RHIZOBIUM

Rhizobium is a genus of Gram-negative soil bacteria that fix nitrogen. Rhizobium species form an endosymbiotic nitrogen-fixing association with roots of legumes and Parasponia.

The bacteria colonize plant cells within root nodules, where they convert atmospheric nitrogen into ammonia using the enzyme nitrogenase and then provide organic nitrogenous compounds such as glutamine or ureides to the plant. The plant, in turn, provides the bacteria with organic compounds made by photosynthesis.This mutually beneficial relationship is true of all of the rhizobia, of which the genus Rhizobium is a typical example.

Kingdom	:	Bacteria
Phylum	:	Proteobacteria
Class	:	Alphaproteobacteria
Order	:	Rhizobiales
Family	:	Rhizobiaceae
Genus	:	*Rhizobium*

Martinus Beijerinck was the first to isolate and cultivate a microorganism from the nodules of legumes in 1888. He named it *Bacillus radicicola*, which is now placed in Bergey's Manual of Determinative Bacteriology under the genus *Rhizobium*.

The word *Rhizobium* comes from the Greek words: "rhiza" which refers to root, and "bios" which refers to life.

Rhizobium Species include:

- *R. leguminosarum*
- *R. alamii*
- *R. lentis*
- *R. japonicum*
- *R. metallidurans*
- *R. smilacinae*
- *R. phaseoli*
- *R. trifolii*

The cross-inoculation groups include:

- Clover groups - *R. trifolii* infects and nodulates plants of genus *Trifolium* (clovers/trefoil)
- Alfalfa groups - *R. meliloti* infects and nodulates the roots of *medicago, melilotus* and *medicago*
- Bean group - *R. phaseoli* infects and nodulates plants of genus Phaseolus (e.g. beans)
- Lupine group - *R. lupine* nodulates lupines and serradella (Ornithopus)
- Pea group - *R. leguminosarum* infects and nodulates pea, sweet pea, lentil, and vetch.
- Soybean group - *R. japonicum* nodulates Glycine such as soybean
- Cowpea group - *Rhizobium* sp. nodulates cowpea, pegionpea, lespedza, groundnut and kudz among a few others.

MORPHOLOGY

- They appear as elongated rods when viewed under the microscope
- Like a number of other bacteria, *Rhizobium leguminosarum* do not form spores in their life cycle
- They posses several flagella on their polar end. This allows them to move from one location to another
- They are aerobic. As such, they need oxygen for respiratory purposes
- There are various strains of the bacteria some of which have granules
- They can tolerate higher temperatures of about 38 degrees Celsius.

