SEMESTER – I

PAPER – I

VIRUSES, PROKARYOTES, ALGAE & BIOFERTILIZERS

Unit-IV: Skill Development: Biofertilizer:

1. **Biofertilizer: Defination, scope and importance**
2. **Various Microbes used As Biofertilizers**

Commercial production of biofertilizers: Rhizobium, Azotobacter, PSB (Phosphate Solubilizing Bacteria, e.g Bacillus polymyxa) and Azolla.

BIOFERTILIZERS

“Biofertilizers are substances that contain microorganisms, which when added to the soil increase its fertility and promotes plant growth.”

Biofertilizers are substance that contains microbes, which helps in promoting the growth of plants and trees by increasing the supply of essential nutrients to the plants. It comprises living organisms which include mycorrhizal fungi, blue-green algae, and bacteria. Mycorrhizal fungi preferentially withdraw minerals from organic matter for the plant whereas cyanobacteria are characterized by the property of nitrogen fixation.
Nitrogen fixation is defined as a process of converting di-nitrogen molecules into ammonia. For instance, some bacteria convert nitrogen to ammonia. As a result, nitrogen becomes available for plants.

Types of Biofertilizers

Following are the important types of biofertilizers:

Symbiotic Nitrogen-Fixing Bacteria

Rhizobium is one of the vital symbiotic nitrogen-fixing bacteria. Here bacteria seek shelter and obtain food from plants. In return, they help by providing fixed nitrogen to the plants.

Loose Association of Nitrogen-Fixing Bacteria

Azospirillum is a nitrogen-fixing bacteria that live around the roots of higher plants but do not develop an intimate relationship with plants. It is often termed as rhizosphere association as these bacteria collect plant exudate and the same is used as food by them. This process is termed associative mutualism.

Symbiotic Nitrogen-Fixing Cyanobacteria

Blue-Green algae or Cyanobacteria from the symbiotic association with several plants. Liverworts, cycad roots, fern, and lichens are some of the Nitrogen-fixing cyanobacteria. Anabaena is found at the leaf cavities of the fern. It is responsible for nitrogen fixation. The fern plants decay and release the same for utilization of the rice plants. Azolla pinnate is a fern that resides in rice fields but they do not regulate the growth of the plant.

Free-Living Nitrogen-Fixing Bacteria

They are free-living soil bacteria that perform nitrogen fixation. They are saprotrophic anaerobes such as *Clostridium beijerinckii*, Azotobacter, etc.

Among all the types of biofertilizers, Rhizobium and Azospirillum are most widely used.

Components of Biofertilizers

The components of biofertilizers include:

Bio Compost

It is one of the eco-friendly product composed of waste material released from sugar industries which are decomposed. It is magnified with human-friendly bacteria, fungi, and various plants.

Tricho-Card

It is an eco-friendly and nonpathogenic product used in a variety of crops as well as in horticultural and ornamental plants, such as paddy apple, sugar cane, brinjal, corn, cotton, vegetables, citrus, etc. It acts as a productive destroyer and antagonistic hyper parasitic against eggs of several bores, shoot, fruit, leaves, flower eaters and other pathogens in the field.

Azotobacter

It protects the roots from pathogens present in the soil and plays a crucial role in fixing atmospheric nitrogen. Nitrogen is a very important nutrient for the plant and about 78% of the total atmosphere comprises nitrogen.

Phosphorus

Phosphorus is one of the essential nutrients for plants growth and development. Phosphate solubilizing microorganisms, hydrolyze insoluble phosphorus compounds to the soluble form for uptake by plants. Many fungi and bacteria are used for the purpose such as *Penicillium, Aspergillus*, *Bacillus*, *Pseudomonas, etc.*

Vermicompost

It is an Eco-friendly organic fertilizer that comprises vitamins, hormones, organic carbon, sulfur, antibiotics that help to increase the quantity and quality of yield. Vermicompost is one of the quick fixes to improve the fertility of the soil.

