Quadrant II – Transcript and Related Materials

Programme: Bachelor of Science (Third Year)

Subject: Microbiology (HONS.) Sem. VI

Course Code : MIC109

Course Title : Agricultural Microbiology

Unit 4: Biofertilizers

Module Name: Steps in mass production of bacterial biofertilizers

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Notes

Biofertilizers are formulated with living or dormant (inactive metabolically) microbial cells. They significantly enhance certain microbial processes to increase the bioavailability of nutrients in a form which can be assimilated by the plant, thereby they aim to supplement the soil with the essential nutrients , improve soil health, soil properties. It also aims to control soil-borne diseases, resulting in higher yields.

Types of bacterial Biofertilizers

The microorganisms that are most commonly used in bio-inoculant preparations are:

- Nitrogen-fixing soil bacteria (*Azotobacter, Azospirillum, Rhizobium*)
- Nitrogen-fixing cyanobacteria (*Anabaena*)
- Phosphate-solubilizing bacteria (*Pseudomonas* sp.)

Laboratory Equipment required

Autoclave – for sterilizing growth medium
Laminar air flow- for culture transfers and inoculation
Incubators - for providing controlled conditions (light, temperature, humidity, etc.) for the growth of microorganisms.
Oven-Dry heat is used to sterilize some materials.
Rotary shakers- provides aeration for growth of cultures.
pH meter- for adjusting the pH of the growth medium.
Refrigerator- for preserving mother cultures required for biofertilizer production.
Fermentor - for pilot plant experiments.

Mass production of Bacterial Biofertilizers

The mass production of carrier based bacterial biofertilizers involves three stages.

• **Selection of a suitable strain of the organism**

a) Ideal Strain / Desirable characteristics

The strains selected for production purposes should meet many requirements:

- Host-rhizobia compatibility & nodulation of the appropriate host plant under a wide range of field conditions.
- They must be capable of effective N_2 fixation
- They should rapidly colonize the roots & compete with compete with indigenous rhizobia for infection
- They should nodulate in the presence of moderate levels of soil N_2& over a range of temperature.
- They should show good growth in broth cultures & survival in carrier.
- Tolerance to high temperature and low moisture
- Have high survivorship in field applications
- They should exhibit resistance to pesticides & tolerance to a range of soil pH.
- Genetic stability

The efficient nitrogen-fixing strains are first selected, maintained and multiplied artificially in the laboratory on the nutrient-rich medium before inoculating it in the seed or soil.

The production of legume inoculant involves 3 independent stages:

- Isolation
- Identification
- Cultivation

A. Isolation of Rhizobia from root nodules

Healthy, unbroken, pink nodules from legumes → wash with water → immerse in 0.1% Hg_2Cl_2 for 4-5 min → wash with sterile water → dip in 70% ethyl alcohol → wash with sterile water → nodule crushed with the help a glass rod → serial dilutions from nodule extract & subsequent plating on CRYEMA or surface spread crushed nodule fluid → incubate for 4-10 days at 26^{o}C → large gummy colonies of bacteria.

B. Identification

i. By microscopy,
ii. Congo red test: 1% congo red dye to YEMA- Rhizobia show white translucent glistening colonies whereas Agrobacterium show pink colonies
iii. Holfers Alkaline broth test: pH of medium is 11. Rhizobium fails to grow at high pH whereas Agrobacterium will grow.
iv. Lactose agar test: Rhizobium cannot utilize lactose whereas Agrobacterium will grow

C. Cultivation

b) Preparation of inoculant/ starter culture

A variety of legume inoculant types are manufactured at the present time & all require broth culture in the first step.

The media used for mass culturing are as follows:

Rhizobium	Yeast extract mannitol broth.
Azospirillum	Dobereiner's malic acid broth supplemented with NH_4Cl
Azotobacter	Waksmann medium No.77
Phosphobacteria	Nutrient glucose broth

The broth is prepared in flasks and inoculum from mother culture is transferred to flasks.

