming areas. Also, as air pollution can damage bio-pesticides, the production should be located away from industrial and urban areas.

5.2 Methods in production of biopesticides

5.2.1 Solid state fermentation

Solid-State Fermentation (SSF) SSF utilizes solid substrates like bran, bagasse, and paper pulp. The main advantage of using these substrates is that nutrient-rich waste materials can be easily recycled as substrates. In this fermentation technique, the substrates are utilized very slowly and steadily, so the same substrate can be used for long fermentation periods. Hence, this technique supports controlled release of nutrients. SSF is best suited for fermentation techniques involving fungi and microorganisms that require less moisture content. However, it cannot be used in fermentation processes involving organisms that require high aw (water activity), such as bacteria. The most used substrates are starchy and lignocellulosic materials. Some of the common substrates used in solid state fermentation are wheat bran, rice and rice straw, hay, fruit and vegetable waste, paper pulp, bagasse, coconut coir, and synthetic media. Spores produced using SSF were more stable and resistant to stress than those produced using liquid culture or on agar. Most of the microorganisms used in SSF are filamentous fungi due to their capability to grow on solid surfaces with low free water, but also some bacteria with biocontrol ability have been produced using SSF.

5.2.2 Liquid state fermentation (lf)/ submerged fermentation (smf)

SmF utilizes free flowing liquid substrates, such as molasses and broths. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly; hence need to be constantly replaced/supplemented with nutrients. This fermentation technique is best suited for microorganisms such as bacteria that require high moisture content. An additional advantage of this technique is that purification of products is easier. Some common substrates used in submerged fermentation are soluble sugars, molasses, liquid media, fruit and vegetable juices, and sewage/waste water.

SmF is primarily used in the extraction of secondary metabolites that need to be used in liquid form. SmF is usually implemented in case of bacterial enzyme production, due to the requirement of higher water potential. SSF is preferred when enzymes have to be extracted from fungi, which require lesser water potential. More than 75% of the industrial enzymes are produced using SmF, one of the major reasons being that SmF supports the utilization of genetically modified organisms to a greater extent than SSF. This is highly critical due to the fact that the metabolism exhibited by microorganisms is different in SSF and SmF, and the influx of nutrients and efflux of waste materials needs to be carried out based on these metabolic parameters. Any slight deviation from the specified parameters will result in an undesirable product.

5.3 Mass production of *Trichoderma*

For mass multiplication of *Trichoderma* the following steps should be followed sequentially as noted below:

1) Take about 200 gm of grains in autoclavable bags [71 (B) x 111 (H) and add equal amount of tap water.
2) After filling the bags, keep a 1.51 inches PVC pipe at the top of the cover and tied it with a rubber band.
3) Close PVC pipe mouth using cotton plug.
4) Boil the grains in a 10-20 liter pressure cooker with water inside it for a period of 40 minutes.
5) The grains are cooled at room temperature after sterilization.
6) Transfer the bags into inoculation chamber.
7) Inoculate with 1-2 bits of *Trichoderma* mother culture in each bag inside the chamber with all the help of inoculation loop/spatula. Shake the bags properly for mixing the fungal culture all over the grains.
8) Keep the inoculated bags at the room temperature (25-30 C)
9) Observe the inoculated bags if there is mycelial growth, do not disturb the inoculated bag. If mycelial growth is not observed, shake the inoculated bag.
10) Once *Trichoderma* sporulation (green color) takes place shake the bags every alternate day for about 5 to 7 days in order to spread and allow the *Trichoderma* growth and further sporulation.
11) Transfer the grains with fully grown *Trichoderma* mycelia & sporulation into cleaned plastic trays and cover it with blotter/newspaper. Keep these plastic trays for further sporulation and drying for about 3-4 days at room temperature. Mix the transferred *Trichoderma* colonized grains once in every day for up to 3-4 days with the help of spatula for enhancing sporulation and drying.
12) The *Trichoderma* will be ready for use as soil application or the grounded fine powder for seed treatment and or foliar application.
13) From 1 kg sorghum grains approximately 500 gm dried biomass of *Trichoderma* including grains can be produced, which could be utilized directly for soil application for one hectare after mixing in kg of well decomposed compost or Farm Yard Manure (FYM). The dry

biomass powder along with 0.5% Carboxy Methyl Cellulose (CMC) can be utilized for seed treatment @ 10 g/kg seed.

5.4 Mass production of *Pseudomonas*

5.4.1 Preparation of mother culture using the king's B medium

Peptone	: 20.0 g
K2HPO4	: 1.5 g
Mg SO4	: 1.5 g
Glycerol	: 10 ml
Distilled water	: 1000 ml

Dispense the above broth into conical flasks and autoclave at 15 lb pressure for 15 minutes. After cooling inoculate with a loop of *Pseudomonas* and incubate for 2 days.

5.4.2 Mass production:

For mass multiplication of *Pseudomonas* the following steps should be followed sequentially as noted below:

1) Prepare Kings B Medium and transfer into conical flask or fermentor depending on the requirement. Sterilize the medium at 15 lb pressure for 15 minutes.
2) The media should be cooled at room temperature after sterilization.
3) Inoculate the conical flask/ fermentor with *Pseudomonas* mother culture @ 5%
4) Keep the inoculated vessels at the room temperature (30- 35^{o}C)
5) Once bacterial growth starts, shake the flasks at every 4-6 hours for about 3 to 4 days in order to spread and allow the bacterial growth.
6) After 7-8 days *Pseudomonas* will be ready to use. Transfer the liquid media with bacterial growth into cleaned plastic trays and add fine talc material @ 1:3 (bacterial media: talc).
7) Mix the material properly and allow them to dry at room temperature.
8) The mixed formulation will be ready for use as soil application or for seed treatment and or foliar application

5.5 Mass production of *Beauveria bassiana* and *Metarhizium anisopliae* in carrot broth

1) Cut the carrot into small pieces (40 g), wash in potable water
2) Transfer the carrot pieces in to conical flask (250 ml) and add 150 ml of water.
3) Plug the conical flasks with cotton and autoclave for 15 min at 15 psi.
4) Allow the flasks to cool and take to laminar flow chamber for inoculation.
5) Transfer the loopful of *B. bassiana* or *M. anisopliae* aseptically.
6) Incubate the flasks at room temperature.
7) Harvest the spores in 12-15 days.

5.6 Mass production of *Bacillus thuringensis*

The fermentation of the different isolates of *B.t*., regardless of subspecies, have some general characteristics in common. They all use sugar (usually glucose, molasses, or starch), producing acid during the fermentation. In general, they have similar requirements for proteins or protein hydrolysates, can use NH_4^+ salts, and respond similarly to minerals. However, the

individual isolates are unique entities, and a particular medium that may support good growth or toxin production by one isolate may be less satisfactory for another. Different isolates of *B.t.* may produce toxins with different spectra of activities.

The "log-phase" of any bacterial fermentation is that period during which the organism is vigorously growing and rapidly dividing. This first phase lasts 16-18 hours. Sporulation is complete 20-24 hours after inoculation, although the cells have not yet lysed. Lysis is complete by 35-40 hours.

5.7 Mass production of nucleopolyhedrosis virus (NPV)

Mass production of Nuclear Polyhedrosis Virus (NPV) on commercial scale is restricted to *in vivo* procedures in host larvae which are obtained by

- Field collection from cotton, pigeon pea and chickpea – *H. armigera*
- Mass culturing in the laboratory in semi synthetic diet – *H. armigera*

Some small scale producers use field – collected larvae for mass production of NPV in spite of the following constraints.

- Collection of a large number of larvae in optimum stage (late IV / early V instars) is time-consuming and can be expensive in terms of labour and transportation costs.
- Wild populations of insects may carry disease causing organisms like microsporidians, cytoplasmic polyhedrosis virus, stunt virus and fungal pathogens which will affect both virus production and quality.
- Transportation of a large number of larvae with cannibalistic behaviour will be a difficult task.
- Parasitized larvae collected from the field will die prematurely yielding little virus.

5.7.1 Production procedure

The NPV of *H. armigera* is propagated in early fifth instar larvae. The virus is multiplied in a facility away from the host culture laboratory. The dose of the inoculum used is 5×10^5 polyhedral occlusion bodies (POB) in 10 ml suspension. The virus is applied on to the semisynthetic diet (lacking formaldehyde) dispensed previously in 5 ml glass vials. A blunt end polished glass rod (6 mm) is used to distribute the suspension containing the virus uniformly over the diet surface. Early fifth instar stage of larvae are released singly into the glass vials after inoculation and plugged with cotton and incubated at a constant temperature of 25^oC in a laboratory incubator. When the larvae exhausted the feed, fresh untreated diet is provided. The larvae are observed for the development of virosis and the cadavers collected carefully from individual bottles starting from fifth day. Approximately, 200 cadavers are collected per sterile cheese cup (300 ml) and the contents are frozen immediately. Depending upon need, cadavers are removed from the refrigerator and thawed very rapidly by agitation in water.

5.7.2 Processing of NPV

The method of processing of NPV requires greater care to avoid losses during processing. The cadavers are brought to normal room temperature by repeatedly thawing the container with cadaver under running tap water. The cadavers are homogenized in sterile ice cold distilled water at the ratio 1: 2.5 (w/v) in a blender or precooled all glass pestle and mortar. The

homogenate is filtered through double layered muslin and repeatedly washed with distilled water. The ratio of water to be used for this purpose is 1: 7.5-12.5 (w/v) for the original weight of the cadaver processed. The left over mat on the muslin is discarded and the filtrate can be semi-purified by differential centrifugation. The filtrate is centrifuged for 30-60 sec. at 500 rpm to remove debris. The supernatant is next centrifuged for 20 min at 5,000 rpm. Then the pellet containing the polyhedral occlusion bodies (POB) is suspended in sterile distilled water and washed three times by centrifuging the pellet in distilled water at low rpm followed by centrifugation at high rpm. The pellet finally collected is suspended in distilled water and made up to a known volume, which is necessary to calculate the strength of the POB in the purified suspension.

6. METHODS OF APPLICATION OF BIOPESTICIDES

The suitable method of application of biopesticides is essential for effective and sustainable pest and disease control. The following are the major methods by which biopesticides are delivered in crop ecosystem for the management of insect pests and diseases.

1) Seed treatment
2) Seedling root dip
3) Soil application
4) Foliar spray

6.1 Method of application of *Trichoderma* and *Pseudomonas*

6.1.1 Seed treatment: Seed treatment is a term that describes both products and processes. The usages of specific products and specific techniques can improve the growth environment for the seed, seedlings and young plants. Seed treatment is an alternative approach to introduce *Trichoderma* spp. and *Pseudomonas* spp. into the soil. This method requires smaller amounts of biological material than soil treatment.

6.1.1.1 Seed dressing: This is the most common method of seed treatment. The seed is dressed with either a dry formulation or wet treated with a slurry or liquid formulation. Dressings can be applied at both farm and industries. Low cost earthen pots can be used for mixing biopesticides with seed or seed can be spread on a polythene sheet and required quantity of biopesticides can be sprinkled on seed lot and mixed mechanically by the farmers.

6.1.1.2 Advantages of seed treatment

1. Protects germinating seeds and seedlings against soil and seed borne pathogens
2. Seed germination enhancement.
3. Early and uniform establishment and growth

6.1.1.3 Seed treatment procedure

1) The powder based or liquid based formulation of the product *Trichoderma* spp. or *Pseudomonas fluorescens* is used @ 10 gm or 10 ml per kg of seed material.
2) Mix the formulation with 250 ml starch/ jaggery solution (5%) to make it sticky.
3) Uniformly coat the seeds with the formulation
4) Dry the coated seeds under shade.
5) Sow the dried seeds on the same day.

6.1.1.4 Recommendation of *Trichoderma* & *Pseudomonas* for seed treatment

Sr. No.	Crop	Disease
1	Sugarcane	Root rot, wilt
2	Rice	Root rot disease, Bacterial sheath blight
3	Chilli	Anthracnose, Damping off
4	Pigeon pea	Wilt, Blight and Root rot
5	Pea	Root rot, White rot
6	Okra	Root knot nematode
7	Tomato	Soil borne fungal disease, Early blight Damping off, Wilt

8	Coriander	Wilt
9	Brinjal	Bacterial wilt
10	Leguminous Vegetables	Soil borne infection, Root knot nematode
11	Wheat	Bunt/False smut/loose smut/covered smut
12	Cruciferous vegetables	Soil / Seed borne diseases (Damping off) Root knot nematode
13	Gram	Wilt, Damping off
14	Capsicum	Root knot nematode

6.1.2 Seedling root dip treatment

a) Mix 20 g or 20 ml of powder based or liquid based formulation of the product *Trichoderma* spp. or *Pseudomonas fluorescens* in one liter of water.
b) Immerse the seedlings root (brinjal, chili, tomato, cabbage, etc.) in the mixture for five to ten minutes before transplanting.

6.1.3 Soil application: *Trichoderma* spp. suppresses the activity of soil borne fungal pathogens, especially *Rhizoctania solani* and *Pythium* spp. and protects transplanted seedlings by colonizing their roots. *Pseudomanas fluorescens* is common non-pathogenic saprophyte that colonises in soil, water and on plant surfaces. It produces a soluble greenish fluorescent pigment. *P. fluorescens* suppress plant diseases by protecting the seeds and roots from fungal infections. Soil application of *Trichoderma* or *Pseudomonas fluorescens* is generally done through FYM enriched with required bioagent.

6.1.4 Preparation of enriched FYM with *Trichoderma* or *Pseudomonas*

1) Uniformly mix 1 kg of *Trichoderma* or *Pseudomonas fluorescens* formulation in 100-250 kg of farmyard manure
2) Cover the mixture and keep it under the shade for 7-8 days
3) Sprinkle the heap with water intermittently.
4) Turn the mixture in every 3-4 days interval
5) Mix the enriched FYM with 1000 kg FYM and broadcast in 1 ha area.

6.1.5 Foliar application: Mix 4-5 g of formulation ($2x10^8$cfu/g) in 1 litre of water and spray in the early morning or late evening hours.

6.2 Method of application of *Beauveria* and *Metarhizium*

6.2.1 Soil application: Soil application of entomopathogens *viz., Beauveria* and *Metarhizium* is done through FYM enriched with required entomopathogen.