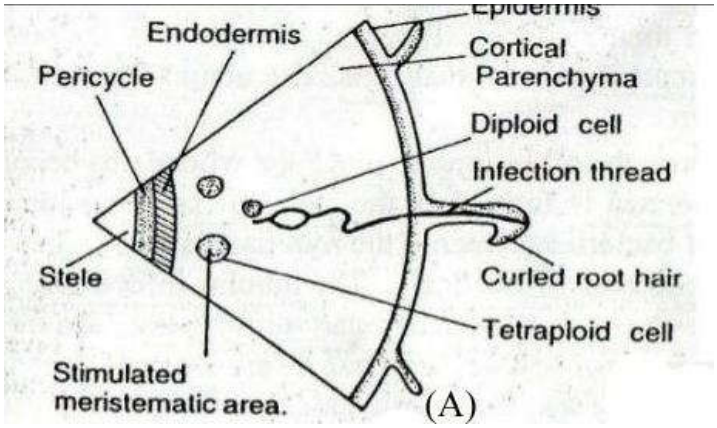
ROOT NODULE FORMATION

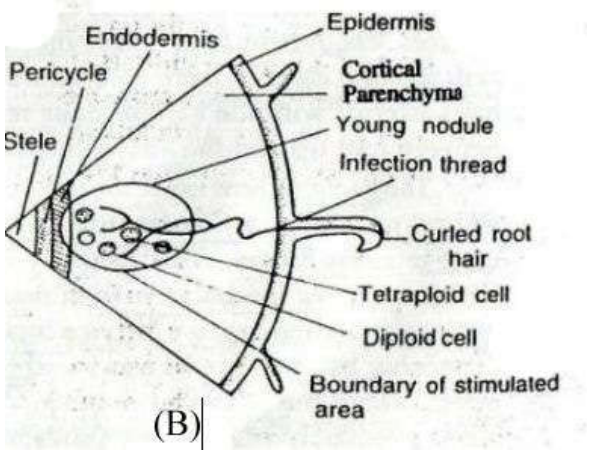
- Rhizobium species can be found in soil. However, the root of leguminous plants (lentil, sweetpea etc) is their primary habitat. In the soil, various leguminous plants release various exudates (dicarboxylic acids etc) that attract *Rhizobium* species.
- Flavanoids have also been shown to play an important role attracting the bacteria given that they are easily absorbed through the membrane of the organisms (passively). Once the bacteria detect these chemicals, they actively swim towards and attach to the legume root.
- In addition to attracting the bacteria, these chemicals (flavanoids in particular) also play an important role of activating genes involved in producing Nod factors. Here, then, attraction to the legume roots is followed by transcription of Nod genes in preparation of the symbiotic relationship.

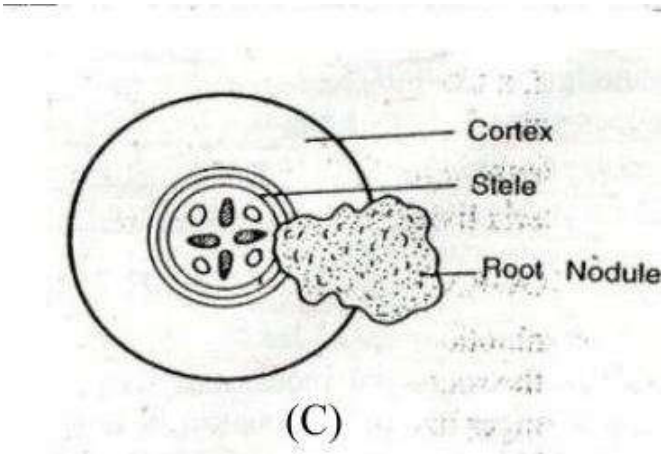
- For the plant, Nod factors stimulate the branching of root hair, hydrolysis of the cell wall as well as deformation of the cell wall. Having attracted the bacteria through the exudates, the changes in the plant roots make it easy for the organism to enter the cells of the root hair for symbiosis.
- When the bacteria comes into contact with the root hair, they cause the plasma membrane of the cells to invaginate. As the bacteria penetrates the cell, the plant produces new cell wall material at the site to not only cover the bacteria, but also allowing them to enter deeper into the root hairs.
- Once the bacteria infects the cells of the root hair, the symbiotic process may produce the following types of nodules
- Determinate nodules - Determinate are found in plants like soybeans, are spherical in shape with large lenticels. Compared to indeterminate nodules, determinate nodules are formed once the tip of the root hairs start growing. However, they lack the persistent meristem found in indeterminate nodules.

- Indeterminate nodules - Compared to determinate nodules, indeterminate nodules are cylindrical in shape and frequently branched. They are often found in plants like peas and alfalfa and start developing even before the growth of the root tip.

