

# Methodology of Nitrogen Biofertilizer Production

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## Abstract

Biofertilizers are defined as preparations containing living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or to soil. They accelerate certain microbial processes in the soil which augment the extent of availability of nutrients in a form easily assimilated by plants. Very often microorganisms are not as efficient in natural surroundings as one would expect them to be and therefore artificially multiplied cultures of efficient selected microorganisms play a vital role in accelerating the microbial processes in soil. Use of biofertilizers is one of the important component of integrated nutrient management, as they are cost effective and renewable source of plant nutrients to supplement the chemical fertilizers for sustainable agriculture

## Microorganisms used as N<sub>2</sub> biofertilizers

### Rhizobium

Rhizobium is a soil habitat bacterium, which can able to colonize the legume roots and fixes atmospheric nitrogen symbiotically. The morphology and physiology of rhizobium will vary from free-living condition to the bacteroid of nodules. They are the most efficient biofertilizer as per the quantity of nitrogen fixed concerned. They have seven genera and highly specific to form nodule in legumes, referred as cross inoculation group. Rhizobium inoculant was first made in USA and commercialized by private enterprise in 1930s. Initially, due to absence of efficient bradyrhizobial strains in soil, soybean inoculation at that time resulted in bumper crops but incessant inoculation during the last four decades by US (United States) farmers has resulted in the build up of a plethora of inefficient strains in soil whose replacement by efficient strains of bradyrhizobia has become an insurmountable problem.

### Azotobacter

Of the several species of azotobacter, *A. chroococcum* happens to be the dominant inhabitant

in arable soils capable of fixing nitrogen (2-15 mg N<sub>2</sub> fixed g<sup>-1</sup> of carbon source) in culture media. The bacterium produces abundant slime which helps in soil aggregation. The numbers of *A. chroococcum* in Indian soils rarely exceeds 10<sup>5</sup> g<sup>-1</sup> soils due to lack of organic matter and the presence of antagonistic microorganisms in soil.

### Azospirillum

*Azospirillum lipoferum* and *A. brasilense* (*Spirillum lipoferum* in earlier literature) are primary inhabitants of soil, the rhizosphere and intercellular spaces of root cortex of graminaceous plants. They perform the associative symbiotic relation with the graminaceous plants. The bacteria of genus azospirillum are nitrogen fixing organisms isolated from the root and above ground parts of a variety of crop plants. They are gram negative, Vibrio or Spirillum having abundant accumulation of polybetahydroxybutyrate (70%) in cytoplasm. Five species of azospirillum have been described to date *A. brasilense*, *A. lipoferum*, *A. amazonense*, *A. halopraeferens* and *A. irakense*. The organism proliferates under both anaerobic and aerobic conditions but it is preferentially micro-aerophilic in the presence or absence of combined nitrogen in the medium. Apart from nitrogen fixation, growth promoting substance production, disease resistance and drought tolerance are some of the additional benefits due to azospirillum inoculation.

### Method of biofertilizer production

The production of carrier based N<sub>2</sub> biofertilizers involves five stages-

- Culturing of microorganisms- starter culture and inoculum production
- Processing of carrier material
- Mixing the carrier and broth culture
- Packing
- Proper storage

Equipments required for N<sub>2</sub> Biofertilizer production are as follows-

Autoclave -It is an apparatus in which materials are sterilized by air free saturated steam (under pressure)



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at a temperature above 100°C.

Laminar air flow chamber- Air borne contamination is avoided in this chamber. It is used for culture transfers and inoculation.

BOD (Biological Oxygen Demand) incubators - Provides controlled conditions (light, temperature, humidity, etc.) required for the growth and development of microorganisms.

Rotary shaker -It is used for agitating culture flasks by circular motion under variable speed control.

Hot air oven- Hot air oven is meant for sterilizing all glassware materials.

pH meter- Useful in adjusting the pH of the growth medium.

Refrigerator - Used for preserving all mother cultures used for biofertilizer production.

Fermentor- It provides the proper environment for the growth of a desired organism. It is generally a large vessel in which, the organism may be kept at the required temperature, pH, dissolved oxygen concentration and substrate concentration.

#### Important steps of biofertilizer production

Biofertilizers are carrier based preparations containing efficient strain of nitrogen fixing or phosphate solubilizing microorganisms. Biofertilizers are formulated usually as carrier based inoculants. The organic carrier materials are more effective for the preparation of bacterial inoculants. The solid inoculants carry more number of bacterial cells and support the survival of cells for longer periods of time.

#### Culturing of microorganisms

Although many bacteria can be used beneficially as a biofertilizer the technique for production of *Rhizobium*, *Azospirillum* and *Azotobacter* are discussed here.

The growth mediums used for mass culturing of different bacterial biofertilizers are as follows:

**Rhizobium** : Grown on Yeast extract mannitol broth

Components	Quantity (g L <sup>-1</sup> )
Mannitol	10.0
K <sub>2</sub> HPO <sub>4</sub>	0.5
Mg SO <sub>4</sub> 7H <sub>2</sub> O	0.2
NaCl	0.1
Yeast extract	0.5
Agar	20.0
Distilled water	1 L

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.