6.2.2 Preparation of enriched FYM with *Beauveria* and *Metarhizium*

1) Uniformly mix 1 kg of formulation of *Beauveria* or *Metarhizium* in 100-250 kg of farmyard manure
2) Cover the mixture and keep it under the shade for 7-8 days
3) Sprinkle the heap with water intermittently.
4) Turn the mixture in every 3-4 days interval

5) Mix the enriched FYM with 1000 kg FYM and broadcast in 1 ha area.

6.2.3 Foliar application: Mix 4-5 g of formulation ($2x10^8$cfu/g) in 1 litre of water and spray in the early morning or late evening hours.

6.2.4 Bio-efficacy of different entomopathogenic fungi against insect pests

Fungus	**Pest & Crop**
Beauveria bassiana	Rice hispa (*Dicladispa armigera*)
	Coffee berry borer (*Hypothenimus hampei*)
	Tea looper caterpillar (*Buzura suppressaria)*
	Sun flower *Helicoverpa armigera*
	Green gram white grubs
Beauveria brongniarti	Sugarcane white grubs *Holotrichia serrata*
	Coconut Rhinoceros beetle (*Oryctes rhinoceros*)
	Sugarcae white grub
	Pigeon pod borer
	Potato White grubs (Brahmina)
Metarhizium anisopliae	Soyabean white grubs *Holotricha longipennis*
Lecanicillium lecanii	Coffee green scale (*Coccus viride*)
	Citrus green scale (*Coccus viride*)
	Indian mustard and Rapeseed- Mustard aphid (*Lipaphis erysimi*)
Nomuraea rileyi	Castor *Spodoptera litura*
	Soybean *Spodoptera litura* *Helicoverpa armigera, Thysanoplusa orichalccea*

6.3 Method of application of *Bacillus thuringiensis*

6.3.1 Foliar application: Mix 4-5 g of formulation ($2x10^8$cfu/g) in 1 litre of water and spray in the early morning or late evening hours.

6.3.2 Recommendation of *Bacillus thuringiensis* against insect pests

Crop	**Insect Pest**
Bacillus thuringiensis* var. *galleriae	
Cabbage & Cauliflower	Diamond back moth (*Plutella xylostella*)
Tomato & Cotton	Fruit borer (*Helicoverpa armigera*)
Okra	Fruit borer (*Earias spp*.)
Chilli	Fruit borer (*Spodoptera litura*)
Rice	Leaf folder (Cnaphalocrocis medinalis)
***Bacillus thuringiensis* Serovar *kurstaki* (3a, 3b, 3c) 5% WP**	
Cotton	American Bollworm, Spotted Bollworm
Red gram	Pod Borer

Cabbage	Diamond back moth
***Bacillus thuringiensis* var. *kurstaki*, Serotype H-39, 3b, Strain Z-52**	
Cotton	Bollworms, *Spodoptera*
Rice	Stem borer, Leaf folder
Gram & Pigeon Pea	*Heliothis*
Soyabean & Tobacco	*Spodoptera*, *Heliothis*, Semilooper, Leafminer
Castor	Hairy caterpillar

6.4 Field application NPV

6.4.1 Directions for use of NPV

1) The recommended dosage is 200 ml of NPV/acre or 500 ml/ha containing 100 and 250 larval equivalent (LE) of NPV respectively as active infective material (one LE = 6 x 10^9 POBs).
2) 100 ml of NPV could be diluted in 200-400 litres of water when high volume sprayer is used and in 50-70 litres of water in case of power sprayers.
3) Preferable to spray using high volume knapsack sprayer. Virus should be sprayed during evening hours. Spray should be initiated as soon as few newly hatched larvae are observed or three to five days after a trap catch of 5 moths per pheromone trap. Subsequent sprays should be made at 7 to10 days intervals depending upon the pest population.

6.4.2 Compatibility of NPV with other insecticides

The viral pathogen seems to be less sensitive to chemical pesticides (organo phosphates). When the combination of pathogen and pesticide is used, sometimes synergistic action is noticed. But is recent years mixing of NPV with insecticides is not advisable due to cross resistance problem.

6.5 Application methods of EPN

Production and application technology is critical for the success of EPNs in biological control. Infective juveniles of EPNs are usually applied using various spray equipment and standard irrigation systems. Nematodes require a film of water around soil particles to move through the soil profile in search of a host. Therefore, it is important to ensure adequate agitation during application,, Enhanced efficacy in EPN applications can be facilitated through improved delivery mechanisms (e.g., cadaver application) or optimization of spray equipment. Entomopathogenic nematodes can be applied with nearly all commercially available aerial or groundspray equipment, including mist blowers, electrostatic sprayers and pressurized sprayers.

6.5.1 Soil application

Soil is the natural habitat for EPNs and thus use of these organisms offers great potential of successful bioocontrol against subterranean insect pests. A series of technology is available for application of EPNs to soil from simple watering cans for small pot or home garden and for large fields or orchards through aerial application. Other methods used in soil application include various irrigation systems such as microjet, overhead etc. Many formulations of EPNs may be used is soil application including activated charcoal, alginate and polyacrylamide gels, baits, clay, vermiculite, peat, polyurethane sponge and water dispersible granules.

The wettable powder formulation containing EPNs can be applied to soil. Mix 2-4 kg WP formulation in 100 kg of any organic manure and apply this mixture in one acre as spot application or broadcast followed by a light irrigation in case soil is dry.

6.5.2 Foliar application

Environmental factors such as ultraviolet rays, desiccation are the key factors in influencing the nematode efficacy on foliage. Conventional equipments are used to apply. Antidesiccants are used to retard evaporation of the nematode suspension and reduce desiccation (Eg. Glycerin 10%). Substantial progress has been made in recent years in developing EPN formulations, particularly for above ground applications, e.g., water-dispersible granules, nematodes on gel, micronized vermiculite, and an aqueous suspension of nematodes. Bait formulations and insect host cadavers can enhance EPN persistence and reduce the quantity of nematodes required per unit area.

Finally, superior bio control applications with EPNs can also be achieved through strain improvement. Improved strains may possess enhanced levels of various beneficial traits such as environmental tolerance, virulence, reproductive capacity, etc. Methods to improve EPNs include strain or species discovery or genetic enhancement via selection, hybridization or molecular manipulation. Many researchers were reported that EPNs can be applied in combination with insecticides and other bio control agents.

6.6 Formulation of biopesticides: part of delivery system

Broadly formulations can be classified into solid (dusts, granules, and powders) and liquid (termed suspensions; oil or water based and emulsions) formulations, Dusts are formulated by the sorption of an active ingredient onto a finely ground solid inert such as talc, clay or chalk with particle size ranging from 50-200 µm.

6.6.1 Wettable powders (WPs)

They consist of 50-80% technical powder, 15-45% filler, 1-10% dispersant and 3-5% surfactant by weight to achieve a desired potency formulation. Fillers are hydrophilic and usually contain silica which resists cake formation and friability during grinding. Dispersant must be added to retain suspension in dispersion; surfactants to overcome surface tension at liquid-solid interface. Among the dried formulations of biopesticides, much attention has been given to WPs because of good miscibility with water and ease of application as sprays with conventional equipment.

6.6.2 Flowables

They are the suspensions of particulates in liquids with 10-40% microorganisms, 1-3% suspender ingredient, 1-5% dispersant, 3-8% surfactant and 35-65% carrier liquid (oil or water)

6.6.3 Additives

They are chemically and biologically active compounds that can alter the formulation physics and kill the targeted species without harming other insects. Important adjuvants are dispersants (amylose), wetting agent (Triton X), spreaders (molasses), stickers (gums), drift control (glycerol), UV radiation screens (Congo red, optical brightener) and phagostimulants (corn meal).

7. METHODS OF QUALITY CONTROL AND TECHNIQUES OF BIOPESTICIDES

7.1 Insecticide act and bio-pesticides

Regulation and policies of bio-pesticide usage and promotion in India

Nearly 500 biopesticides are available in the Indian market duly registered by Central Insecticide Board, but quality control is a major issue in most of the products**.** Indian government is promoting several rules, regulations, schemes and policies for the promotion of production, research, adoption and registration of bio-pesticides. Insecticide Act 1968 has been amended so as to simplify the registration process and allow quick development and invention of bio-pesticides.

Indian government has made certain policies and acts for the promotion and regulation of pesticides including bio-pesticides.

7.2 The Destructive Insects and Pests Act, 1914: an act to prevent the introduction into and the transport from one state to another in India of any insects, fungus or other pest which is or may be destructive to crops.

7.3 The Insecticide Act, 1968: This act regulates the import, manufacture, sale, transport, distribution and use of insecticides with a view to prevent risk to human beings or animals, and for matters connected therewith.

7.4 Insecticides Rules, 1971: The main objectives are the functioning of board, registration committee and laboratory, registration of insecticides, grant of licenses, packaging and labeling, appointment of insecticide analysts and insecticide inspectors, transport and storage of insecticides in transit by road, water and rail. These rules also take into account the provisions regarding protective clothing, equipment and other facilities for workers during the manufacture of insecticides.

7.5 Promotion of Integrated Pest Management, 1991: promotion of use of bio-pesticides: neem based pesticides, bacillus based bio-pesticides, insect pathogen as alternative to chemical pesticides.

7.6 Biopesticides registered under section of 9(3) of the insecticides act, 1968 for the use in country (as on 29.02.2020)

Sr.No	Biopesticide	Registered as
1.	Azadirachtin (Neem Product)	Botanical
2.	Pyrethrin (Pyrethrum)	
3.	*Trichoderma viride*	Antagonistic fungi
4.	*Trichoderma harzianum*	
5.	*Ampelomyces quisqualis*	
6.	*Pseudomonas flourescens*	Antagonistic bacteria
7.	*Bacillus subtilis*	

8.	*Metarhizium anisopliae*	Entomopathogenic fungi
9.	*Beauveria bassiana*	
10.	*Lecanicillum* (*Verticillium*) *lecanii*	
11.	*Bacillus sphericus*	Entomotoxic bacteria
12.	*Bacillus thringiensis var. kurstaki*	
13.	*Bacillus thringiensis var. galleriae*	
14.	*Bacillus thringiensis var. israelensis*	
15.	NPV of *Helicoverpa armigera*	Baculovirus
16.	NPV of *Spodoptera litura*	

7.7 Indian standards for different biopesticides available in India

Biopesticide	Parameters to be tested	Parameter specification	Biopesticides Registered
Antagonistic Fungi	Moisture content	8 % maximum	***Trichoderma viridae*** ***Trichoderma harzianum*** ***Ampelomyces quisqualis***
	pH	6.5 to 7.5	
	CFU/g of the product by serial dilution	2 X 10^6 minimum	
	Antagonistic ability	Minimum 60 %	
	Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and such other microbials	Should be nil.	
	Other microbial contaminants	1 X 10^4 maximum	
	Chemical/botanical pesticide contaminants	Should be nil.	
	Bioassay based on diseased severity and root colonization	>70% germination < 30 % disease severity.	
Antagonistic Bacteria	**Colony Forming Unit (CFU)**	1 X 10^8 CFU/g	***Pseudomonas flourescens***

	Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and such other microbials	Nil	***Bacillus subtilis***
	Other microbial contaminants	1 X 10^4 CFU/g max.	
	Chemical/botanical pesticide contaminants	Absent	
	Antagonistic capability on target organism	At least 35 %	
	Bioassay for diseased severity and root colonization	>70% germination < 30 % disease severity.	
Entomopathog-enic Fungi	Colony Forming Unit (CFU)	1 X 10^8 CFU/g minimum	***Beauveria bassiana*** ***Metarhizium anisopliae*** ***Lecanicillium* (*Verticillium*) *leccanii***
	Pathogenic contaminants such as gram negative bacteria *Salmonella*, *Shigella*, *Vibrio* and such other microbials	Nil	
	Other contaminants	1 X 10^4 maximum	
	Chemical/botanical pesticide contaminants	Nil	
	Entomopathogenic capability on target insects by bioassay.	LC-$_{50:}$ Not more than 2.00X10^6 spores/ml (3.0X10^3 spores/mm^2) against *S.litura.* $LC_{50:}$ Not more than 4.00X10^6 spores/ml (6.0X10^3 spores/mm^2) against *H. armigera*	

Entomotoxic bacteria	Moisture content	< 5 %	***Bacillus sphaericus*** ***Bacillus thuringiensis* var. *israelensis*** ***Bacillus thuringiensis* var. *kurstaki*** ***Bacillus thuringiensis* var. *galleriae***
	Beta exotoxin content by housefly bioassay	Negative	
	Potency of product by bioassay (LC50)	15,000 ITU/mg for ***Bacillus thuringiensis* var. *israelensis*,** 1,700 ITU/mg for ***Bacillus sphaericus*** 16,000 ITU/mg for ***Bacillus thuringiensis* var. *kurstaki***	
	Bt Delta endotoxin content by ELISA	Minimum 2 %	
	Pathogenic contaminants	Should be nil	
	Other microbial contaminants	1 X 10^4	
	Chemical/botanical pesticide contamination	Should be nil	
Baculoviruses – Nuclear Polyhedrosis Virus (NPV)	Viral Unit: NPVs (*Helicoverpa* & *Spodoptera*)	1x10^9 POB/ml or gm	***Helicoverpa* Nuclear Polyhedrosis Virus (Ha NPV)** **Spodoptera Nuclear Polyhedrosis Virus (SNPV)**
	Bioassay of NPV for determining LC50 against *Helicoverpa* or *Spodoptera*	HaNPV <0.5 POB/mm^2 S NPV <20 POB/mm^2	
	Pathogenic contaminants	Nil	
	Other microbial contaminants	1 X 10^4 maximum	
	Chemical/botanical pesticide contaminants	Nil	

8. IMPEDIMENTS AND LIMITATION IN PRODUCTION AND USE OF BIOPESTICIDES

Biopesticides control pests in an ecofriendly and nontoxic manner. These offer the following advantages:

1) Zero or little residual effect: Residues of microbial bio-pesticides produce no adverse effects on living beings.
2) Applicability in organic farming and potential to be used as a part of Integrated Pest Management (IPM).
3) Fewer chances to develop resistance by pests: As alternatives to conventional chemical pesticides, they can help reduce the selection pressure for the evolution of pesticide resistance in pest populations.
4) Biodegradable in nature.
5) Effective in low concentration and provide pest control over generations.
6) These do not affect ecofriendly insects such as pollinators and soil micro flora.
7) Biopesticides often have good compatibility with biological pest control agents (natural enemies) and with few conventional chemical pesticides, so they can be readily incorporated into IPM programmes.