Diagram showing steps involved in root nodule formation







COMPOSITION OF YEAST EXTRACT MANNITOL AGAR MEDIUM

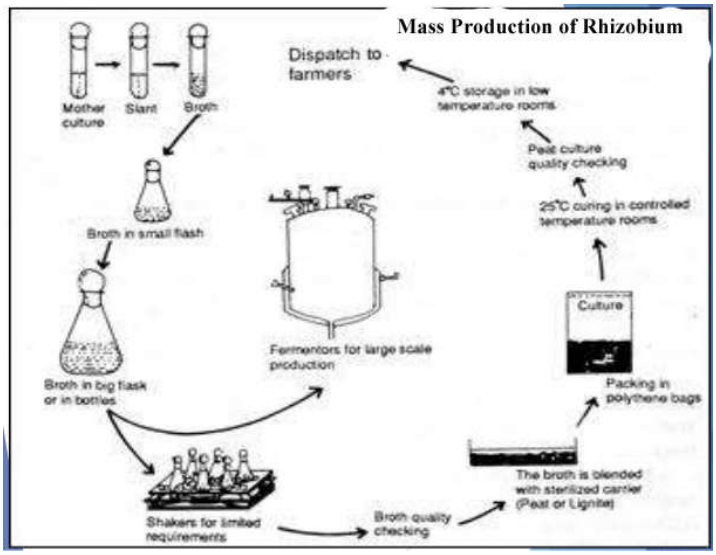
Mannitol	-	10.0g
K_2HPO_4	-	0.5g
$Mg\ SO_4.7H_2O$	-	0.2g
Nacl	-	0.1g
Yeast Extract	-	0.5g
Agar	-	20 g
Distilled water	-	1000 ml

Add 10 ml of Congo red stock solution (dissolve 250 mg of Congo red in 100ml water) to 1 liter after adjusting the pH to 6.8 and before adding agar.

Rhizobium forms white, translucent, glistening, elevated and comparatively small colonies on this medium

Moreover, *Rhizobium* colonies do not take up the colour of congo red dye added in the medium.

Those colonies which readily take up the congo red stain are not *rhizobia* but presumably *Agrobacterium*, a soil bacterium closely related to *Rhizobium*.



Inoculum preparation

- Prepare appropriate media for specific to the bacterial inoculant in 250 ml, 500 ml, 3 litre and 5 litre conical flasks and sterilize.
- The media in 250 ml flask is inoculated with efficient bacterial strain under aseptic condition
- Keep the flask under room temperature in rotary shaker (200 rpm) for 5- 7 days.
- Observe the flask for growth of the culture and estimate the population, which serves as the starter culture.
- Using the starter culture (at log phase) inoculate the larger flasks (500 ml, 3 litre and 5 litre) containing the media, after obtaining growth in each flask.
- The above media is prepared in large quantities in fermentor, sterilized well, cooled and kept it ready.
- The media in the fermentor is inoculated with the log phase culture grown in 5 litre flask. Usually 1-2 % inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks.
- The cells are grown in fermentor by providing aeration and given continuous stirring.
- It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at 40° C number of viable cells begins to decrease.
- The broth is checked for the population of inoculated organism and contamination if any at the growth period.
- The cells are harvested with the population load of 109 cells / ml after incubation period.
- There should not be any fungal or any other bacterial contamination at 10-6 dilution level

Processing of carrier material

The use of ideal carrier material is necessary in the production of good quality biofertilizer.

1.Peat soil

2.Lignite

3.Vermiculite

4.Charcoal

5.Press mud

6.Farmyard manure and soil mixture can be used as carrier materials.

The neutralized peat soil/lignite are found to be better carrier materials for biofertilizer production.

Selection of ideal carrier material.

1. Cheaper in cost
2. Should be locally available
3. High organic matter content
4. No toxic chemicals
5. Water holding capacity of more than 50%
6. Easy to process, friability and vulnerability.

Preparation of carrier material

The carrier material (peat or lignite) is powdered to a fine powder so as to pass through 212 micron IS sieve. The pH of the carrier material is neutralized with the help of calcium carbonate, since the peat soil / lignite are acidic in nature

(pH of 4 - 5) The neutralized carrier material is sterilized in an autoclave to eliminate the microorganisms

Mixing the carrier and the broth culture and packing

Inoculant packets are prepared by mixing the broth culture obtained from fermenter with sterile carrier material as described below:

Preparation of Inoculants packet

- The neutralized, sterilized carrier material is spread in a clean, dry, sterile metallic or plastic tray.
- The bacterial culture drawn from the fermentor is added to the sterilized carrier and mixed well by manual (by wearing sterile gloves) or by mechanical mixer.
- The culture suspension is to be added to a level of 40- 50% water holding capacity depending upon the quantityof carrier material

- ❖ The inoculant packet of 200 g quantities in polythene bags, sealed with electric sealer and allowed for curing for 2-3 days at room temperature.