Importance of Biofertilizers

Biofertilizers are important for the following reasons:

•Biofertilizers improve the soil texture and yield of plants.

•They do not allow pathogens to flourish.

•They are eco-friendly and cost-effective.

•Biofertilizers protect the environment from pollutants since they are natural fertilizers.

•They destroy many harmful substances present in the soil that can cause plant diseases.

•Biofertilizers are proved to be effective even under semi-arid conditions.

Applications of Biofertilizers

Following are the important applications of biofertilizers:

Seedling root dip

This method is applicable to rice crops. The seedlings are planted in the bed of water for 8-10 hours.

Seed Treatment

The seeds are dipped in a mixture of nitrogen and phosphorus fertilizers. These seeds are then dried and sown as soon as possible.

Soil Treatment

The biofertilizers along with the compost fertilizers are mixed and kept for one night. This mixture is then spread on the soil where the seeds have to be sown.

Microbes As Biofertilizers

The following microorganisms are used as biofertilizers:

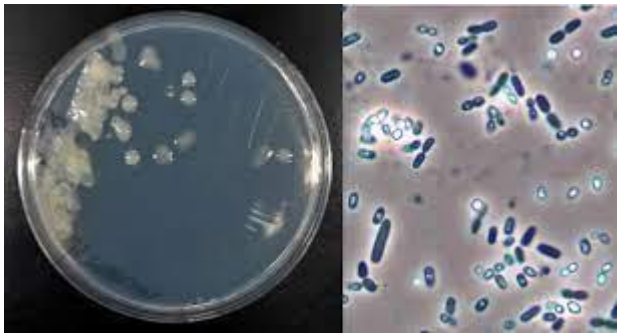
•**Rhizobium:** They form root nodules in leguminous plants and fix the atmospheric nitrogen into an organic form. Rhizobium also has no negative effect on soil quality and improves the quality, nutrient content, and growth of the plant.

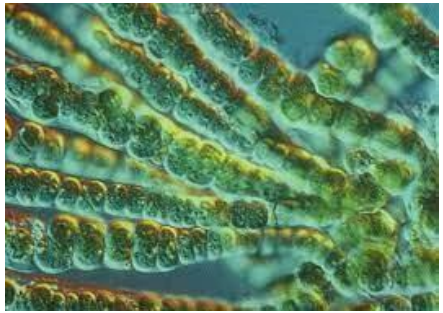
•**Azotobacter:** These are free-living nitrogen fixers found in all types of upland crops. These not only fix nitrogen but also provide certain antibiotics and growth substances to the plant.

•**Azospirillum:** Unlike Azotobacter, these can be used in wetland areas. They are found inside the roots of the plant (non-free-living) where they fix the atmospheric nitrogen.

•**Blue-green algae:** These are free-living nitrogen-fixing Cyanobacteria that are present only in wet and marshy lands. However, they do not survive in acidic soil.

Mycorrhiza: It is a symbiotic association between the fungi and the roots of a plant. The mycorrhizal fungi play an important role in binding the soil together and improves the activity of the microbes. The fungi draw water and nutrients from the soil thereby increasing the plant productivity. It also helps the plant to survive under various environmental stresses.





Commercial production of biofertilizers: Rhizobium

Rhizobium is a soil habitat Gram-negative bacterium, which can able to colonize the legume roots and fixes atmospheric nitrogen symbiotically. There is symbiotic association between plant and bacteria, initiated when bacteria in the soil attach to root hairs. This highly specific attachment process is mediated by plant proteins,the lectins that bind the bacteria to the surface of the root hairs and then penetrated by the microbes.The infected root cells divide and form a nitrogen fixing nodule which provides the anaerobic environment necessary for nitrogen fixation. Pulse legumes are attributed to their ability to biologically fix nitrogen in symbiosis with certain types of bacteria (e.g. Rhizobium, Bradyrhizobium). These bacteria are able to convert atmospheric nitrogen into nitrogen compounds to the tune of 72 to 350 kg of nitrogen per ha per year.To improve the food and nutritional security the food legume has a major role.The dried seeds of legume have ability to be stored for long periods without compromising their nutritional value, thereby, increased food availability till the new arrivals. The legume growers have both the options of self- consumption and also cash crops to fetch income.

