RAMA UNIVERSITY

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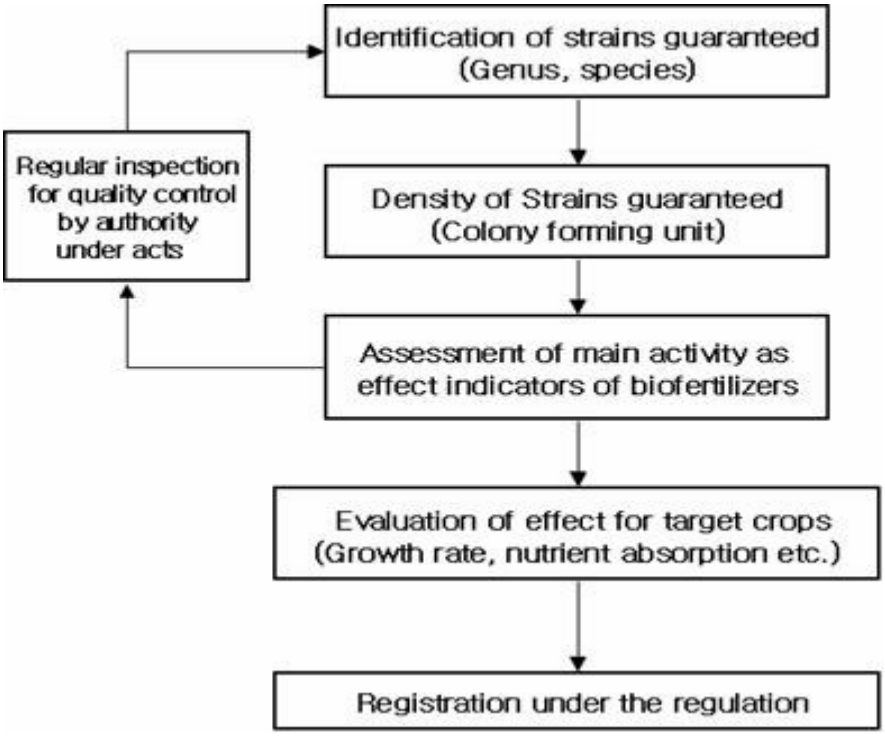
FACULTY OF AGRICULTURAL

1. Quality Management

Quality management is very essential, and must be performed continually to control the microbial products in favor of the customers. The guidelines used for evaluating quality are limited to the density of the available microorganisms and viability and preservation of the guaranteed microorganisms. It is important to set control plots that do not contain available microorganisms, but whose other compositions are the same as the final microbial products. Also it is highly desirable that the biofertilizer manifests the major effects for quality management of the final biofertilizer products. The major effects are used as indicators for the biofertilizer. Also, the effects are included as guaranteed activities of the biofertilizer. It is an indispensable requirement to distinguish between the available microorganisms and the supplementary compositions on the effects of the biofertilizer guaranteed by the suppliers. If the final results of the two experimental plots are the same or cannot be confirmed statistically, then the product is only an organic matter. This means that the effects of microbial products have to originate from the guaranteed microorganisms, and the target of the matters should be presented in details as a prescription. It is essential to evaluate precisely the functions under the given usage manifested by the applicant



Biofertilizers, known as microbial products, act as nutrient suppliers and soil conditioners that lower agricultural burden and conserve the environment. Good soil condition is imperative to increased crop production, as well as human and/or animal health welfare. Thus, the materials used to sustain good soil condition, are treated as environmental matters. However, as mentioned earlier, there are still some problems to be met on the use of microbial products. More precise

quality control must be made in favour of the customers. With this in mind, we will do our best to develop better production techniques and to improve the management system for microbial products. Although the effects of biofertilizers are different among nations due to variances in climate and soil conditions, the importance of biofertilizer on environmental conservation in the 21st century, must not be ignored. In the same manner, various biotechnologies should be accepted for increasing the biofertilizer effects with concern for the environment. Biofertilizers lessen the environmental burden emanating from the chemical compounds. Our viewpoints on biofertilizers are the same for biocontrol and bioremediation, because we are members of an ecosystem related to the world wide web of foods.

2. Procedures for Quality Control of Biofertilizer

2.1. *Rhizobium*

Quality checks on *Rhizobium* biofertilizer can be divided into three parts:

1. Mother culture test
2. Broth test
3. Peat test

2.1.1. Mother culture test

Before producing *Rhizobium* biofertilizer, the mother culture should be checked on the following:

1.1 Growth
1.2 Purity
1.3 Gram stain

Growth

By streaking a mother culture on yeast mannitol + congo red agar (YMA) plates, checking the growth of rhizobia. Fast–growing rhizobia colonies will appear in 3-5 days, and a slow–growing rhizobia will appear in 5-7 days.