Curing can be done by spreading the inoculant on a clean floor/polythene sheet/ by keeping in open shallow tubs/ trays with polythene covering for 2-3days at room temperature before packaging.

Specification of the polythene bags

- ❁ The polythene bags should be of low density grade.
- ❁ The thickness of the bag should be around 50 - 75 micron.
- ❁ Each packet should be marked with the
- ❁ name of the manufacturer,
- ❁ name of the product
- ❁ strain number
- ❁ the crop to which recommended
- ❁ method of inoculation
- ❁ date of manufacture
- ❁ batch number,
- ❁ date of expiry
- ❁ price
- ❁ full address of the manufacturer and storage
- ❁ instructions to farmers

Storage of biofertilizer packet

- ✓ The packet should be stored in a cool place away from the heat or direct sunlight.
- ✓ 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 carrierinoculant packet may be determined at 15 days interval.
- ✓ There should be more than 109 cells / g of inoculant at the time of preparation and 107 cells/ g on dry weight basis before expiry date.

Application of *Rhizobium*

1. Seed treatment or seed inoculation
2. Seedling root dip
3. Main field application

Seed treatment

- ✓ One packet of the inoculant is mixed with 200 ml of rice to make a slurry.
- ✓ The seeds required for an acre are mixed in the slurry so as to have a uniform coating of the inoculant over the seeds and then shade dried for 30 minutes.
- ✓ The shade dried seeds should be sown within 24 hours.
- ✓ One packet of the inoculant (200 g) is sufficient to treat 10 kg of seeds.

Seedling root dip

- This method is used for transplanted crops.
- Two packets of the inoculant is mixed in 40 litres of water.
- The root portion of the seedlings required for an acre is dipped in the mixture for 5 to 10 minutes and then transplanted.

Main field application

Four packets of the inoculant is mixed with 20 kgs of dried and powdered farm yard manure and then broadcasted in one acre of main field just before transplanting.

AZOTOBACTER

Azotobacter is a genus of usually motile, oval or spherical bacteria that form thick-walled cysts and may produce large quantities of capsular slime. They are aerobic, free-living soil microbes that play an important role in the nitrogen cycle in nature, binding atmospheric nitrogen, which is inaccessible to plants, and releasing it in the form of ammonium ions into the soil (nitrogen fixation). In addition to being a model organism for studying diazotrophs, it is used by humans for the production of biofertilizers, food additives, and some biopolymers. The first representative of the genus, *Azotobacter chroococcum*, was discovered and described in 1901 by Dutch microbiologist and botanist Martinus Beijerinck. *Azotobacter* species are Gram-negative bacteria found in neutral and alkaline soils, in water, and in association with some plants.

TAXONOMY

Kingdom	:	Bacteria
Phylum	:	Proteobacteria
Class	:	Gammaproteobacteria
Order	:	Pseudomonadales
Family	:	Pseudomonadaceae/Azotobacteraceae
Genus	:	*Azotobacter*

Distribution

Azotobacter species are ubiquitous in neutral and weakly basic soils, but not acidic soils. They are also found in the Arctic and Antarctic soils, despite the cold climate, short growing season, and relatively low pH values of these soils. In dry soils, *Azotobacter* can survive in the form of cysts for up to 24 years.

Representatives of the genus *Azotobacter* are also found in aquatic habitats, including fresh water and brackish marshes. Several members are associated with plants and are found in the rhizosphere, having certain relationships with the plants. Some strains are also found in the cocoons of the earthworm *Eisenia fetida.*

Morphology

Cells of the genus *Azotobacter* are relatively large for bacteria (2–4 μm in diameter). They are usually oval, but may take various forms from rods to spheres. In microscopic preparations, the cells can be dispersed or form irregular clusters or occasionally chains of varying lengths. In fresh cultures, cells are mobile due to the numerous flagella. Later, the cells lose their mobility, become almost spherical, and produce a thick layer of mucus, forming the cell capsule. The shape of the cell is affected by the amino acid glycine, which is present in the nutrient medium peptone.