Limitations and constraints of using bio-pesticides

1) Biopesticides show slow rate of control as compared to chemical insecticides Most of the microbial bio-pesticides cannot be used alone for the complete replacement of conventional pesticides. Therefore, these bio-pesticides are used as components of Integrated Pest Management (IPM).
2) Lower efficacy and shorter persistence.
3) Specific to the life cycle of the pest.
4) Greater susceptibility of biopesticides to adverse environmental conditions.
5) Lack of highly virulent strains.
6) As bio-pesticide is totally a live organism, it is important to maintain the vigor and microbial load.
7) Sophisticated equipments are required for the production of quality biopesticides.
8) Their market performance is very poor as the quality of bio-pesticides produced is not good.
9) In many of the cases, bio-pesticides that are sold in the market are contaminated and microorganism count is also low which results in poor performance.
10) The market for bio-pesticides is not well established like markets for chemical pesticides.
11) Due to lack of wide spread studies and consistent results, farmers get confused whether they should adopt bio-pesticides or not.
12) Pesticide storage requires specific instruments and environmental conditions which are very costly and cannot be afforded by farmers, shopkeepers and sellers.
13) Budget required for bio-pesticides production is high.

14) Import and export of bio-pesticides is much difficult as compared to chemical pesticides/ Regulatory and marketing constraints.

The registration of biopesticides often poses a particular challenge to regulatory authorities and the special biological properties of these natural control agents should be taken into account. For small biopesticides companies aiming to development a range of niche products, the cost, represent a serious constraint to registering new biopesticide products.

The development of a more balanced regulatory system for biopesticides production would be beneficial for natural enemies and other biopesticides for agricultural development without risk to people or environment, and the process of registration could be speeded up and the cost might be cut down. The regulatory system should be more flexible and it must have the full and justified trust of the public.

Remedies for bio-pesticide production, usage and marketing

1) Adulteration should be avoided during packaging of bio-pesticides.
2) Lyophilized and dried preparations of bio-pesticides should be used to achieve viability and stability of biological products.
3) The registration of bio-pesticides is expensive compared to their production which poses major hurdle in their development. The problems related to their registration should be addressed.
4) The developed bio-pesticide strain should be monitored extensively to access its threats to the consumer and environment.
5) Microbial bio-pesticides should be protected from contamination in order to improve the shelf life.
6) Sustainable and controlled release of biopesticide is also necessary.
7) Pathogenicity and virulence of some microbial strains can be improved using biotechnological tools.
8) There are some aspects such as resistance, potential of dispersion and persistence should be studied thoroughly.

PART II: BIOFERTILIZERS

1. BIOFERTILIZER: INTRODUCTION, STATUS AND SCOPE

1.1 Introduction

Biofertilizers may be defined as "substances which contain living microorganisms that colonize the rhizosphere or the interior of the plants and promote growth by increasing the supply or availability of primary nutrients to the target crops, when applied to soils, seeds or plant surfaces".

Biofertilizers can fix atmospheric nitrogen through the process of biological nitrogen fixation (BNF) and solubilize plant nutrients like phosphates, potash; in addition, it also stimulates plant growth through synthesis of different growth promoting substances and has C: N ratio 20:1 indicating its stability.

Biofertilizers can be categorized into five groups based on their nature and activity as described below. Recently, the potash mobilizers like *Frateuri aaurentia*, zinc and sulphur solubilizers like *Thiobacillus sp*. and manganese solubilizer fungal culture like *Pencillium citrinum* have also been identified for commercial operations.

Only a few prokaryotic organisms are able to fix nitrogen directly through a biological process. Annual biological nitrogen fixation (BNF) is estimated to be around 175 million tones of which close to 79 % is accounted for by terrestrial fixation.

1.1.1 History of Biofertilizers

- **In the 19th century** scientists had understood the value of mineral nutrition of plants and few others suspected that plants could obtain nitrogen from the atmosphere.
- Two German chemists, **Hellriegel and Wilfarth** (1988), showed innate ability of legumes to fix elemental nitrogen in the atmosphere. Since, neither plants nor microorganisms can fix nitrogen independently; the process has been called **symbiotic nitrogen fixation**.
- The root nodule bacteria were isolated by **Beijerinck** in 1888 and were grouped in the genus *Rhizobium* ("Rhizo" means "root" in Greek). These are gram negative, rod shaped, motile, aerobic, non spore forming bacteria.
- **Bond** in United Kingdom and **Quispel, Silver and Becking** in Europe made considerable headway on nitrogen fixation in non-leguminous nodulated plants between 1950 and 1970.
- **Date, Brockwell and Roughley** worked on the development of techniques involved in inoculant production and its application to seed, referred to as "Bacterization".
- The Brazilian group of workers was headed by **Dobereiner** who introduced the concept of 'associative symbiosis' or 'diazotrophic biocoenosis' while working with the association between *Azospirillum* and many graminaceous plants.

1.1.2 History of biofertilizer use in India

In India, systematic study on biofertilizers was started by N. V. Joshi in 1920. *Rhizobium* was the first isolated from various cultivated legumes and this was followed by extensive research by Gangulee, Sarkaria and Madhok on the physiology of the nodule bacteria along with its inoculation for better crop production. The milestones in research, production and promotion of biofertilizer in India are given below.

1920- First study of legume-*Rhizobium* symbiosis by **N.V. Joshi**.

1939- Discovery of Blue-green algae (BGA) in rice fields by P.K. Dey

1960-First isolation of non symbiotic N-fixing bacteria *Derxia gummosa* in the World by P.K. Dey and R. Bhattacharya

1979- Initiation of All India Coordinated project on Biological Nitrogen Fixation.

1983- Setting up of National Project on Development and use of Biofertilizer by Ministry of Agriculture, Govt. of India.

1988- Setting up of National Facility Centre of BGA at IARI.

1.1.3 Different groups of biofertilizers

Sl. No.	Groups	Examples
1. Nitrogen (N_2) fixing Biofertilizers		
I	Free-living	*Azotobacter*, *Clostridium*, *Anabaena*, Nostoc
Ii	Symbiotic	*Rhizobium*, *Frankia*, *Anabaena azollae*
Iii	Associative Symbiotic	*Azospirillum*
2. P-Solubilizing Biofertilizers		
I	Bacteria	*Bacillus megaterium* var. *phosphaticum*, *Bacillus circulans*, *Pseudomonas striata*
Ii	Fungi	*Penicillium sp.*, *Aspergillus awamori*
3. P-Mobilizing Biofertilizers		
I	Arbuscular mycorrhiza	*Glomus sp.*, *Gigaspora sp.*, *Acaulospora sp.*, *Scutellospora sp.*, *Sclerocystis sp.*
Ii	Ectomycorrhiza	*Laccaria sp.*, *Pisolithus sp.*, *Boletus sp.*, *Amanita sp.*
Iii	Orchid mycorrhiza	*Rhizoctonia solani*
4. Biofertilizers for Micro nutrients		
I	Silicate and zinc solubilizers	*Bacillus sp.*
5. Plant Growth Promoting Rhizobacteria		
I	Pseudomonas	*Pseudomonas fluorescens*

Source: Biofertilizers- types and their application, KrishiSewa

1.2 Status of Biofertilizer in India

Estimated annual requirement of *Rhizobium* inoculum varies from 1,250 to 15,000t. Based on crop area in India, the present requirement of biofertilizers is around 5,50,000 metric tons and there is an ample potential to increase it to 50,000-60,000 tons by 2020; however, the total production in our country is much less than requirement which points out the inevitability of increase in biofertilizer production.

Now, the government of India is boosting the biofertilizer industries by providing subsidies to a maximum of 20 lakh rupees and awarding a national productivity award to the efficient biofertilizer production unit. Agro Industries Corporation has the maximum production capacity which is followed by State Agriculture Departments, National Biofertilizers Development Centers, State Agricultural universities and private sectors.

1.2.3 Marketed biofertilizers in India

- Nitrogen fixer, e.g. *Rhizobium*, *Bradyrhizobium*, *Azospirillum*, *Azotobacter*, Acetobacter, Azolla and BGA.

- Phosphorus solubilizer, e.g. *Bacillus*, *Pseudomonas* and *Aspergillus*.
- Phosphate mobilizer, e.g. VA-mycorrhiza (VAM) like *Glomus.*
- K-solubilizer, e.g. *Frateuria aurantia*.
- Silicate solubilzer, e.g. *Thiobacillus thiooxidans*.
- Plant growth promoting biofertilizers, e.g. *Pseudomonas sp*.

1.2.4 Environmental Limitations for Application of Bio-fertilizer

1. Unavailability of suitable carrier Resource constraint
2. Market level constraints and lack of awareness of farmers
3. Lack of quality assurance and limited resource generation for Biofertilizers production
4. Seasonal and un assured requirement
5. Soil and climatic factors and inadequate experienced staff
6. Native microbial population, faulty inoculation techniques and mutation during fermentation.

1.3 Scope of Biofertilizer

Biofertilizers make nutrients available that are naturally abundant in soil and atmosphere to plants. These to be effective and cheap inputs, free from any environmental hazards. In a nutshell, it provides "ecofriendly" organic agro-input which has the ability to convert nutritionally important elements from unavailable to available form through biological processes. So, it can be expected to reduce the use of chemical fertilizers and pesticides by introducing biofertilizers.

The microorganisms in biofertilizers reestablish natural nutrient cycle, maintain optimum nutrient level in soil and also increase soil organic matter content as a result healthy plants can be grown, while upholding sustainability and fertility of the soil. Therefore, they are extremely advantageous in enriching soil fertility.

Different biofertilizers provide growth-promoting factors to plants through secretion of different vitamins, phytohormones and by successfully facilitating composting and controlling attack of pest and soil borne diseases. It not only saves chemical fertilizers but also help in its effective utilization and results in higher yield rates.

Dryland agriculture constitutes a very large part of agricultural area in and more than 90% of coarse cereals, 80% of groundnut and 85% of pulses come from these regions. Dryland agriculture is characterized by low productivity, unpredictable climatic swings and low dosage of chemical fertilizers and in this situation biofertilizers, particularly *Rhizobium*, *Azotobacter*, and PSB could be a bridge between removals and additions to soil nutrients where farmers can scarcely afford costly inputs.

It is an established fact that due to fixation in acidic and alkaline soils, the efficiency of phosphatic fertilizers is very low (15-20%) and unfortunately both soil types are prevailing in India. On that account, the inoculation of phosphate solubilizing bacteria in soils is needed to restore and maintain the effective microbial populations for solubilization of chemically fixed phosphorus as well as availability of other macro and micronutrients to harvest good sustainable yield of various crops.

2. MECHANISM OF NITROGEN FIXATION

Biological Nitrogen Fixation: The biological nitrogen fixation is carried out by some bacteria, cyanobacteria and symbiotic bacteria. In symbiotic association, the bacterium provides fixed nitrogen (NH_3) to the host and derives carbohydrates and other nutrients from the latter.

Mechanism of biological N-fixation

Biological nitrogen fixation occurs in the presence of the enzyme nitrogenase, which is found inside the nitrogen fixing prokaryote. In addition to this enzyme, a source of reducing equivalents (ferredoxin (Fd) or flavodoxin), ATP and protons are required.

The overall reaction of biological nitrogen fixation (BNF) is represented by the following equation:

$$N_2 + 8H^+ + 8e^- + 16\ ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

The **enzyme nitrogenase** is in-fact an enzyme complex which consists of two metallo-proteins. None of these two components alone can catalyse the reduction of N_2 to NH_3. Both the components are extremely sensitive to O_2.

(i) **Fe-protein or iron-protein**

(ii) **Fe Mo-protein or iron-molybdenum protein**

2.1 Symbiotic N-fixation: Formation of Root Nodules in Leguminous Plants

The rhizobia occur as the free-living organisms in the soil before infecting their respective host plants to form root nodules. The rhizobia migrate and accumulate in the soil near the roots of the legume plant in response to the secretion of certain chemicals such as **flavonoids and betaines** by the roots. Root hairs of legume produce **specific** sugar binding proteins called as **lectins.** These lectins are activated by **Nod factors** to facilitate the attachment of rhizobia to the root hairs whose tips in turn become curved.

Rhizobia now secrete enzymes which degrade the cell walls of root hairs at the point of their attachment for entry into the root hair. From root hairs, the rhizobia enter into the cells of inner layers of cortex through **infection threads**.