In addition to their nutritive value, by virtue of broad genetic diversity in food legumes and climate resilience to sustain well in adverse weather situations. Inclusion of legumes in dominated cereal-based cropping system increased the yield of subsequent cereal crops and reduce the fertilizer cost too resulting decrease in cost of production and increase profitability.

Isolation of Rhizobium

a) Assessment of nodules

1. Wash the roots,free off adhering dirt
2. Cut 10 nodules randomly and note the colour of the juice coming out from nodules on crushing,if pink in colour it means it is effective, otherwise not.
3. Record the percentage of effective and ineffective nodules.
4. Record the percentage of nodules of main and lateral roots.
5. Note the colour, size, shape, abundance and location of the characteristics nodules

b) Examination of nodules for bacteroids

1. Sterilize the nodules with 0.1 per cent acidified mercuric chloride for 2 minutes and wash in sterile water.

2. Crush the nodule in a drop of water on a clean slide,mix well with a loop.
3. Transfer several loop-full to another clean slide.
4. Dry and fix the slide on gentle heat ad stain with rose bengal stain for 5 minutes.
5. Identify bacteroids having irregular shapes and an uneven distribution of protoplasmic contents.

c) Isolation of Rhizobium from nodules

1. Wash the soil from the root nodules and carefully cut a nodule from the root, leaving a small portion of the root attached.
2. Place the nodule in petri plate containing 0.1 per cent acidified HgCl (1 ml conc. HCl/L) and 2 keep it immersed for 5 minutes.
3. Use sterile forceps to transfer the nodule to a petri plate containing sterile water. Wash at least six times and place in 95 per cent ethyl alcohol for 3 minutes. Remove to a petri plate containing sterile water and rinse the nodule twice.
4. Add 1 ml of sterile water to each of six numbered sterile petri plates. Crush these sterilized nodules with forceps in petri plate No.1.Mix the nodular tissue with water.

5. Transfer one loop full of the suspension to plate 2 and dilute by mixing with 1.0 ml of water. Repeat the loop dilution procedure for plate's No.3,4,5 and
6. 0 6. Pour plates (No. 2 - 6) with Congo red mannitol agar and incubate the plates at 28 C for 5-7 days.
7. After incubation examine the plates, note typical Rhizobium colonies, raised, moist with round edges, later changing to opaque while. Stain with carbolcrythrosin (5 minutes) and compare the bacteria with those found in the nodule

Large Scale Production of Liquid Rhizobium Biofertilizers

Liquid Rhizobium biofertilizers production technology includes isolation or procurement of bacterial strains for required purpose, selection of suitable effective strain, preparation of mother culture, broth preparation, addition of cell protectants and their mixing, followed by packaging, storage and dispatch. The Rhizobium strains most adopted and found suitable in Marathwada region are produced at biofertilizer production unit, Vasantrao Naik Krishi Vidyapeeth,Parbhani.

Steps to be followed in preparation of liquid Rhizobium biofertilizers

1. Preparation of mother or starter cultures Starter cultures of selected strains are obtained after ascertaining their performance in laboratory, pot culture experiments and at field levels. The pure culture of efficient strain of particular microorganism is grown on respective agar medium on slant and maintained in the laboratory. A loopful of inoculum from the slant is transferred in a 250 ml capacity conical flask containing liquid medium, keep the conical flak on rotary shaker for at least 72 hrs depending whether they are fast growing or slow growing.The content of these flasks usually attain a load of 6 7 10 -10 cells per ml called mother culture or starter culture. This mother culture is further multiplied in larger flasks called as broth preparation.
2. Preparation of broth cultures Prepare liquid medium for Rhizobium. Distribute equal quantity in big conical flasks (1000 ml). Sterilize it in autoclave for half an hour at 15 lbs pressure. After sterilization each flask containing suitable broth is inoculated with the mother culture in 1:5 proportions aseptically under laminar flow.