Purity

Check purity by streaking culture on glucose peptone agar plate, and incubate for 24 hours at 30 ºC. No growth or poor growth should be obtained on GPA. Good growth and color changes can be expected from contaminants.

Gram stain

A loop of mother culture is checked by Gram staining. Rhizobial cell is Gram–negative, retains safranin color. Cells should appear red and not violet when observed under the microscope.

2.1.2. Broth test

The following qualities of the broth samples must be checked to make sure that the broths are in good condition:

2.1 pH
2.2 Staining
2.3 Optical density
2.4 Total count
2.5 Viable number

pH

Slow–growing rhizobia such as rhizobia for soybeans, mungbean and peanut produce a little basic compounds. After incubation, the pH will increase. (example, pH before growing = 6.0, after growing pH = 6.1 – 6.2). If broth pH decreases, it means some contaminants occur; lower pH indicates presence of contaminants.

Staining (Gram stain or Fuchsin stain)

Rhizobial cells are stained for observation of shape and size of the cells. Cells of rhizobia are rod–shaped, with one or two cells sticking together. They do not appear in long–chain. Long–chained cells are indicative of contaminants.
Gram-stained cells should appear red, not violet. Fuchsin staining is an easier and faster method. Rhizobial cells can be routinely checked using Fuchsin stain.

Optical Density
Broth culture with active rhizobial growth will become turbid in 3-4 days. Broth turbidity, or optical density using spectrophotometer (at 540 nm) will show readings of 0 –to 1.0 O.D. The value of O.D. correlates to number of cells. If O.D. values are high then cells number are also high. We can measure cells from 107 – 109 per milliliter; if the number of cells are low this method is not accurate. This method has its limitations: (i). It gives a direct count (viable + dead cells), (ii). Polysaccharide production in different media gives different results, and (iii). Limitation from the instrument itself.

Total count
Total count includes viable cells and dead cells by using Petroft-Hausser counter At least 10 small squares all around the total area are counted, and not only in one large square.
Precautions: 1. Cells have to be homogeneous.
2. Clumping of cells (use non–ionic detergent).
3. It gives total count only.
4. Petroft, cover slip must be properly positioned to get uniform depth.

Viable count
The number of living cells is counted by spread plate or drop plate methods. Doing spread plate by making serial dilutions from 10-1 – 10-6 or 10-7 (depend on concentration) then three replicates of 0.1 milliliter of broth from 10-6 and 10-5 are spread over the YMA + CR plates. Plates are incubated in incubator (28 – 30ºC) or at room temperature for 7 days. Colonies of rhizobial cells are round, opaque and have smooth margin. They are white and do not absorb red color as well as the other bacteria. Calculation of the number of rhizobia per ml; no. of cells/ml = no. colonies x dilution factor vol. of inoculum
For example, no. of cells/ml = 32 x 106 = 32 x 107 0.1

2.1.3. Peat test
For the peat inoculant, we check these qualities:
1. pH
2. Moisture content
3. Viable number
4. Plant infection method (MPN)

pH
Maintain neutral pH for the inoculant. Since peat is acidic the pH has to be increased with CaCO3. Weigh 10 g of inoculant, pour 20 ml of distilled water, mix well with glass rod, incubate at least 30 minutes, and then measure with pH meter.

Moisture content
The optimum moisture content of peat-inoculant is between 40 – 50 %. At low moisture rhizobia will die rapidly. If moisture is high, inoculant may stick to the plastic bag and, thus, not good for rhizobial growth.

Viable number

The number of viable rhizobia is counted by spread-plate method as in the broth test. It is more difficult when analyzing non-sterile peat. Colonies may sometimes be contaminated by other bacteria. The well trained staff is needed to conduct this microbiological analysis.

2.2. Non-symbiotic N2-fixer

In the laboratory, microbial growth may be represented by the increment in cell mass, cell number or any cell constituent. Utilization of nutrients or accumulation of metabolic products can also be related to growth of the organism. Growth, therefore, can be determined by numerous techniques based on one of the following types of measurement: (a) cell count, directly by microscopy or by an electronic particle counter, or indirectly by colony count, (b) cell mass, directly by weighing or measurement of cell nitrogen, or indirectly by turbidity; and (c) cell activity, indirectly by relating the degree of biochemical activity to size of the population.