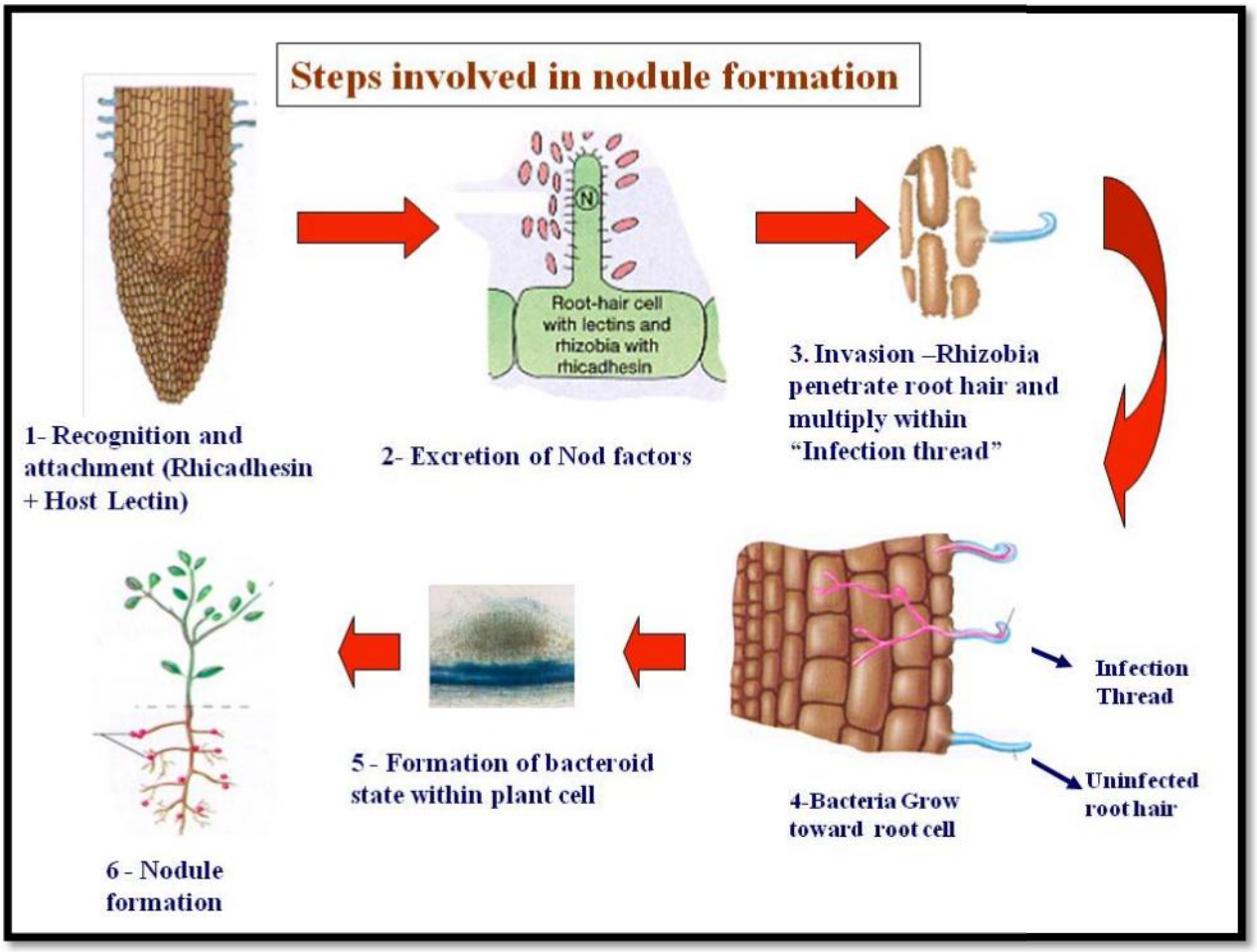
The rhizobia continue to multiply inside infection thread and are released into cortical cells in large numbers, where they cause cortical cells to multiply and ultimately result in the formation of nodules on the upper surface of the roots. After their release into cortical cells, the rhizobia stop dividing and enlarge. The enlarged and non-motile groups of bacteria inside the membranes are called as bacteroids.

The nodule has its own **vascular system** which is connected with vascular system of the root to facilitate transfer of fixed nitrogen i.e., NH_3 to the host and carbohydrates and other nutrients from the host to the bacteroids.

In root nodules of leguminous plants, a red pigment- an oxygen binding heme protein which is very much similar to hemoglobin of red blood corpuscles is found. This pigment is called as **leghemoglobin** and occurs in cytosol of infected nodule cells. Leghemoglobin gives pinkish-red colour to the nodules. The globin part of this pigment is synthesized in host plant

genome in response to the bacterial infection, while its heme portion is synthesized by bacterial genome. It (i) protects the nitrogenase inside the bacteroids from deterimental effect of oxygen and (ii) maintains adequate supply of oxygen to the bacteroids, so that through respiration ATPs continue to be generated which are required for nitrogen fixation.

After its formation inside bacteroids, ammonia (or NH_4^+) is released into cytosol of infected nodule cells where it is converted into **amides** or **ureids**. These amides or ureids are translocated to shoots of host plant through xylem, where they are rapidly catabolized to NH_4^+ for entry into mainstream of ammonium assimilation.



2.2 Asymbiotic N_2-fixation

Some of the bacteria and most of the cyanobacteria comprise this class of microorganisms and are also known as free-living diazotrophs. Both aerobic and anaerobic bacteria are free-living diazotrophs. Water, oxygen, nutrients are required in optimum amount, so that, the microorganism can grow.

Cyanobacteria grow mainly in the crop fields. The site of nitrogen fixation in the cyanobacteria is the heterocyst because the enzyme (nitrogenase) required for nitrogen fixation acts under anaerobic condition. Then the question arises how unicellular and non-heterocystous

cyanobacteria fix nitrogen? Some cells in these microorganisms become specialized i.e. have oxygen level reduced. Typically, they fix nitrogen in dark and photosynthesize in light.

Bacterial types fixing nitrogen asymbiotically

Aerobic bacteria	Anerobic bacteria	Facultative anerobic bacteria	Photosynthetic bacteria
Azomonas Azotobacter, Beijerinckia, Derxia, Methylomonas, Mycobacterium	*Clostridium, Desulfovibrio*	*Bacillus, Enterobacter, Klebsiella*	*Chlorobium, Chromatium, Rhodomicrobium, Rhodopseudomonas, Rhodospirillum*

Many free-living/non-symbiotic heterotrophic bacteria live in the soil and fix significant levels of nitrogen without the direct interaction with other organisms. Examples of this type of nitrogen-fixing bacteria include species of *Azotobacter*, *Bacillus*, *Clostridium*, and *Klebsiella*. As previously noted, these organisms must find their own source of energy, typically by oxidizing organic molecules released by other organisms or from decomposition or may be chemolithotrophs which utilize inorganic compounds as a source of energy. Because nitrogenase can be inhibited by oxygen, free-living organisms behave as anaerobes or microaerophiles while fixing nitrogen.

The biological fixation of nitrogen is the most important way to access N to organisms, this process requires a high proportion of the ATP. The *Nitrogenase* enzyme is sensitive to O_2, that irreversible inactivates the enzyme. Diazotrophs must employ mechanisms, which permit the supply of O_2 required for energy generation and protect *Nitrogenase* from the deleterious effect of O_2. They have developed **several strategies for limiting O_2 access to Nitrogenase:**

1) It could avoid O_2 and live in environments, which are permanently anaerobic (*Clostridium*).
2) It could generate a physical barrier (heterocyst) around its nitrogenase and in this way prevent O_2 from diffusing to the enzyme (cyanobacteria).
3) The microorganism, by accelerating (speeding up) its metabolism, reduce the concentration of O_2 within the vicinity of nitrogenase (*Azotobacter*).
4) They could modify its nitrogenase in such manner as to render it resistant to inactivation by O_2 (conformational protection) (*Azotobacter*).

Difference between Symbiotic and Non Symbiotic Nitrogen fixation

Sr. No.	Symbiotic Nitrogen fixation	Non Symbiotic Nitrogen fixation
1.	A part of a mutualistic relationship in which plants provide a niche and fixed carbon to bacteria in exchange for fixed nitrogen	A process of biological nitrogen fixation performed by a group of autotrophic bacteria living free in the soil either aerobically or anaerobically and not dependent on plant.
2.	Symbiotic nitrogen fixing bacteria live in	Non-symbiotic nitrogen fixing bacteria

	a mutualistic relationship with plants	are free-living in the soil
3.	Ammonia, amino acids and ureides are formed	Ammonia, nitrites and nitrates are formed
4.	Symbiotic bacteria fix and make available nitrogen for its host	Non-symbiotic bacteria make nitrogen available in the soil
5.	Examples: *Rhizobium, Bradyrhizobium, Frankia* etc.	Examples: *Azotobacter, Azosprillum, Beijerinckia* etc.

3. STRUCTURE AND CHARACTERISTIC FEATURES OF BACTERIAL BIOFERTILIZERS

3.1 Bacteria:

- Symbiotic nitrogen fixers: *Rhizobium, Frankia spp.*
- Associative nitrogen fixer: *Azospirillum spp.*
- Free living nitrogen fixers: *Azotobacter spp.*
- Algal biofertilizers: BGA in association with *Azolla Anabena, Nostoc, Ocillatoria*
- Phosphate solubilising bacteria: *Pseudomonas, Bacillus spp.*

3.1.1 *Rhizobium*

- *Rhizobium*, who's name comes from Greek (Riza = Root and Bios = Life), is a nitrogen fixing bacteria that can be found in the soil or in the roots of legumes.
- It has been used, since the time of the **Egyptians**.
- *Rhizobium* is one of the important nitrogen-fixing bacteria, which helps the legume to maintain the soil fertility by way of their symbiotic association and nitrogen fixation.
- A legume plant having effective root nodules **not only can meet its own nitrogen requirement but can also enrich the soil nitrogen content** and thus improving soil fertility and sustainability.
- Legumes are cultivated widely in different parts of India and the practice of seed inoculation with *Rhizobium* is popular among farmers.

History

- Egyptians-Unknowingly used *Rhizobium*
- **1888-Martinus Beijerinck** was the first person to separate Rhizobia and named it ***Bacillus radicicola***
- **1889- Frank** named them ***Rhizobium***
- **1959-Pommer** became the first person to get an isolated *Rhizobium*
- 1980's- **Johanna Döbereiner** discovered **specific species** of *Rhizobium*

- Rhizobia (fast-growing *Rhizobium* spp. and slow-growing *Bradyrhizobium* spp.) or root nodule bacteria are medium-sized, rod-shaped cells, 0.5-0.9 µm in width and 1.2-3.0 µm in length.
- They do not form endospores, are Gram-negative, and are mobile by a single polar flagellum or two to six peritrichous flagella.
- Rhizobia are predominantly aerobic chemoorganotrophs and are relatively easy to culture. They grow well in the presence of O_2 and utilize relatively simple carbohydrates and amino compounds.

- Normally, they are not found to fix N in free-living form. Some strains of rhizobia require vitamins for growth. Optimal growth of most strains occurs at a temperature range of 25-30°C and a pH of 6.0-7.0.
- Despite their usual aerobic metabolism, many strains are able to grow well under microaerophilic conditions.
- Most rhizobia produce white colonies when cultured in yeast-mannitol (YM) medium.
- Most rhizobia only weakly absorb congo red dye, which is included in culture media for isolating rhizobia.

Free-living rhizobia in the soil

- Rhizobia are facultative microsymbionts that live as normal components of the soil microbial population when not living symbiotically in the root nodules of the host legume.
- Outside the root nodule, rhizobia are mostly found on the root surface (rhizoplane), soil around and close to the root surface (rhizosphere), and, to a lesser extent, nonrhizosphere soil.

Rhizobia as symbionts

- The free-living rhizobia in the soil enter the root hairs of the susceptible host legume by a complex series of interactions known collectively as the infection process.
- This begins with adhesion of specific rhizobia to the surface of the root hair. Adhesion is followed by deformation and curling of the root hair, which results in the characteristic shepherd's crook appearance.
- The rhizobia are released from the tip of the infection thread into the cytoplasm of the host cells, where multiplication takes place.
- The rhizobia divide and differentiate into the form known as bacteroids. The forms of bacteroids encountered in the nodules of legumes vary considerably. The bacteroids may be X-and V-shaped, pear-shaped, or spherical depending upon the type of species.
- The presence of leghemoglobin gives a pink/red color to the nodule interior.
- The enzyme nitrogenase is a complex of two enzymes, an Fe-containing protein and an Fe-Mo protein. It is responsible for the conversion (reduction) of atmospheric N into NH_4^+, and is synthesized in the cytosol of the bacteroids.
- Legume utilizes NH_4^+ to convert certain precursor metabolites (e.g., α-ketoglutarate, phosphoenopyruvate) into amino acids, which, in turn, are synthesized into proteins.
- The complex biochemical reactions whereby the inert atmospheric nitrogen is enzymatically reduced into a utilizable form for the plant by the nitrogenase enzyme complex of the bacteroids is called **biological nitrogen fixation (BNF**).

Classification of the rhizobia

- The ability of certain rhizobia to infect and nodulate particular group(s) of legume species is important in the classification of rhizobia.
- Rhizobia are generally classified according to a host-based system. In this host-based system, legume(s) have been assembled into cross-inoculation groups, which are useful in organizing the diverse legumes and their rhizobial partners.

- Essentially, a cross-inoculation group consists of a collection of legume species that will develop effective nodules when inoculated with the rhizobia obtained from the nodules from any member of that legume group.
- Rhizobia belong in the family Rhizobiaceae, which consist of the following genera: Genus I-Rhizobium; Genus II-Bradyrhizobium; Genus III-Agrobacterium; and Genus IV-Phyllobacterium.
- Only Genera I and II fix N symbiotically in the root nodules of legumes. The species of rhizobia in Genera I and II, and the cross-inoculation groups of legumes nodulated by these rhizobia are summarized in Table.
- **In Genus I are the fast-growing acid producers** that develop pronounced turbidity in liquid media within 2-3 days and have a mean doubling time of 2-4 h. The cells are motile by two to six peritrichous flagella. They can grow on a wide range of carbohydrates, but usually grow best on glucose, mannitol or sucrose.
- **In Genus II are the slow-growing**, **alkali-producing rhizobia**, collectively known as bradyrhizobia. They require 3-5 days to produce moderate turbidity in liquid media and have a mean doubling time of 6-8 h. Most strains in this group grow best with pentoses as their C source. The cells are motile by a single polar or subpolar flagellum.