3. Keep the flaks on rotary shaker for 72 hours or in sterile fermenter until the viable count per ml 12 14 reaches to 10 -10 cells. The broths become thicker in consistency. This broth culture with population of 12 at least 10 cells per ml should be stored at suitable temperature and condition.
4. Preparation of liquid Rhizobium biofertilizer with cell protectant To prepare the liquid Rhizobium biofertilizers from the above prepared broth, all the ingredients required should be mixed thoroughly under laminar flow to avoid the contamination.
5. Filling and packaging of bottles After preparation of liquid Rhizobium biofertilizer as mentioned above the requisite quantity as per need should be filled in the sterile auto-lock high-density polyethylene (HDPE) plastic bottles under laminar flow to avoid contamination.

Commercial production of biofertilizers: Azotobacter

Usually Azotobacter is grown on a solid medium free of nitrogen. After some times (6 months) old growth of Azotobacter is transferred to a fresh solid medium to renew the growth. This procedure is repeated periodically so that the culture can be maintained in good condition.

Production of azotobacter:

i. Mother culture: A pure growth of any organism on a small scale is called as a mother culture. Mother culture is always prepared in a conical flask of 500 or 1000 ml. Capacity and then this mother culture is used for further production.

For this purpose, one liter conical flasks are taken to which 500 ml of broth of nitrogen free medium is added and these flasks are then plugged with non-absorbent cotton, sterilized in an auto slave for 15-20 minutes at 75 lbs pressure for 15 minutes. Flasks are then inoculated with mother culture with the help of inoculating needle aseptically. The flasks are transferred to shaker and shaking is done for 72-90 hours so as to get optimum growth of bacteria in broth.

Bacteria are multiplied by binary method i.e. cell division. After about 90 days, the number of per milliliters comes to about 100 crores. Total growth of bacteria in this broth means starter culture or mother culture, which should carefully be done, since further purity of biofertilizer or quality of biofertilizer depends upon how mother culture is prepared.

ii. Production on a large scale: Azotobacter is multiplied on a large scale by two ways viz. Fermenter and Shaker. The fermenter is most automatic and accurate method of multiplication of any micro-organism. In this method, the medium is taken in a fermenter and then sterilized. After this pH of the medium is adjusted and 1% mother culture is added. In order to get an optimum growth of the Azotobacter required temperature and oxygen supply is adjusted so that concentrated broth is made. This concentrated broth of the culture is then mixed with a carrier previously sterilized and bio-fertilizers are prepared. Depending upon the demand and supply suitable fermenter is selected.

In the 2nd method i.e. shake method, a suitable medium is prepared transferred to conical flask of suitable capacity. These flasks are then sterilized in an autoclave at 15 lbs pressure for 15 minutes. Each flask is inoclulated with 10 ml mother culture and they are transferred to shaker for multiplication where they are kept for 72-90 hours. This broth is mixed with a suitable carrier previously sterilized. Thus biofertilizer is prepared, filled in plastic bags and stored in cool place.

Selection of carrier:

A carrier is nothing but a substance which has high organic matter, higher water holding capacity and supports the growth of organism. In order to transport the biofertilizer and becomes easy to use the suitable carrier is selected. Generally Lignite cool, compost and peat soil are suitable carriers for Azotobacter. Out of these carriers lignite is most suitable for this organism, since it is cheaper, keeps organism living for longer period and does not lower the quality of bio-fertilizers.

The lignite comes in clouds and hence it is ground in fine powder by grinding machine. Its finesses should be 250-300 mesh. The pH of the carrier is adjusted to neutral by adding CaCO3. The lignite naturally has a variety of micro-organism and hence it is sterilized in autoclave at 30 lbs. Pressure for 30 minutes. After this the broth is mixed with lignite 1:2 proportion by following method.