Under magnification, the cells show inclusions, some of which are colored. In the early 1900s, the colored inclusions were regarded as "reproductive grains", or gonidia – a kind of embryo cells. However, the granules were later determined to not participate in the cell division. The colored grains are composed of volutin, whereas the colorless inclusions are drops of fat, which act as energy reserves.

Cysts of the genus *Azotobacter* are more resistant to adverse environmental factors than the vegetative cells; in particular,they are twice as resistant to ultraviolet light. They are also resistant to drying, ultrasound, and gamma and solar irradiation, but not to heating.

The formation of cysts is induced by changes in the concentration of nutrients in the medium and addition of some organic substances such as ethanol, n-butanol, or β-hydroxybutyrate. Cysts are rarely formed in liquid media. The formation of cysts is induced by chemical factors and is accompanied by metabolic shifts, changes in catabolism, respiration, and biosynthesis of macromolecules it is also affected by aldehyde dehydrogenase and the response regulator AlgR.

The cysts of *Azotobacter* are spherical and consist of the so-called "central body" – a reduced copy of vegetative cells with several vacuoles – and the "two-layer shell". The inner part of the shell is called intine and has a fibrous structure. The outer part has a hexagonal crystalline structure and is called exine.

Physiological properties

Azotobacter respires aerobically, receiving energy from redox reactions, using organic compounds as electron donors,and can use a variety of carbohydrates, alcohols, and salts of organic acids as sources of carbon.

Azotobacter can fix at least 10 μg of nitrogen per gram of glucose consumed. Nitrogen fixation requires molybdenum ions, but they can be partially or completely replaced by vanadium ions. If atmospheric nitrogen is not fixed, the source of nitrogen can alternatively be nitrates, ammonium ions, or amino acids. The optimal pH for the growth and nitrogen fixation is 7.0 –7.5, but growth is sustained in the pH range from 4.8 to 8.5.

Azotobacter can also grow mixotrophically, in a molecular nitrogen-free medium containing mannose; this growth mode is hydrogen-dependent. Hydrogen is available in the soil, thus this growth mode may occur in nature.

While growing, *Azotobacter* produces flat, slimy, paste-like colonies with a diameter of 5–10 mm, which may form films in liquid nutrient media. The colonies can be dark-brown, green, or other colors, or may be colorless, depending on the species. The growth is favored at a temperature of 20–30°C.

Bacteria of the genus *Azotobacter* are also known to form intracellular inclusions of polyhydroxyalkanoates under certain environmental conditions (e.g. lack of elements such as phosphorus, nitrogen, or oxygen combined with an excessive supply of carbon sources).

Pigments

Azotobacter produces pigments.For example,*Azotobacter chroococcum* forms a dark-brown water-soluble pigment melanin.This process occurs at high levels of metabolism during the fixation of nitrogen, and is thought to protect the nitrogenase system from oxygen. Other *Azotobacter* species produce pigments from yellow-green to purple colors, including a green pigment which fluoresces with a yellow-green light and a pigment with blue-white fluorescence.

ISOLATION OF *Azotobacter*

The soil samples were collected from various fields and serial dilutions were done. The organism were isolated by the analysis of the characteristics according to the morphological and biochemical characteristics. The various biochemial tests conducted were citrate utilization, catalase, urease, indole, methyl red,vogues prokauer, H_2S and nitrate reduction test were performed and confirmed. Then using the specific medium Pikovskaya's medium for phosphobacter and Asbhy's agar for *Azotobacter* were used to grow the organism for the mass production.

MASS PRODUCTION OF *AZOTOBACTER*

For mass production of *Azotobacter*, bacterial strain is isolated from various regions and grown on slants for preservation as per need culture from slant were transferred to liquid broth of selective as well as optimized medium in the rotary shaker for 4 days to prepare starter culture. Later on the starter cultures is transferred to the fermenter in batch culture mode with proper maintenance of 300C and continuous agitation for 4-9 days, when cell count reached to 108-109 cells/ml, the broth used as inoculant. For easy handling, packing, storing and transporting broth is mixed with an inert carrier material which contains sufficient amount of cells. After proper mixing carrier containing inoculant was left for 7days and above formulated microbial inoculants used as biofertilizer.