Table: Cross-inoculation grouping of *Rhizobium*

***Rhizobium* spp.**	**Cross-inoculation grouping**	**Legume types**
Genus I - *Rhizobium*		
R. leguminosarum bv. *viceae*	Pea group	*Pisum, Vicia, Lathyrus, Lens*
R. leguminosarum bv. *trifolii*	Bean group	*Trifolium*
R. leguminosarum bv. *phaseoli*	Cover group	*Phaseolus vulgaris*
R. meliloti	Alfalfa group	*Melilotus, Medicago, Trigonella*
R. loti	Lotus group	*Lotus, Lupinus, Orinthopus*
R. galagae	Galagae group	*Galagae orientalis*
R. *fredii*	Soybean group	*Glycine max*
R. sp.	Chickpea group	*Cicer arietunum*
R. spp.	-	*Leucaena, Glyricidia, Sesbania, Prosopis, Acacia*
Genus II - *Bradyrhizobium*		
Bradyrhizobium japonicum	Soybean group	*Glycine max*
B. spp.	Cowpea group	*Arachis hypogaea, Cajanus cajan, Vigna unguiculata, Vigna mungo, Vigna radiata, Acacia, Cyamopsis tetragonoloba, cowpea, Dalbergia, Dolichos, Aeschynomene, Indigofera, Tephrosia*

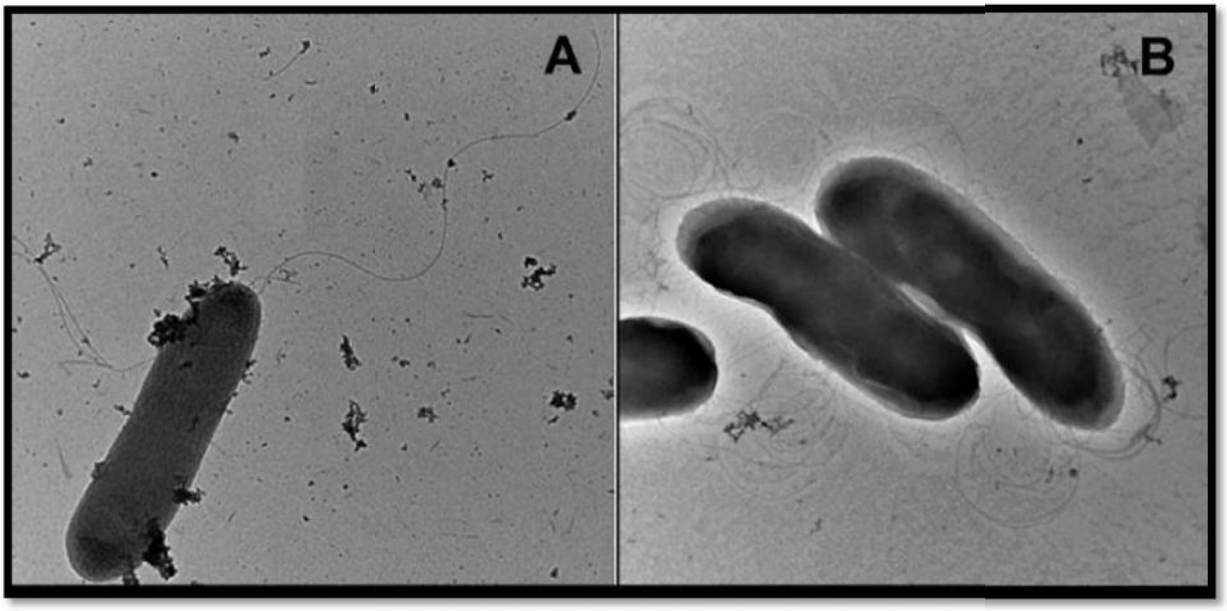
3.1.2*Frankia*

- *Frankia* are Gram-positive, filaments, aerobic, spore forming, heterotrophic, nitrogen-fixing bacteria having generation time of 15 hours. They grow in hyphal form and live in symbiosis with actinorhizal plants.
- This genus was originally named by Jørgen Brunchorst, in 1886 to honor the German biologist Albert Bernhard Frank. Hendrik Beking redefined the genus in 1970 and created the family Frankiaceae within the Actinomycetales. He retained the original name of *Frankia* for the genus.
- During growth, **Frankia** produces three **cell** types: sporangiospores, hyphae, and diazo-vesicles (spherical, thick walled, lipid-enveloped **cellular structures**). Diazo-vesicles are responsible for supply of sufficient N to the host plant during symbiosis.
- **Membranes** of *Frankia* **diazo-vesicles** surrounded by lipid components called **hopanoids, which contain nitrogenase, an oxygen-sensitive enzyme**.
- Hopanoids thicken and stabilize the walls, which help to keep oxygen away from the nitrogenase.
- *Frankia* strains can also fix N_2 in the free-living state. Under fixed-N limitation and aerobic conditions, special organs for N_2-fixation (**spherical vesicles**) are formed at the ends of hyphae. Vesicles are usually swollen structures formed at the ends of filaments under conditions of low oxygen concentration. Where present they are the site of *nitrogenase* activity and the thickness of their lipid-based walls is adjusted to prevent oxygen reaching the enzyme.
- **Actinorhizal Plants**: Plants nodulated by *Frankia* are collectively referred to as actinorhizal plants. The nodules formed by Frankia show indeterminate growth, most are perennial, and older parts may become very woody, with the active regions confined to the tips of nodule lobes.
- Actinorhizal plants are mainly woody in nature, most being shrubs or trees. They are found worldwide from the arctic to the tropics on high mountains and in lowland bogs. The amounts of nitrogen fixed by them in their natural environment are related to soil and climatic conditions (up to approximately 100 kg N / h per year).

3.1.3 *Azospirillum*

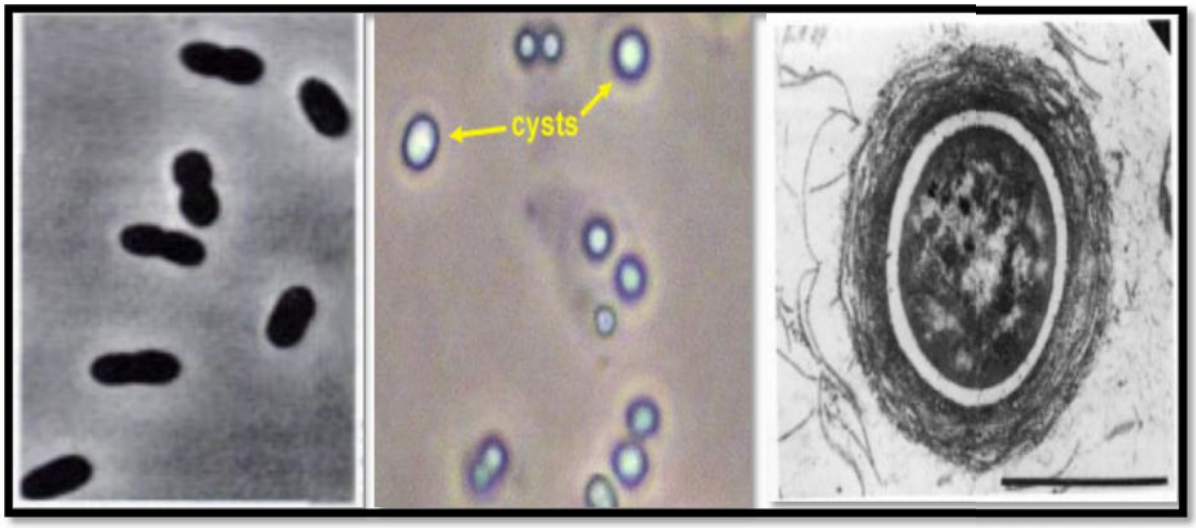
- Azospirilla are one of the earliest discovered and best-characterized associative N_2-fixing bacteria.
- The genus *Azospirillum* owes its name to its N_2-fixing capability (Az*o*-) and the spiral movements of the cell (-*spirillum*).
- These short rod-shaped, slightly curved Gram-negative highly motile bacteria were first isolated from soil in the Netherlands in 1925.
- Not many reports followed this first isolation until the group of Dr. Johanna Döbereiner 'rediscovered' *Azospirillum* in the mid-1970s.

- They are mainly present in cereal plants. Inhabits both root cells as well as surrounding of roots forming associative symbiotic relation and increasing nitrogen-fixing potential of the cereal plant.
- *Azospirillum* is recognized as a dominant soil microbe. It fixes nitrogen in the range of 20- 40 kg/ha in the rhizosphere in non-leguminous plants such as cereals, millets, Oilseeds etc.
- Considerable quantity of nitrogen fertilizer up to 25-30 % can be saved by the use of *Azospirillum* inoculant. These species have been commercially exploited for the use as nitrogen supplying Biofertilizers.
- Presently eight *Azospirillum* species have been described: *A. brasilense, A. lipoferum, A. halopraeferens, A. irakense, A. largimobile, A. doebereinerae, A. oryzae* and *A. amazonense*.
- *A. brasilense, A. lipoferum* and *A. irakense* display a mixed pattern of flagellation. *A. halopraeferens* and *A. amazonense* only display the polar flagellum.
- Motility offers the bacterium the advantage of moving towards favorable nutrient conditions. *Azospirillum* exhibit positive chemotaxis towards organic acids, sugars, amino acids and aromatic compounds as well as towards root exudates.
- Another feature of *Azospirillum* is the directed movement towards optimal oxygen concentrations, called aerotaxis. This behavioral response can be advantageous to guide the bacteria to optimal niches for nitrogen fixation.
- *Azospirillum* has been shown to positively influence plant growth, crop yields and N content of the plant. This plant stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms, including biological N_2 fixation and production of plant-growth-regulating substances particular auxins, which increase the number of lateral roots and enlarge the root hairs.



3.1.4 *Azotobacter*

- *Azotobacter* spp. are Gram negative, heterotrophic free-living/non-symbiotic, obligate aerobe, short rods with size ranges from 2-10 µm long and 1-2 µm wide, that form thick-walled cysts, present in alkaline and neutral soils.
- There are around six species in the genus *Azotobacter*, some of which are motile by means of peritrichous flagella.
- The *Azotobacter* genus was discovered in 1901 by Dutch microbiologist and botanist **Beijerinck** (**Founder of environmental microbiology**). *A. chroococcum* is the first aerobic free-living nitrogen fixer discovered.
- Apart from its ability to fix atmospheric nitrogen in soils, it can also synthesize growth promoting substances such as auxins and gibberellins and also to some extent the vitamins.
- Many strains of *Azotobactor* also exhibit fungicidal properties against certain species of fungus. Response of *Azotobactor* has been seen in rice, maize, cotton, sugarcane, pearl millet, vegetable and some plantation crops.
- *Azotobacter* is heaviest breathing organism and requires a large amount of organic carbon for its growth.
- These bacteria utilize atmospheric nitrogen gas for their cell protein synthesis. This cell protein is then mineralized in soil after the death of *Azotobacter* cells thereby contributing towards the nitrogen availability of the crop plants.
- *Azotobacter* has beneficial effects on crop growth and yield through, biosynthesis of biologically active substances, stimulation of rhizospheric microbes, producing phyopathogenic inhibitors.
- The various species of *Azotobacter*: *Azotobacter vinelandii, A. chroococcum, A. paspali, A. beijerinckii, A. armeniacus, A. macrocytogenes, A. nigricans*.



3.1.5 *Bacillus*

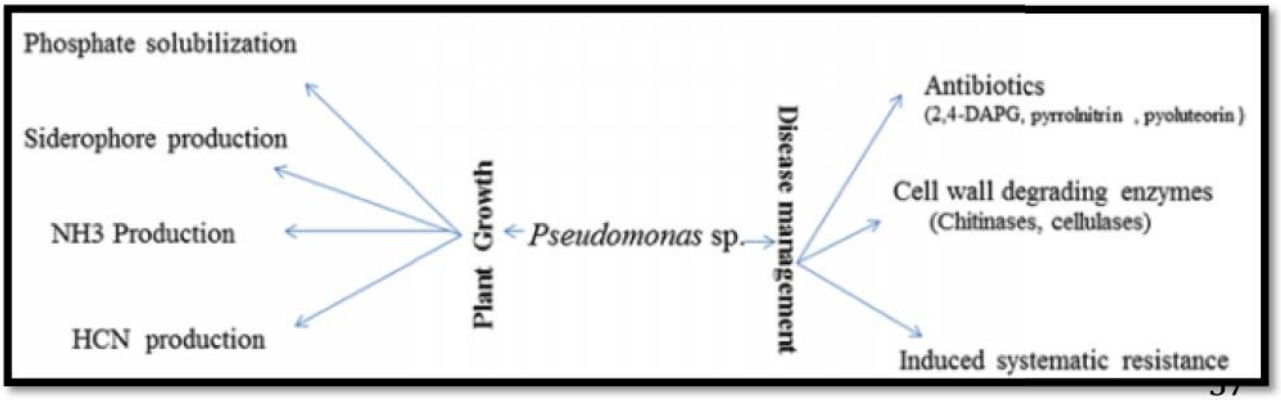
- Bacillus are Gram positive, aerobic, 3- 4 µm in size, spore former (resistant to heat) bacteria.
- The cells are often arranged in chains with rounded or square ends and usually have a single endospore, which are very resistant to adverse conditions.

Role of *Bacillus* in Agriculture:

- ***Bacillus* spp. promotes plant growth by**: (1) excreting cytokinins into rhizosphere and (2) stimulating the synthesis of phytohormones, such as IAA and GA_3.
- *Bacillus* spores act as biological control agents by inhibiting the growth of various pathogenic microbes.
- Studies have shown that the impact of *Bacillus* spp. varies among crop species and that the application of *Bacillus* can improve agronomic traits of crop plants and impart enhanced tolerance to some pathogens.
- Treatment with *Bacillus* spp. elicited ISR in most of the plant species evaluated and also altered secondary metabolite biosynthesis in plants; both effects contributed to protection against plant diseases.
- In contrast to *Pseudomonas*, using *Bacillus* strains to trigger the ISR pathway in plants is dependent on the ethylene and jasmonate pathways.

3.1.6 *Pseudomonas*

- *Pseudomonas* is Gram-negative, aerobic, motile (one or several polar flagella), non-spore forming straight or slightly curved rods.
- Another important *Pseudomonas* feature is production of variety of pigments. Character of pigmentation is significant factor as diagnostic traits of *Pseudomonas*.
- *Pseudomonads* can produce diffusible pigments that fluoresce in short wavelength (254 nm) ultraviolet light.
- Some of these pigments, like yellow-green pyoverdine (fluorescein), are siderophores that play an important physiological role in satisfying the iron requirement.
- Other pigments produced by species of *Pseudomonas* include pyocyanin (*P. aeruginosa*, blue color), pyorubin (*P. aeruginosa*, red color), chlororaphin (*P. chlororaphis*, green color), pyomelanin (*P. aeruginosa*, brown/black color). *P. mendocina* is able to produce carotenoid pigment.
- *Pseudomonas* can grow in minimal media with ammonium ions or nitrate as N-source and a single organic compound as the sole C and energy source, not requiring organic growth factors.
- Optimal temperature for growth is approximately 28ºC, although some species can grow at 4ºC or 41ºC. Most species can't tolerate acid conditions (pH 4.5 or lower).



***Pseudomonas* in plant growth and disease management**

- In general, *Pseudomonas* spp. show good colonization in numerous ecological niches including soil, water, and plant surfaces and can inhibit the growth of plant pathogens and promote plant growth.
- *Pseudomonas* strains can promote plant growth by producing plant hormones such as IAA and ACC deaminase and function as biocontrol agents by producing various pathogen-deterrent compounds, including antibiotics, polysaccharides and siderophores.
- *Pseudomonas* can induce ISR and strains of *Pseudomonas* can significantly reduce disease caused by the many plant pathogens.

4. CYNOBACTERIAL BIOFERTILIZER- ***Anabaena, Nostoc***

Cyanobacteria (blue-green algae) are a diverse group of photosynthetic, Gram negative prokaryotes and have the ability to grow in a variety of aquatic and terrestrial environments. They occur freely or in symbiotic associations with a wide range of lower and higher plants or in microbial mats. Cyanobacteria are well known for having nitrogen fixing ability and other agriculturally important potentialities.