Galvanized trays are sterilized and used. To these trays, previously sterilized lignite is transferred and broth is then added (lignite2: broth 1) and mixed properly. Trays are then kept one above the other for 10-12 hours for allowing the organism to multiply in the carrier. This mixture is then filled in plastic bags of 250 g or 500 g capacity. Plastic bags are properly. Trays are then kept one above the other for 10-12 hours for allowing the organism to multiply in the carrier. This mixture is then filled in plastic bags of 250 g or 500 g capacity. Plastic bags are properly sealed. All the required information such as name of biofertilizer, method of use expiry date, etc. is printed on plastic bags. In this way biofertilizer is ready to sell or use. If biofertilizer is used immediately then bags are stored in cool place otherwise they should be stored in cold storage in order to keep biofertilizer in good quality.

As per ISI standards, one gram of biofertilizer immediately after it is prepared should have one crore cells of bacteria and 15 days before expiry date one gram of biofertilizer should have 10 lakh bacteria. If biofertilizer is stored at 15-20 0C then it will remain effective for 6 months. However, at 0 to 4 0C (cold storage) the bacteria will remain active for 2 years. The storage periods are decided after testing the biofertilizer for that particular storage conditions, such temperature and humidity.

Use of Azotobacter as Biofertilizer:

Plant needs nitrogen for its growth and Azotobacter fixes atmospheric nitrogen non-symbiotically. Therefore, all plants, trees, vegetables, get benefited. However, especially cereals, vegetables, fruits, trees, sugarcane, cotton, grapes, banana, etc. are known to get addition nitrogen requirements from Azotobacter. Azotobacter also increases germination of seeds. Seeds having less germinating percent if inoculated can increase germination by 20-30%.

Commercial production of biofertilizers: Phosphate Solubilizing Fertilizers (PSB)

A group of heterotrophic microorganisms solubilize this fixed phosphorous by producing organic acids and enzymes and make them available to the crops. This group of microorganism is called Phosphorous Solublising Microorganisms (PSM). Phosphate solubilizing bio-inoculants/biofertilizers are prepared from the bacteria or fungi which solubilize fixed form of phosphate in the soil.The Phosphate solubilising bacterial strains in the starter cultures were needed to be grown in large scale for which their mass production were required.

Mass production of inoculant:

So larger conical flasks of 1000 ml were taken and then again starter cultures were transferred to these larger conical flaks containing the appropriate growth media in aseptic conditions for small scale production and for large scale production again 1 litre of the starter cultures were put into the fermenter. Finally continuous agitation and proper aeration was done for about 1 week. The flasks were checked for time to time for the growth of the cell mass and that they were free of any contamination. After 1 week the cell population increased up to 10^9 cells/ml or 10^9 cfu/ml load in the larger conical flasks.

Then the conical flasks were stored in cool temperatures so that they can be mixed with proper carrier materials. Moreover, it is not advisable to keep the conical flasks for long time in storage because of the loss of cell load.

Carrier material preparation

The carrier should have the following characteristics a) It should have high organism matter content b) Low soluble salts less than 1% c) High moisture content capacity. In this experiment for the inoculation to be made charcoal, cow compost and vermi-compost was used as carrier material. There are many steps for preparation of the carrier material. The steps are discussed below**:**

First about 1 kg of dried cow dung and black coal was brought from different areas. Then by the help of mortar and pestle the entire coal was crushed to dried powdered form. After crushing also the remaining pieces were further powdered by the help of mixer and grinder. The dust form of coal as charcoal was made and to it 1% calcium carbonate and wooden charcoal or activated charcoal was mixed and neutralized so that no contaminants are present A) Similarly the cow dung was also crushed and powdered with the help of mixer and grinder. B) Some amount of vermi-compost was also added as a carrier material.

Preparation of inoculum with carrier material

The mass produced bacterial cell cultures of both Bacillus spp. and Pseudomonas spp. were taken out of storage and then the cell cultures were mixed with the sterilized carrier materials in individual beakers. The mixing of the carrier materials and the production media were in the ratio 2:1 where 1 part of production media was mixed with 2 parts of carrier material or in other words 30:60 ratio of both. It was done manually and under aseptic conditions. The cell count of that carrier mixed culture was found to be 10^8 CFU/gm. The biofertilizers were packed in polythene bags which are advised to be of 250 gm. Then the packets were left at room temperature for curing.