The multiplication of *Azospirillum* is expected to have reached its maximal at 3-5 days after inoculation. Inoculants in autoclaved carriers are not expected to contain many inoculants. The recommended counting technique for BIO-N inoculant utilizing known volume of serial dilutions is the drop-plate method (Miles & Mistra). Plate dilutions are ranging from 10-4 to 10-7. If proper aseptic procedures are not fully observed, contaminants may be accidentally introduced during the injection of the broth culture and during serial dilution and plating. Such contaminants will usually be detectable on these indicator media and their number should be reported together with their number of viable cells as additional measure of the quality.

Procedure:

A. Dilution

1. Weigh 10 g of BIO-N inoculant and inoculate it on 95 ml of distilled water
2. Shake vigorously and set aside.
3. Make serial dilution of the 95 ml inoculated with diluted BIO-N. To achieve this, set out 7 tubes each containing 9 ml of sterile diluents.
4. Use a fresh pipette tips for each dilution.

B. Plating

1. Use sterile Enriched Nutrient Agar plates which are at least 3 days old or have dried at 37 oC for 2 hours.
2. Plate dilutions 10-4, 10-5, 10-6, 10-7
3. Allow the drops to dry by absorption into the agar; then invert and incubate at room temperature. Wrap the plates with sterile paper.
4. After 3-5 days of incubation with daily observations count the colonies of the respected organisms of the BIO-N inoculants.
5. Preferred counting range should be 10-30 colonies.

2.3. Mycorrhiza – the arbuscular mycorrhizal fungi, AMF

The article is an adaptation from INVAM publication. Quality control in the production of AMF inoculum is essential for product consistency, reliability and reproducibility. This is applied to the laboratory, preparation room, growth room, storage room and the greenhouses, taking care into the design, to achieve the most efficient control in inoculum production.

2.3.1. Laboratory quality control

i. Spores are extracted from selected batches of monospecific spore cultures in the preparation room.

ii. The spores are transported in petri dishes to the laboratory and placed in a refrigerator before examination.

iii. The petri dishes are examined under stereoscopic microscopes.

iv. Description of the spores from each petri dish are recorded.
v. Petri dishes are the cleaned and dried.

2.3.2. Preparation room quality control

i. This room has to be isolated from the greenhouse and growth room, and should not receive unsterilised soil or potting media samples..
ii. Stored materials (cultures; sterilized growth media) are clearly labeled and placed in specific containers.
iii. Floor should always be clean, avoiding sweeping, which encourages distribution of dust.
iv. Benches and other surfaces are cleaned with wet towels.
v. Containers are surface-sterilized with 10% sodium hypochlorite.

2.3.3. Growth room quality control

i. The growth room should be temperature controlled (22 ºC), and air is exhausted to the outside (no recycling of stale air)
ii. Bench tops should be painted with anti-microbial paint.
iii. All surfaces should be sterilized periodically e.g. monthly.
iv. All samples are checked for contaminants and pathogens.
v. Watering is done manually, with great care to avoid cross-contamination.

2.3.4. Storage room quality control

i. All sampled stored are placed in plastic bags, with proper labelling, and surface of bags should be wiped clean before storage.
ii. Floors and bench tops are wiped regularly, and dusting or sweeping should be avoided to prevent generation of dust.

2.4. Phosphate Solubilizers

As discussed in the definition of biofertilizer, phosphate solubilizers (PS) must contain phosphate solubilizing bacteria or fungi. Commercially produced PS biofertilizers (PSB) must be certificated with guaranteed components such as type of strains, microbial density, and biological activity. If possible the rate of phosphorus absorption of target crops is more valuable as fertilizer. It is suggested that the procedure shown in next figure would be used for the quality control of biofertilizer.

2.4.1. Inoculation on the Media

○ Dilute inoculants using diluents
○ Inoculate suitable diluted solution on selective agar medium
- Plate dilution method

2.4.2. Count of Colony Forming Units

○ Count colonies forming clear zones on agar plate (Fig. 3)
○ Calculate colonies as per unit (g or ml)
When average number of colonies is 45 on 10-5 series.
The total colony number is 45x105 / g dry matter.]

2.4.3. Analysis of Organic Acid

○ Cultivation of isolate on solution medium contained unsoluble phosphate
○ Check the solubility on medium
- Transparence or clearance
○ Analysis of the organic acids in solution by the HPLC

2.4.4. Test of Quality Certificated

○ Effects of inoculant which be proposed and certificated by manufacturers

- Plant growth rate, nutrient absorption of crops etc.