4.1 Important nitrogen fixing cyanobacterial genera

Form of Cyanobacteria	**Cyanobacterial members**
Unicellular	*Aphanothece, Synechococcus, Gloeocapsa, Gloeothece*, Myxosarcina, Pleurocapsa*, Xenococcus*
Filamentous heterocystous	***Anabaena*****, Anabaenopsis, Aulosira, Calothrix*, Chlorogloea, Cylindrospermum, Fischerella*, Gloeotrichia, Hapalosiphon, Mastigocladus, Nodularia,* ***Nostoc*****, Rivularia, Scytonema*, Stigonema, Tolypothrix, Westiellopsis*
Filamentous non-heterocystous	*Lyngbya, Microcoleus, Myxosarcina, Oscillatoria, Plectonema, Pseudanabaena, Schizothrix, Trichodesmium*

*These Cyanobacteria may exist in symbiotic associations also.

4.2 Cyanobacteria as Bio-fertilizers

Cyanobacteria fix atmospheric N2 by forms, i.e. free-living and symbiotic associations with partners such as water fern *Azolla, cycads, Gunnera*, etc.

Some Cyanobacterial members are endowed with the specialized cells known as **heterocyst** (thick-walled modified cells), which are considered site of nitrogen fixation by nitrogenase enzyme. The enzyme is a complex, catalyzes the conversion of the molecular N2 into reduced form like ammonia.

Cyanobacteria can contribute to about 20–30 kg N/ha as well as the organic matter to the soil. Many Asian countries like China, Vietnam, India, etc., have been utilizing Cyanobacteria in paddy cultivation as the alternative to nitrogen fertilizers.

The following benefits to the agro-ecosystem are offered through use of cyanobacteria:

- Enhanced solubilization and mobility of nutrients of limited supply.
- Complexing of heavy metals and xenobiotics to limit their mobility and transport in plants.
- Mineralization of simpler organic molecules such as amino acids for direct uptake.
- Protection of plants from pathogenic insects and diseases as bio-control agents.
- Stimulation of the plant growth due to their plant growth promoting attributes.
- Improving the physico-chemical conditions of soils.

4.3 Cyanobacteria as Plant Growth Promoters

Cyanobacteria release extracellular plant growth promoting substances; some described as hormones like gibberellins, cytokinin, auxin or abscisic acids. Others are explained as

vitamins, particularly vitamin or amino acids, antibiotics and toxins. Cyanobacteria related to paddy crop revealed that cyanobacterial inoculation could enhance rice seed germination, root and shoot growth.

The fast cyanobacterial cell growth and simple nutritional requirements mainly water, sunlight and CO_2 provides a wide scope for the commercial application of cyanobacterial species as plant growth promoters.

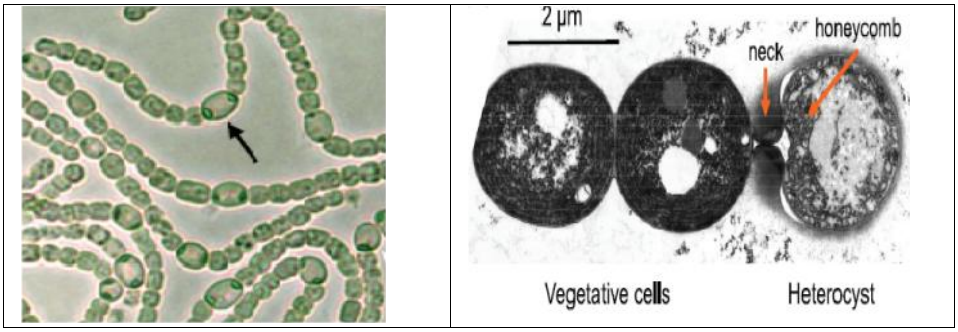
4.4 *Anabaena*

Characteristics of *Anabaena*

- Anabaena is a genus of filamentous cyanobacteria that exist as plankton. Its filament resembles the filament of *Nostoc*.
- They perform oxygenic photosynthesis.
- They are heterocyst forming and photoautotrophic.
- During times of low environmental nitrogen, about one cell out of every ten will differentiate into heterocyst.
- They are known for nitrogen-fixing abilities, and they form symbiotic relationships with certain plants, such as the Azolla.
- **Akinetes formation:** Akinetes are formed during unfavorable conditions. **Akinetes are thick walled spores with a large amount of reserved food material.** Their wall is two to three layers thick. They have granular protoplasm. Akinetes are capable of forming new filaments.
- They are one of four genera of cyanobacteria that produce neurotoxins, which are harmful to local wildlife, as well as farm animals and pets.

Importance of *Anabaena*:

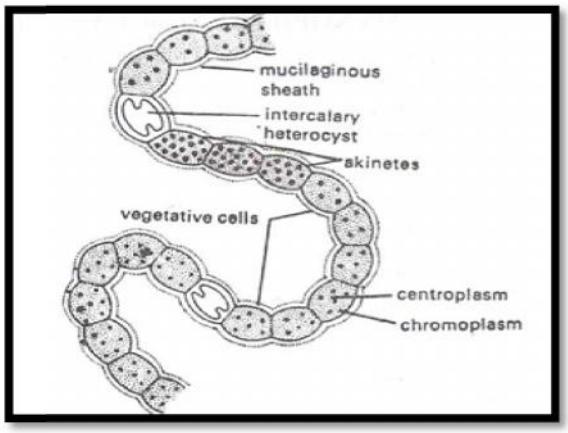
- It is believed that cyanobacteria on Earth are responsible as being the producer for most of the **oxygen** in the atmosphere.
- The *Anabaena* has been extensively studied because it also undergoes a process where it produces **hydrogen gas** by using sunlight. This product could provide a reusable source of energy.
- Certain species of Anabaena have been used on rice, paddy fields. They act as natural fertilizer.
- The cyanobacteria seem to have been the foundation of changing the Earth's atmosphere because it takes care of half of the Earth's photosynthesis.



4.5 *Nostoc*

Characteristics of *Nostoc*

- Common in fresh water ponds with free floating large colonies and appears as circular balls.
- It can also be found in soil, or moist rocks, at the bottom of lakes and springs (fresh and salt water), but rarely in marine habitat.
- It can also show symbiosis, with plant tissues like in hornworts and provide nitrogen to its host through heterocyst. It may also found as a part of lichen.
- **Cells** of ***Nostoc*** *spp*. are spherical, barrel-shaped, or oval forming unbranched filaments. The filaments (trichomes) may contain both heterocysts (thick walled, specialized N-fixing cells) and akinetes (thick walled cell that functions as a resting cell).
- **Cell structure**- It is prokaryotic type. Cell wall is made up of cellulose.
- **Pigments**- The cells are blue in color due to presence of phyccocyanine (blue pigment), other pigments include chlorophyll, carotene and phycorythrin.



5. FUNGAL BIOFERTILIZER- Arbuscular Mycorrhiza

5.1 Introduction:

- Mycorrhizae are mutualistic symbiotic associations formed between the roots of higher plants and fungi.
- It is a Greek word, myco: fungi and rhiza: root.
- Fungus root association was discovered first time by the German botanist A. B. Frank in the last century (1855) in forest trees such as pine.
- In nature, approximately 90-95 % of plants are infected with mycorrhizae. Convert insoluble form of phosphorous in soil into soluble form.
- The fungal network around the root increase the contact surface area between roots and particles of soil & absorbs nutrients from long distance away.

5.2 Types of mycorrhizae:

- On the basis of morphological and anatomical features, mycorrhizae are divided into the three types: 1) Endomycorrhizae 2) Ectomycorrhizae 3) Ectendomycorrhizae.

Endomycorrhiza:

- Endomycorrhizae is a mycorrhizal association in which the fungal hyphae are present on root surface as individual threads that may penetrate directly into root hairs, other epidermal cells & into cortical cells.
- **Endomycorrhizae** further classified in to five types: 1) **AM fungi (Arbuscular Mycorrhizae**) 2) Orchidoid mycorrhizae 3) Monotropoid mycorrhizae 4) Ericoid Mycorrhizae 5) Arbutoid mycorrhizae

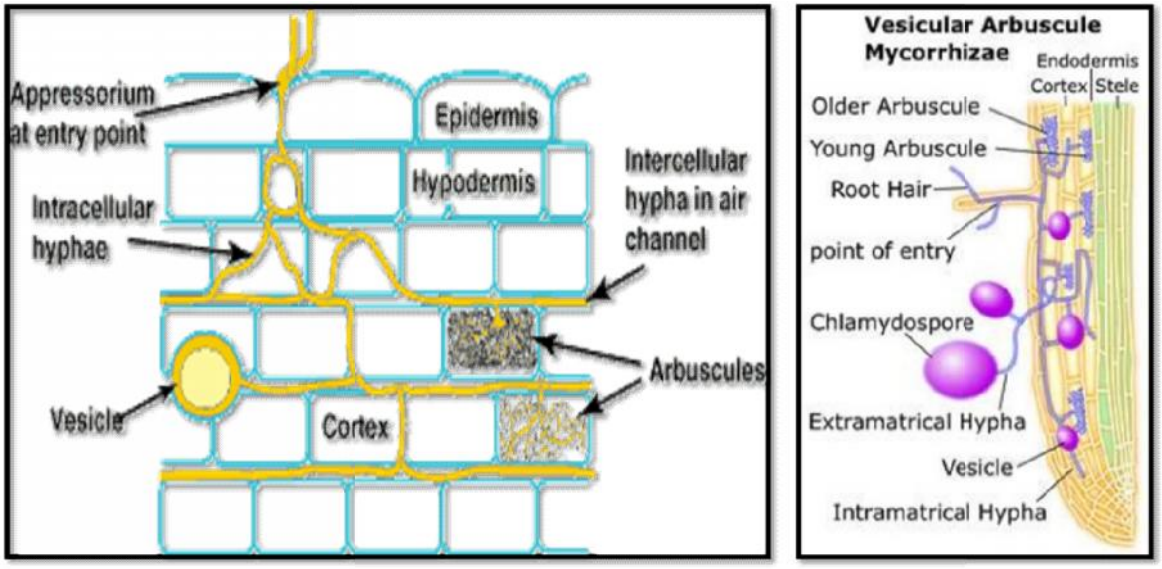
Arbuscular Mycorrhizae:

- Fungi formed association with plants belongs to *Ascomycetes, Basidiomycetes* and *Zygomycetes*.
- All AM fungi are obligate biotrophic, as they are completely dependent on plants for their survival.
- **Arbuscules** - Highly branched structures, site of nutrient transfer, short-lived structures.
- **Vesicles** – Oval shaped, darkly staining structures, thin walled, lipid filled structures that form in intercellular spaces, function as storage organ.
- **Root penetration of fungal hypha:** Hypha grows intra-cellular and penetrates the cell walls of cortical cells, causing invagination of the plasma membrane.

5.3 Applications of Mycorrhizae:

- Significant role in nutrient recycling. Increase nutrient uptake of plant from soil.
- Increases absorption of phosphate by crops, uptake of zinc also increases. Increases uptake of water from soil. Increases uptake of sulphur from the soil. Increases the concentration of cytokinins and chloroplast in plants. They protect plants during stress condition.
- Increase diversity of plant. Produce uniform seedling.
- More tolerant to adverse soil chemical constraints which limit crop production.

- Increase plant resistance to diseases and drought.
- Stimulate the growth of beneficial microorganisms.
- Improve soil structure. Stable soil aggregate – hyphal polysaccharides bind and aggregate soil particles.



5.4 Preparation of Mycorrhizal biofertilizer:

1. **Isolation**:

A) Sieving method: Soil sample + sterile water → Hot water → Filter and sieve (719µm → 250µm → 50µm → 45µm) → Spores separated from soil particles→ Mix with carrier material→ Use when required as biofertilizer.

B) Floatation method: Soil sample + sterile water → Separate the soil particles using membrane filter→ Centrifuge (Density gradient centrifuge = at 3000rpm for 30 min) → Spores separated from soil particles → Mix with carrier material → Use when required as biofertilizer.

2. **Mass production:**

Spores + antibiotic solution (streptomycin of 220 ppm concentration for 15 min) → Wash spores with mercuric chloride → Wash with distilled water → Inoculate the plant pots [Guinea grass (*Panicum maximum*) or Bahiya grass (*Paspalum notatum*)] → Keep in green house for 3 - 4 weeks → Uproot the plants → Check for colonization → Again keep for field growth (1 – 1½ months) → Macerate the root → Check for moisture content (only 5 % should be there) → **Use as biofertilizer.**

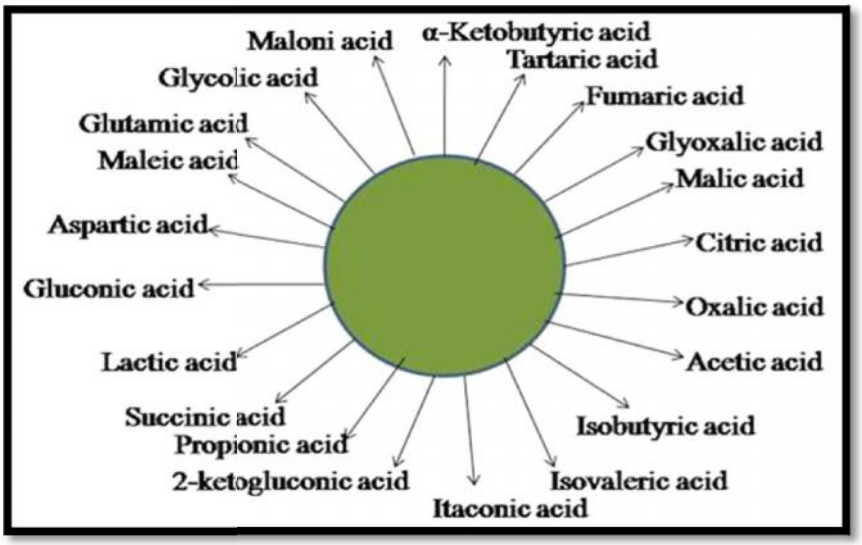
6. MECHANISMS OF PHOSPHATE AND POTASH SOLUBILIZATION AND MOBILIZATION

- Bacteria are more effective in phosphorus solubilization than fungi. Among the whole microbial population in soil, PSB constitute 1 to 50 %, while phosphorus solubilizing fungi are only 0.1 to 0.5 % in P solubilization potential.
- Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi and mycorrhiza are the most powerful P solubilizers.