- The culture is grown under shaking conditions (200 rpm) for 5- 7 days at 30±2°C as submerged culture.
- The culture is incubated until maximum cell population of 10^{10} to 10^{11}cfu ml^{-1} is produced.
- Under optimum conditions this population level could be attained:
- ✓ 4 to 9 days for *Rhizobium*
- ✓ 5 to 7 days for *Azospirillum*
- ✓ 2 to 3 days for phosphobacteria 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 fermenter

The media is prepared in large quantities in fermenter
Sterilized and cooled well
Media in fermentor is inoculated with the log phase of culture grown in large flask (usually 1-2 % of inoculum is sufficient, however inoculation is done up to 5% depending on the growth of the culture in the larger flasks) by providing aeration & continuous stirring.

Checking of broth

Broth is checked until required level of cell count is reached.
Cells are harvested when the population reaches 10^9 cells/ml.
It is not advisable to store the broth after fermentation for periods longer than 24 hours, as the viability of cells begins to decrease even at 4° C.

Properties of inoculant

1. The inoculant should be a carrier based one.

2. The inoculant should contain a minimum of 10^{8-9} viable cells of *rhizobium*/g of the carrier on dry mass basis within 15 days of manufacture & 10^7 within 15 days before the expiry date marked on the packet when the inoculant is stored at 25-30°C

3. The inoculant should have maximum period of 6 months expiry from the date of its manufacture.

4. The inoculant should not have any contamination with any other microorganism.

5. The pH of the inoculant should be between 6-7.

6. The inoculant should show effective nodulation on all those species/cultivars listed on the packet before the expiry date. The total dry mass of the inoculated plants should be significantly higher than that of the uninoculated controls & atleast 50% more than the controls.

Carrier material : Conventionally carrier–based biofertilizers are preferred but liquid formulation technology, has also been developed which has more advantages than the carrier-based inoculants.

Preparation/ Processing of carriers for inoculants: **Various types of material** are used as carrier for seed or soil inoculation. In the case of seed inoculation, the carrier must be milled to fine powder with particle size of 10 -40 µm. Peat, soil with charcoal, soil with soyabean meal, lignite, vermiculite, decomposed saw dust, perlite with rice husk, compost, ground rock phosphate with coffee husk compost, bagasse soaked in water and press mud can be used as carrier materials. Neutralized peat soil/lignite are found to be better carrier materials for seed inoculation.

Properties of peat may affect the multiplication of *Rhizobium* population & their survival during storage. Peat is usually acidic & must be neutralized to pH 6.5 – 7.0 using $CaCO_3$. After pH adjustment, the wet peat should be dried to a moisture content of 5-10%. Coarse particles (0.5-1.2 mm) are used for granulated inocula. Peat is sterilized by autoclaving below 100^{o}C, radiation, flash drying, chemical sterilization (ethylene oxide & methyl bromide). Gamma-irradiation at 50 kGy (5 Mrads) is the most suitable way of carrier sterilization, because the sterilization process makes almost no change in physical and chemical properties of the material.

To achieve a tight coating of inoculant on the seed surface, use of an adhesive, such as gum arabic, methylethylcellulose, sucrose solutions and vegetable oils, is recommended.

Selection of ideal carrier material.

A good carrier should have the following qualities:

- Highly absorptive (water-holding capacity of more than 50%.)
- non-toxic to plant and microorganisms;
- easy to sterilize by autoclaving or gamma-irradiation
- Available locally in adequate amounts;
- easy to process and free of lump-forming materials
- Provide good adhesion to seeds;
- Has good buffering capacity;
- High organic matter content
- Inexpensive

- The carrier used with the inoculant should be in the form of a powder (capable of passing through 75-106 µ sieve)

Mixing the carrier and the broth: The culture broth is mixed with the carrier material so as to adjust the final moisture level to 35 to 40% on wet basis.

• Broth having excess cell population can be suitably diluted before mixing.

• The neutralized, sterilized carrier material and broth are mixed either manually or mechanically and left in trays for 2 to 5 days for curing.

• 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 - 3 days at room temperature before packaging.

•Cell count from 10^9 to 10^{10} per g can be reached at the time of curing.

Packing Finally, the bio-inoculant is packed in polythene bags of low density (50-75 µ). Each packet should be labelled with the product information like name of the product, type of carrier, batch number, DOM, DOE, net quantity and storage instructions etc. Each packet should be ISI (BIS) certified.

Storage

• At normal room temperature the inoculant can be stored upto 3 months.

• The shelf life could be extended to 12 months when stored at 4°C.

• The inoculant packets should be stored in a cool place away from heat or direct sun light