Storage of biofertilizers

The polythene packets containing biofertilizers were stored in cool place away from direct sunlight. The biofertilizers were then sent to the hilly regions for application on the proper fields of biofertilizer plots.

Commercial production of biofertilizers: Azolla

Several cost-effective methods can be used for the cultivation of azolla as a livestock feed**.** a method for cultivating azolla that is easy and economical for livestock farmers. One of its attractions is that the dung produced by livestock is used to help fertilize the azolla plants which, in turn, provide nutrition for the livestock.

• A water body is made, preferably under the shade of a tree, with the help of a silpauline sheet. Silpauline is a polythene tarpaulin which is resistant to the ultra violet radiation in sunlight. A pit of 2 x 2 x 0.2 m is dug as a first step.

• All corners of the pit should be at the same level so that a uniform water level can be maintained. The pit is covered with plastic gunnies to prevent the roots of the nearby trees piercing the silpauline sheet, which is spread over the plastic gunnies.

• About 10 – 15 kg of sieved fertile soil is uniformly spread over the silpauline sheet. Slurry made of 2 kg cow dung and 30 g of Super Phosphate mixed in 10 litres of water, is poured onto the sheet. More water is poured on to raise the water level to about 10 cm.

• About 0.5 – 1 kg of fresh and pure culture of azolla is placed in the water. This will grow rapidly and fill the pit within 10 – 15 days. From then on, 500 – 600 g of azolla can be harvested daily.

A mixture of 20 g of Super Phosphate and about 1 kg of cow dung should be added once every 5 days in order to maintain rapid multiplication of the azolla and to maintain the daily yield of 500 g.

• A micronutrient mix containing magnesium, iron, copper, sulphur can also be added at weekly intervals to enhance the mineral content of azolla.

Summary of NARDEP's method of azolla production

• It is important to keep azolla at the rapid multiplication growth phase with the minimum doubling time. Therefore biomass (around 200 g per square meter) should be removed every day or on alternate days to avoid overcrowding.

• Periodic application of cow-dung slurry, super phosphate and other macro and micronutrients except nitrogen, will keep the fern multiplying rapidly.

• The temperature should be kept below 25°C. If the temperature goes up the light intensity should be reduced by providing shade. If possible, it is best to place the production unit where it is shady.

• The pH should be tested periodically and should be maintained between 5.5 and 7.

• About 5 kg of bed soil should be replaced with fresh soil, once in 30 days, to avoid nitrogen build up and prevent micro-nutrient deficiency.
• 25 to 30 percent of the water also needs to be replaced with fresh water, once every 10 days, to prevent nitrogen build up in the bed.
• The bed should be cleaned, the water and soil replaced and new azolla inoculated once every six months.
• A fresh bed has to be prepared and inoculated with pure culture of azolla, when contaminated by pest and diseases.
• The azolla should be washed in fresh water before use to remove the smell of cow dung.

Harvesting and preparing azolla as livestock feed

• Harvest the floating azolla plants using a plastic tray having holes of 1 cm2 mesh size to drain the water.
• Wash the azolla to get rid of the cow dung smell. Washing also helps in separating the small plants which drain out of the tray. The plants along with water in the bucket can be poured back into the original bed.

For use as a livestock feed, the fresh azolla should be mixed with commercial feed in 1:1 ratio to feed livestock. After a fortnight of feeding on azolla mixed with concentrate, livestock may be fed with azolla without added concentrate.

- For poultry, azolla can be fed to egg layers as well as broilers.
- In case of severe pest attack the best option is to empty the entire bed and lay out a fresh bed in a different location.

Cost

The cost of producing azolla using NARDEPS' method is less than Rs 0.65 per kilogram (approximately 0.015 US dollars, or 1½ cents per kg).

THE END