6.1 Mechanism of P Solubilization:

6.1.1. Lowering Soil pH

- The principal mechanism for solubilization of soil P is lowering of soil pH by microbial production of organic acids or the release of protons.
- In alkaline soils, phosphate can precipitate to form calcium phosphates, including rock phosphate, which are insoluble in soil. Their solubility increases with decreases in soil pH.
- PSMs increase P availability by producing organic acids that lowers the soil pH.
- Strong positive correlation has been reported between phosphate solubilization and organic acids produced.
- PSMs are also known to create acidity by evolution of CO_2, as observed in solubilization of calcium phosphates.
- Production of organic acid coupled with the decrease of the pH by the action of microorganisms resulted in P solubilization.
- As the soil pH increases, divalent and trivalent forms of inorganic P, and $HPO4^{-2}$, $HPO4^{-3}$, occur in the soil.



Schematic representation of the organic acids that may be produced by PSM and used to solubilize inorganic forms of phosphate

6.1.2. Chelation

- Organic and inorganic acids produced by PSM dissolve the insoluble soil phosphates by chelation of cations and competing with phosphate for adsorption sites in the soil.
- The hydroxyl and carboxyl groups of the acids chelate the cations bound to phosphate, thereby converting it into soluble forms. These acids may complete for fixation sites of Al and Fe insoluble oxides, on reacting with them, stabilize them, and are called "chelates". 2-ketogluconic acid is a powerful chelator of calcium.
- Production of inorganic acids, such as sulphuric, nitric, and carbonic acid, has been reported.
- Nitric and sulphuric acids react with calcium phosphate and convert them into soluble forms.

6.1.3. Mineralization

- Organic phosphate is transformed into utilizable form by PSM through process of mineralization, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds such as nucleic acids, phospholipids, sugar phosphates, phytic acid, polyphosphates, and phosphonates.
- PSMs produce phosphatases like phytase that hydrolyze organic forms of phosphate compounds, and release inorganic phosphorus that will be utilized by plants. Alkaline and acid phosphatases use organic phosphate as a substrate to convert it into inorganic form.
- The commonly reported phytase-producing fungus are: Aspergillus candidus, Aspergillus *fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Penicillium rubrum*, *Penicillium simplicissimum*, *Trichoderma harzianum*, and *Trichoderma viride*.
- Soil *Bacillus* and *Streptomyces* spp. mineralize complex organic phosphates through production of extracellular enzymes like phosphoesterases, phosphodiesterases, phytases, and phospholipases. Mixed cultures of PSMs (*Bacillus*, *Streptomyces*, and *Pseudomonas*) are most effective in mineralizing organic phosphate.
- Some PSM produces **siderophores**, which hydrolyze the organic P in the soil resulting in P availability.

6.2 Mechanisms of Potassium Solubilization

- The Rhizospheric microorganism that mainly solubilizes the insoluble K to soluble forms of K are *Frateuria aurantia, Bacillus mucilaginosus, B. edaphicus, B. circulans, Acidothiobacillus ferrooxidans, Paenibacillus, Aspergillus terreus* etc.
- In general, the most important mechanisms known in K mineral solubilization by KSMs are: (i) Lowering the pH; (ii) Enhancing chelation of the cations bound to K; and (iii) Acidolysis of the surrounding area of microorganism.
- Organic matter after decomposition produces acids like citiric acid, formic acid, malic acid, oxalic acid. These organic acids produced, enhance the dissolution of potassium compounds by supplying protons and by complexing Ca^{2+} ions.
- Organic acids produced by microorganisms such as acetate, citrate and oxalate can increase mineral dissolution in soil.
- Solubilization of potassium occurs by complex formation between organic acids and metal ions such as Fe^{2+}, Al^{3+} and Ca^{2+}.

7. PRODUCTION TECHNOLOGY OF BIOFERTILIZERS

Strain: According to the first edition of Bergey's Manual of Systematic Bacteriology `A strain is made up of the descendants of a single isolation in pure culture and usually is made up of a succession of cultures ultimately derived from an initial single colony '.

Criteria for strain selection: The following are the criteria for strain selection of biofertilizer agents. A more efficient strain is that which fulfill most of these criteria:

- Efficient N-fixation/Phosphate solubilization in addition to several other beneficial PGPR attributes.
- Should be able to adapt to a particular environment and soil type.
- It should be easy to mass multiply them under controlled cultural conditions in defined media.
- It should maintain its viability during the tenure of storage.
- Should be able to colonise effectively and in more number in the rhizosphere and should be able to compete with the natural microflora in the rhizosphere.
- The other beneficial PGPR attributes are:
  - Production of plant growth promoting hormones like auxins, gibberellins, cytokinins, should reduce the ethylene production by the production of enzyme ACC deaminase.
  - Should increase the availability of other macro and micronutrients.
  - Should inhibit the plant pathogens through production of various antibiotics, siderophores, HCN etc.
  - Should be able to induce ISR (Induced systemic resistance) in the plants against infection from plant pathogens.

The efficient strains are selected and then multiplied on the nutritionally rich artificial medium before inoculating in the seed and soil. In soil, the strain has to survive and multiply to compete for infection site on roots against hostile environment in soil.

7.1 Sterilization techniques:

Sterilization is defined as complete removal or killing of all living cells including their spore from the material being sterilized.

1. Hot-air oven:

It is equipment used for dry heat sterilization. It is most commonly used for sterilizing glassware like Petri dishes, test tubes, pipettes and metal instruments that can tolerate prolonged heat exposure. Sterilization is accomplished by exposure of materials / articles usually at 160^{o} for 2.0 hr or at 180^{o}C for 1.5 hr. An oven consists of an insulated cabinet, thermostat, thermometer and a fan.

Exposure time is counted from when objects to be sterilized have reached the desired temperature inside the oven. Glassware should be perfectly dried before placing in a hot air oven since wet glassware may break. Objects, such as a glass petriplate, should be placed in sealable metal box or double layered brown paper to prevent recontamination. After the sterilization

process, the oven and its contents should be allowed to cool before opening the doors to prevent breakage and recontamination by cool air rushing into the chamber.

2. Autoclave:

Moist heat is usually provided by saturated steam under pressure in an autoclave or pressure cooker and it is the most reliable method of sterilization for most of the materials. It is an apparatus in which saturated steam under pressure effects sterilization (autoclaving). Most of the microorganisms are killed at 121^{o}C (*i.e.*, 15 lbs/in^{2}) in 20 min. The autoclave or pressure cooker should be equipped with pressure gauges, thermometer, automatic pressure control valves and exhaust valves. It is used mostly for media sterilization.

3. Flame sterilization:

Flame sterilization is used for metal objects, such as transfer needles and the tips of forceps and glass objects, such as the lips of flasks and culture tubes, microscope slides and cover slips. The object to be sterilized is held at a 45^{o} angle in the upper portion of a flame from a Bunsen burner or alcohol (spirit) lamp. Tempered metal can be heated to "red hot" and remains sterile as long as it is hot.

4. Ultraviolet rays:

It has lower energy content than ionizing radiation and is capable of producing a lethal effect in exposed cells (range 210-300 nm). The most lethal wavelength is 265 nm, which corresponds to the optimal absorption wavelength of DNA. UV light induces aberrant thymine dimer bonds between adjacent thymine nucleotide bases in the nucleic acid, which results in a deletion mutation.

Because of the low penetration capacity, the UV light is used as a disinfecting agent and has a very limited application as a sterilizing agent. It is effectively used to sterilize the air (wavelengths of 250-265 nm) inside laminar flow, isolation chamber, and operating rooms in hospitals.

7.2 Production technology of carrier based biofertilizers

A. Culturing in the small flasks containing broth

The isolated strain is inoculated in the small flasks containing suitable medium for inoculums production. Now, the carrier is autoclaved at 15 psi at 121°C for 20 min. The culture broth is mixed with the carrier at 30%, that is, for 1 kg carrier; 300 ml of culture broth is used. The mixture is spread on a plastic sheet in a closed room for air-drying. The biofertilizer is packed in sterile plastic air tight bags and stored. For large-scale production of inoculums, culture fermenters are used.

B. Mass production

Isolated bacterial cultures are sub-cultured in to nutrient broth. The cultures are grown under shaking condition at 30±2°C. The culture incubated until it reaches maximum cell population of 10^{10} to 10^{11}. Under optimum condition, this population level could be attained within 4-5 days for *Rhizobium*, 5-7 days for *Azospirillum*, and 6-7 days for *Azotobacter*. The culture obtained in the flask is called Starter culture. For large-scale production, inoculum from

starter culture is transferred in to large flasks / fermentor and grown until required level of cell count is reached. The following media are used for mass multiplication of various biofertilizers.

***Rhizobium*:** CRYEMA (yeast extract mannitol Agar + congo red)
***Azospirillum*:** Dobereiner's (Semi-solid N-free bromothymol blue (Nfb) malate) medium
***Azotobacter*:** Waksman's No. 77 broth medium; Jenson's broth
Phosbacteria (PSM): Pikovaskaya broth
***Pseudomonas*:** Kings B broth
***Trichoderma*:** Potato Dextrose Broth

Prepare appropriate media specific to bacterial inoculant in required quantity. The medium is inoculated with specific bacterial strain under aseptic condition. Incubated at 30±2ºC for 5-7 days in rotary shaker. Observe growth of the culture and estimate the population (starter culture). The above the media is prepared in large quantities in fermentor. Sterilized and cooled media in a fermentor is inoculated with the log phase of culture grown in large flask (usually 1-2 % of inoculum is sufficient).

C. Culturing in the Fermentor

- Large-scale multiplication of microorganisms often requires large vessels, commonly called **fermentors**. Industrial fermentors are designed to provide the best possible growth.
- These vessels must be strong enough, should be resistant to corrosion by the fermentation product and should not contribute toxic ions to the growth medium.
- It must have provisions for rapid incorporation of sterile air into the medium as the fermentation of microorganism is to occur aerobically.
- Some form of stirring should be available in the fermentor. The fermentor should provide for the intermittent addition of antifoam agents as demanded by the foaming status of the medium.
- A mechanism for detecting pH values of the culture medium and for adjusting these values during growth is often required.
- There must also be an outlet pipe in the bottom of the fermentor for removing the completed fermentation broth from the tank.
- Fermentors are available in varying sizes. These sizes are usually stated based on the total volume/capacity of the fermentor. However, the actual operating volume in a fermentor is always less than that of the total volume, because a "head space" must be left at the top of the fermentor above the liquid medium to allow for splashing, foaming and aeration of the liquid. This headspace usually occupies one fifth to one fourth or more of the volume of the fermentor.
- Small laboratory fermentors have a total volume of one to two litres of medium with a maximum of about 12 to 15 lit pilot plant fermentors, which are used in large scale studies of fermentations.

- Pure culture fermentation usually requires that the medium be sterilized. In small laboratory fermentors, the medium is placed directly in the fermentor and the fermentor is then autoclaved.

Carrier material: Various types of material are used as carrier for seed or soil inoculation. For preparation of seed inoculant, the carrier material is milled to fine powder with particle size of 10 - 40μm. The properties of a good carrier material for seed inoculation are:

(1) Non-toxic to inoculant bacterial strain or the plant.
(2) Good moisture absorption capacity.
(3) Easy to process and free of lump-forming materials.
(4) High organic matter content.
(5) Locally available.
(6) Inexpensive.
(7) Good adhesion to seeds.
(8) Good pH buffering capacity.

Essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered:

(1) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil.
(2) Survival of the inoculant bacteria during the storage period.
(3) Survival of the inoculant bacteria in soil. After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa. Such carrier materials that offer the available nutrient and/or habitable micro-pore to the inoculant bacteria will be desirable

Sterilization of carrier: Carrier sterilization is autoclaving. Carrier material is packed in partially opened, thin-walled polypropylene bags and autoclaved for 60 min at 121 ºC.

Preparation of inoculants packet: Neutralized and sterilized carrier material is spread in a clean, dry, sterile metallic or plastic. Bacterial culture drawn from the fermentor is added to the sterilized carrier and mixed well by manual or mechanical mixer. Inoculants are packed in polythene bags sealed with electric sealer.

Specification of the polythene bags

- Polythene bags should be of low-density grade.
- Thickness of bag should be around 50-75 micron.
- Packet should be marked with the: Name of the manufacture, Name of the product, Strain number, The crops to which recommended, Method of inoculation, Date of manufacture, Batch number, Date of expiry, Price, Full address, storage instruction.

7.3 Production of liquid Formulations of biofertilizers

7.3.1 Liquid biofertilizers - A step forward to biofertilizer technology

Liquid biofertilizers are suspension containing desired microorganisms and special cell protectants or chemicals that encourage formation of latent spores or cysts for longer shelf life and tolerance to adverse environments.

Liquid formulations use liquid materials as carrier, which is usually water, oil or some solvents in form of suspension, concentrates or emulsions. Most popular liquid inoculant formulations contain *particular organism's broth 10-40%*, *suspender* ingredient 1-3%, dispersant 1-5%, surfactant 3-8% and carrier liquid (oil and/or water) 35-65% by weight. Viscosity is adjusted at equal to the setting rate of the particles, which is achieved by the use of colloidal clays, polysaccharide gums, starch, cellulose or synthetic polymers.

The **advantages** of liquid biofertilizers over powder based are that:

- Microorganisms have longer shelf life up to 2 years,
- generally they circumvent the effect of high temperature,
- Maintain high CFU more than 10^9/ml up to 12 months,
- Better survive on seeds and soil,
- Liquid biofertilizers are easy to use, handling and storage by farmers,
- The dosage is ten times less than that of powder form; it can be packed in different volumes and save carrier materials.

7.3.2 Advantages/Disadvantages of the production technology of biofertilizers

Carrier-based	**Liquid-based**
Advantages	
Cheap	Longer shelf-life
Easier to produce	Easier to produce
Less investment	Temperature tolerant
	High cell counts
	Contamination-free
	More effective
	Product can be 100% sterile
Disadvantages	
Low shelf-life	High cost
Temperature sensitive	Higher investment for production unit
Contamination prone	
Low cell counts	
Less effective	
Automation difficult	

7.4 FCO specifications and quality control of Biofertilizer

Quality control

Till 2006 although **BIS standards** were followed for assessment of quality for four types of biofertilizers, but it was voluntary in nature. The Government of India brought four biofertilizers namely *Rhizobium*, *Azotobacter*, *Azospirillum* and PSB under the ambit of **Fertilizer (Control) Order 1985 (FCO)** during 2006. With the picking up of mycorrhizal biofertilizer production through tissue culture technique, the same was also brought under the FCO with separate specifications. Recently, two more biofertilizers, namely potash mobilizing and zinc solubilizing biofertilizers have also been incorporated under FCO.