AZOSPIRILLUM

The prefix “Azo-” comes from the French word “azote”, which means nitrogen. This prefix is used to denote the ability of the bacteria to fix atmospheric nitrogen. The ending “-spirillum” refers to the shape of the bacteria which is similar to spiral-shaped bacteria in the genus

Discovery and Reclassification

The first species described in the genus was originally named *Spirillum lipoferum* in 1925 by M.W. Beijerinck. In Brazil, during the 1970s, similar strains of this species were found associated with the roots of grain plants by scientists lead by Dr. Johanna Döbereiner. Her group discovered that these bacteria had the ability to fix nitrogen. Due to this discovery, *Spirillum lipoferum* was reclassified in 1978 as *Azospirillum lipoferum* by Jeffery Tarrand, Noel Krieg, and Döbereiner, who also added *Azospirillum brasilense* to the genus. By 2020, twenty-one species of *Azospirillum* had been described.

Azospirillum is a Gram-negative, microaerophilic, non-fermentative and nitrogen-fixing bacterial genus from the family of Rhodospirillaceae. *Azospirillum* bacteria can promote plant growth.

TAXONOMY

Kingdom	:	Bacteria
Phylum	:	Proteobacteria
Class	:	Alphaproteobacteria
Order	:	Rhodospirillales
Family	:	Rhodospirillaceae
Genus	:	*Azospirillum*

MORPHOLOGY

The genus *Azospirillum* belongs in the alpha-Proteobacteria class of bacteria. *Azospirillum* are Gram-negative, do not form spores, and have a slightly-twisted oblong-rod shape. *Azospirillum* have at least one flagellum and sometimes multiple flagella, which they use to move rapidly *Azospirillum* are aerobic, but

many can also function as microaerobic diazotrophs, meaning, under low oxygen conditions, they can change inert nitrogen from the air into biologically useable forms.

At least threespecies,*A.melinis*, *A.thiophilum*,and *A.humicireducens* are facultative anaerobes, and can live, if necessary, without oxygen. Growth of *Azospirillum* is possible between 5°C and 42°C and in substrates with a pH of 5 to 9, with optimal growth occurring around 30°C and 7 pH. Microbiologists use nitrogen-free semi-solid media isolate *Azospirillum* from samples. The most commonly used media is called "NFb".

Isolation of *Azospirillum*

25 mL test tubes with 5 mL of NFB semi-solid medium were inoculated with one gram rhozophere soil were inoculated with malate semisolid medium Bacterial smears preparation Flame heat fixed smears prepared from light suspension of cells, flooded with crystal violet solution for 1 minute and washed for 30 seconds in the running water. Rinse off excess water and flooded in iodine solution for 1 min and again washed in running water for 30 seconds. Slides were passed through iodinated alcohol solution for removing excess stain and washed with tap water for five seconds. The excess water removed and flooded with counter stain (safranin) for 1 minute and again washed in tap water, air dried and examined under microscope.

Mass production of *Azospirillum*

The isolated strains were used for large scale multiplication. The isolated strain selected for the preparation of azospirillum inoculums. These strains were inoculated into BMS agar slants. They are called starter culture. These starter cultures were transferred into 100mL NFB liquid medium. containing 250mL conical flask. These cultures were incubated at 28+2'c for 4 days with occasional shaking of the conical flasks, for proper aeration. After 4 days, the cultures were mixed with carrier materials. Lignite was used as carrier for Biofertilizer production, 4kg lignite used per liter of broth culture for mixing. After mixing 2% CaCO3 was added and the mixed inoculums covered by polythene sheets for curing for 24 hours. After 24 hours the carrier based inoculums were pocketed into polythene bags.The polythene bags were stored into the store room at 28=2c. These culture pockets were used for field application.