**Azospirillum** : Grown on Dobereiner's malic acid broth with NH<sub>4</sub>Cl (1g per liter)

Components	Quantity
Malic acid	5.0g
Potassium hydroxide	4.0g
Dipotassium hydrogen orthophosphate	0.5g
Magnesium sulphate	0.2g
Sodium chloride	0.1g
Calcium chloride	0.2g
Fe-EDTA (1.64% w/v aqueous)	4.0 mL
Trace element solution	2.0 mL
BTB (0.5% alcoholic solution)	2.0 mL
Agar	1.75 g
Distilled water	1000 mL
pH	6.8

#### Trace element solution

Sodium molybdate	200 mg
Manganous sulphate	235 mg
Boric acid	280 mg
Copper sulphate	8 mg
Zinc sulphate	24 mg
Distilled water	200 mL

**Azotobacter**: Grown on N-free Mannitol agar medium

Components	Quantity (g L <sup>-1</sup> )
Mannitol	10.0
CaCO <sub>3</sub>	5.0
K <sub>2</sub> HPO <sub>4</sub>	0.5
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2
NaCl	0.2
Ferric chloride	Trace
MnSO <sub>4</sub> ·4H <sub>2</sub> O	Trace
N-free washed Agar	15.0
pH	7.0
Distilled Water	1 L

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The above broths are prepared in separate flasks and inoculum from respective mother culture is transferred to flasks. The culture is grown under shaking conditions at  $30\pm 2^{\circ}\text{C}$  as submerged culture. The culture is incubated until maximum cell population of  $10^{10}$  to  $10^{11}$  cfu  $\text{mL}^{-1}$  is produced. Under optimum conditions this population level could be attained with in 4 to 5 days for rhizobium; 5 to 7 days for azospirillum; and 6-7 days for azotobacter. The culture obtained in the flask is called starter culture. For large scale production of inoculant, inoculum from starter culture is transferred to large flasks/seed tank fermentor and grown until required level of cell count is reached.

### 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 (passing sterile air through compressor and sterilizing agents like glass wool, cotton wool, acid etc.) and given continuous stirring.
- 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  $10^9$  cells per mL after incubation period.

- There should not be any fungal or any other bacterial contamination at  $10^6$  dilution level.
- It is not advisable to store the broth after fermentation for periods longer than 24 hours. Even at  $4^{\circ}\text{C}$  number of viable cells begins to decrease.

### Processing of carrier material

The use of ideal carrier material is necessary for the production of good quality biofertilizer. Peat soil, lignite, vermiculite, charcoal, press mud, 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. The following points are to be considered in the selection of ideal carrier material.

- Cheaper in cost
- Should be locally available
- High organic matter content
- No toxic chemicals
- Water holding capacity of more than 50%
- 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 (1:10 ratio), 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 contaminants.

Mixing the carrier and the broth culture and packing

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

### Preparation of Inoculants packet

- The neutralized and 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 (after 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 population.
- The inoculant packet of 200 g quantities in polythene bags, sealed with electric sealer and



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allowed for curing for 2 -3 days at room temperature (curing can be done by spreading the inoculant on a clean floor/polythene sheet or by keeping in open shallow tubs/ trays with polythene covering for 2 -3 days at room

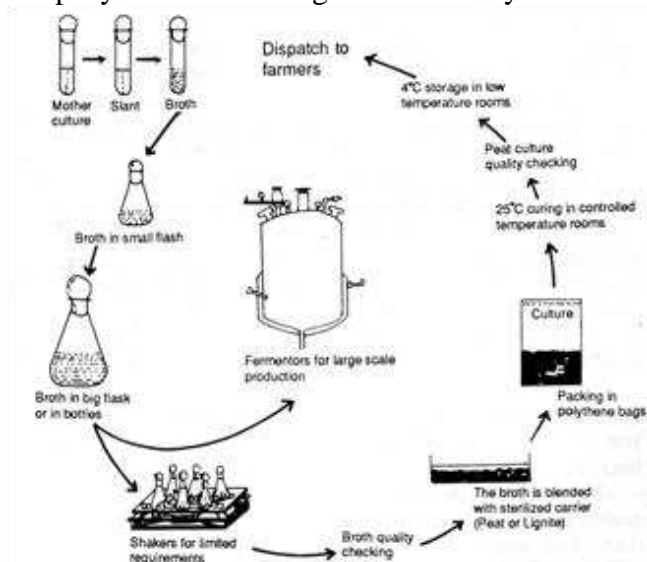


Figure1 Process flow for production of biofertilizers

### ***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(s) to which recommended, method of inoculation, date of manufacture, batch number, date of expiry, price, full address of the manufacturer and storage instructions etc.,

### ***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 carrier inoculant packet may be determined at 15 days interval. There should be more than  $10^9$  cells per gram of inoculant at the time of preparation and  $10^7$  cells per gram on dry weight basis before expiry date.