Under the statutory provisions of FCO, biofertilizer production and its sales have been regulated and is a mandatory requirement of registration for every manufacturing unit with the **State Fertilizer Controller** (who is generally the Commissioner or Director of Agriculture Department). In every district, some officers of the Agriculture Department have been declared as **Fertilizer Inspectors**, who are authorized to inspect production and storage facilities and draw samples for quality analysis.

National Centre of Organic Farming (NCOF), Ghaziabad and its **nine Regional Centres** located at Bangalore, Bhubaneswar, Gandhinagar, Gaziabad, Imphal, Jabalpur, Nagpur, Panchkula and Patna have been declared as notified testing laboratories. Under the provisions of the act, State Governments can also develop their own quality control laboratories and notify them under the FCO 1985.

Minimum specifications of selected biofertilizers as specified in FCO are given below:

Specifications of *Rhizobium* biofertilizer

S.No.	Parameter	Requirements
(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cells/g of powder, granules or carrier material or $1x10^8$ cells/ml of liquid
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5
(v)	Particles size (for carrier based)	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Maximum moisture % by wt.(for carrier based)	30-40%
(vii)	Efficiency character	Should show effective nodulation on all the species listed on the packet

*Type of carrier: The carrier materials such as peat, lignite, peat soil, humus, wood charcoal or similar material favouring growth of organism

Specifications of *Azotobacter* Biofertilizer

S.No.	Parameter	Requirements
(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cells/g of powder, granules or carrier material or $1x10^8$ cells/ml of liquid
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5
(v)	Particles size (for carrier based)	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Maximum moisture % by wt.(for carrier based)	30-40%
(vii)	Efficiency character	The strain should be capable of fixing at least 10 mg of nitrogen per g of sucrose consumed

*Type of carrier: The carrier material such as peat, lignite, peat soil, humus, wood charcoal or similar material favouring growth of the organism

Specifications of Phosphate Solubilizing Bacterial Biofertilizer

S.No.	Parameter	Requirements
(i)	Base	Carrier based* in form of moist/dry powder or granules, or liquid based
(ii)	Viable cell count	CFU minimum $5x10^7$ cells/g of powder, granules or carrier material or $1x10^8$ cells/ml of liquid
(iii)	Contamination level	No contamination at 10^5 dilution
(iv)	pH	6.5-7.5 for moist/dry powder, granulated carrier based and 5.0-7.5 for liquid based
(v)	Particles size (for carrier based)	All material shall pass through 0.15-0.212mm IS sieve
(vi)	Maximum moisture % by wt.(for carrier based)	30-40%
(vii)	Efficiency character	The strain should have phosphate solubilizing capacity in the range of minimum 30%, when tested spectrophotometrically. In terms of zone formation, minimum 5mm solubilization zone in prescribed media having at least 3mm thickness.

Specifications of Mycorrhizal Biofertilizers

S.No.	Parameter	Requirements
(i)	Form/base	Fine powder/tablets/granules/root biomass mixed with growing substrate
(ii)	Particle size for carrier based	90% should pass through 250 micron IS sieve (60 BSS)

	powder formulations	
(iii)	Moisture content, max.	8 -12%
(iv)	pH	6.0-7.5
(v)	Total viable propagules/g of product, minimum	100/g of finished product
(vi)	Infectivity potential	80 infection points in test roots/g of mycorrhizal inoculum used

7.5 Biofertilizer-Storage & shelf life

Biofertilizer packets need to be **stored** in cool and dry place away from direct sunlight and heat. The packets may be stored at room temperature or in cold storage conditions in lots in plastic crates or polythene / gunny bags. The population of inoculant in the carrier inoculant packet may be determined at 15 days interval. There should be more than 10^9 cells / g of inoculant at the time of preparation and10^7 cells/ g on dry weight basis before expiry date.

Liquid biofertilizer formulation could be considered as one potential strategy for improving the shelf-life of biofertilizer. Unlike solid carrier based biofertilizers, liquid formulations allow the manufacturer to include sufficient amount of nutrients, cell protectant, and inducers responsible for cell/spore/cyst formation to ensure prolonged shelf-life. The shelf-life of common solid carrier based biofertilizers is around six months; however, it could be as high as two years for a liquid formulation. Further, solid carrier based biofertilizers are less thermo-tolerant whereas; liquid formulations can tolerate the temperature as high as 55°C. Hence, improved shelf-life could be achieved by the application of a liquid biofertilizer formulation.

Quality Control of Biofertilizer

The proper quality control mechanism of biofertilizer production and application covers the whole experimental process: from microorganism isolation, through laboratory screening of the isolated strains for plant growth; greenhouse screening for plant growth promotion; field screening of the most effective microbes in cropped soil; readjustment and refining of inoculants; environmental impact test and, finally, production.

The quality specifications of biofertilizers differ from country to country and may contain the following parameters:

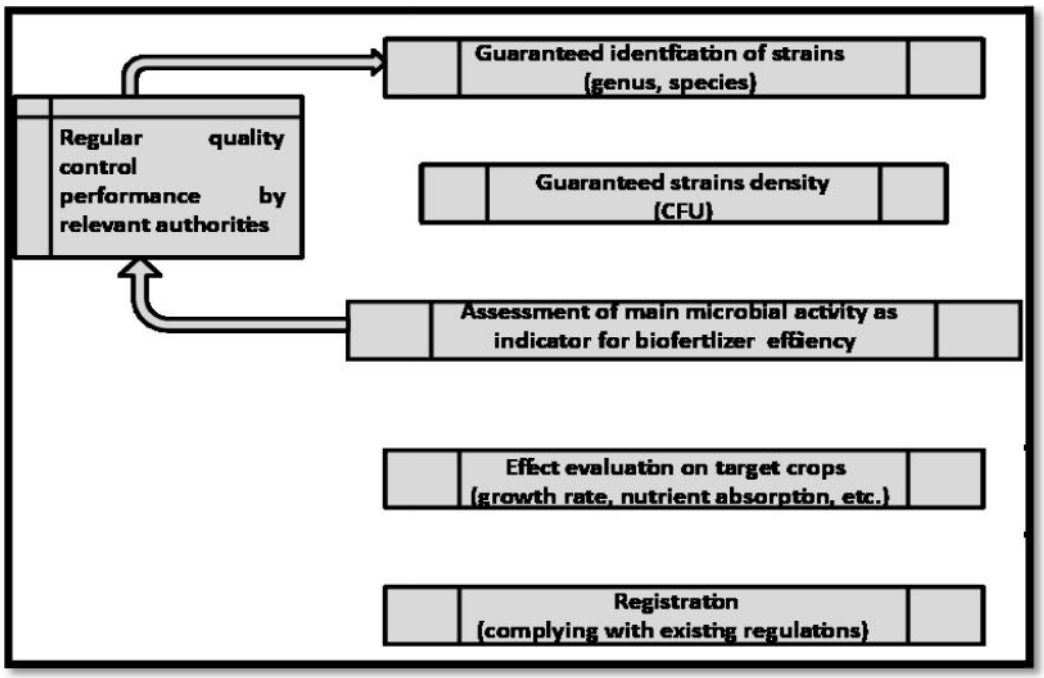
- The microbial strain(s) used; the quality of biofertilizers is usually defined in terms of two important characteristics: presence of a recommended strain in the required quantity and in active form.
- Microbial density at the time of manufacture and at the time of expiry: the number of selected microorganisms in the active form per gram or milliliter of biofertilizer. The guidelines used are limited to the density of the available microorganisms and their viability and preservation.

- The permissible contamination; it is important to set control schemes that account for putative contaminating microorganisms.
- The expiry period;
- The pH, the moisture and the carrier
- Each packet of biofertilizer should have the information eg. name of the product, leguminous crop for which intended, name and address of the manufacturer, type of carrier, batch or manufacture no, expiry date.
- Each packet should also be marked with the ISI mark. The biofertilizer should be stored in the cool place and keep away from direct heat.

Quality has to be controlled **at various stages of production** as well: during the mother culture stage, carrier selection, broth culture stage, mixing of broth and culture, packing and storage. The main quality parameters of biofertilizers are as follows:

- Appearance
- Living target bacteria: *Rhizobium sp.*, PSB, *Pseudomonas, Azotobacter* etc.
- Multi-strain biofertilizer
- Water content
- Size
- Organic matter
- pH
- Non-target bacteria (contaminants)
- Shelf-life

General procedure for quality control of biofertilizers



8. METHODS OF APPLICATION OF BIOFERTILIZERS

There are four ways for application of solid biofertilizer.

(1) The most extensively used method is the Seed treatment. The biofertilizer is applied at the rate of 200gm per 8Kg of seeds. The per acre application rate is determined from the amount of seed to be sown in a field. Before the application, biofertilizer is mixed in water (1:2) to form slurry. The slurry is poured in container with seeds to be sown. The combination is mixed properly such that each seed is coated by biofertilizer. The seeds are dried under the shade and then sown. This method is recommended for pulses, oilseeds and fodder crops.

(2) The second method is Seedling treatment. Dose wise diluted formulation is required for seedling treatment. About 1 part of biofertilizer in 10 parts of water is prepared. The roots of seedlings to be transplanted in field are dipped in biofertilizer solution for 30 minutes. After the treatment, the seedlings are immediately planted in field without drying. This method is recommended for crops like tomato, brinjal, potato, cabbage, onion, paddy and chilly which are replanted at seedling stage.

(3) The third method of choice is the Set treatment. For this treatment the ratio of biofertilizer to water is 1:50. The explants or cut pieces of planting material are immersed in biofertilizer mixture for 30 minutes. The treated pieces are dried in shade and then planted in field. The crops like sugarcane, banana, grapes and strawberries are recommended to be treated by Set treatment.

(4) Biofertilizers can also used for intermittent application for the standing crop or soil treatment before plantation or sowing. For such direct soil applications, biofertilizer is mixed with carriers like soil, compost, farmyard manure, rice husks or lignite (1kg per 25kg of carrier) and then directly put in the soil. The applied area needs to be irrigated immediately.

There are three ways for application of solid biofertilizer

1. Seed treatment:

- Take 5-7 ml of liquid biofertilizer / kg of seed and mix it well with seeds.
- Allow the coated seed to dry for 20 – 30 minutes under shade.
- After 30 minutes check whether the seed are properly dried or not, if not turn the material and allow them to dry for another 10-15 minutes.
- After shade drying the coated seed are ready for sowing.

2. Seedling treatment:

- Prepare a suspension of 50 ml of biofertilizer in 10 liters of water.
- Dip the seedlings for 20-30 minutes and transplant treated seedlings immediately.

3. Soil treatment:

- Prepare the mixture of 500 ml of biofertilizer in 40-50 kg of soil/compost.
- Broadcast the mixture in one acre (0.2 ha) either at sowing or 24 hours before sowing.
- Broadcasting may be done in standing crops just before irrigation.
- One liter biofertilizer in 120-150 kg of soil/compost should be used for long duration crops.
- Do not mix chemical fertilizers, insecticides or pesticides directly with biofertilizers.

9. FACTORS INFLUENCING THE EFFICACY OF BIOFERTILIZER

The critical factors which are responsible for the effectiveness of a particular biofertilizer are as follows:

1. Suitability of the species to the target crop.
2. Suitability of the strain: There are specific strains of *Rhizobium* for different leguminous species like Cowpea, Red gram, Soybean, Alfalfa etc. Biofertilizer specific culture should be used for specific crop.
3. Identification of strains as suited to the agro eco system, particularly the soil pH, moisture conditions.
4. Rhizo-competence of the microbes for that crop.
5. The aseptic conditions of manufacturing, the cell count of living organism present the carrier material, purity and level of contamination.
6. The conditions of carrier material in which the culture is packed and the quality of packing material, which determine the shelf life.
7. The conditions, in which the packed materials are stored, distributed and kept with farmers before it is applied.
8. Soil conditions particularly pH, organic matter content, moisture level and agronomic practices, soil salinity/sodicity/acidity etc.

Marketing constraints for wide scale use of biofertilizers

1. Retail fertilizer dealers do not keep biofertilizers mainly because of the short shelf life, limited demand and lack of storage facilities.
2. These are major constraints in the availability of biofertilizers in the market. Shelf life of carrier based inoculants, which are currently being produced, is usually not more than six months.
3. Further due to poor awareness among framers as well as development staff (Extension Staff) demand of biofertilizers is not increasing.
4. Instability of the inputs and outputs markets
5. Lack of developed marketing channels and infrastructure
6. Initiatives for promotion of biofertilizer business sector

General constraints for wide scale adoption of biofertilizers

- Unawareness regarding the biofertilizers' utility, short shelf-life, lack of ready availability in time and in the desired quality, inconsistency in results with their application.
- Different methods of inoculation application.
- No visual difference in the crop growth immediately after biofertilizer application is observed in comparison with that of inorganic fertilizers.
- Socio-psychological constraints that lead to unawareness of biofertilizer technology: lack of motivation form extension agencies; low credibility of source of biofertilizers; farmers'

belief that chemical fertilizers are more effective than biofertilizers; lack of use of biofertilizers by fellow farmers or their application being not permitted in farmers' culture.

- Lack of awareness of biofertilizers is a major challenge for farmers.
- Insufficient understanding of the technology of agro-dealers, extension services and policy makers.

Can we use biofertilizers with chemical fertilizers?

There is a huge difference in the application amount and the actual availability of chemical fertilizers to the plants. Biofertilizers have been reported to enhance the availability of these inorganic inputs to the plants. Thus Biofertilizers can be used along with chemical fertilizers but the care should be taken to avoid direct contact of chemical based inputs with Biofertilizers which is likely to reduce the microbial population of Biofertilizers.

Disadvantages of biofertilizers

- Biofertilizers require special care for long-term storage because they are alive.
- Must be used before their expiry date.
- If other microorganisms contaminate the carrier medium or if growers use the wrong strain, they are not as effective.
- Biofertilizers lose their effectiveness if the soil is too hot or dry.