

V. Quality Control of Biofertilizers

1. General Concept of Quality Control

1.1. Definition of Biofertilizer

Biofertilizer is still an unclear term. It can be easily found that biofertilizers are identified as plant extract, composted urban wastes, and various microbial mixtures with unidentified constituents, and chemical fertilizer formulations supplemented with organic compounds. Likewise, the scientific literature has a very open interpretation of the term biofertilizer, representing everything from manures to plant extracts.

However biofertilizer is most commonly referred to the use of soil microorganisms to increase the availability and uptake of mineral nutrients for plants. So it is necessary to define the term “biofertilizer”. There is a proposal that “biofertilizer” be defined as a substance which contains living microorganisms which colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrient and/or growth stimulus to the target crop, when applied to seed, plant surfaces, or soil.

Whether the existence of a microorganism increases the growth of plants by making nutrients more available or replacing soil nutrients or increasing plant access to nutrient, as long as the nutrient status of the plant has been enhanced by the microorganisms, the substance that was applied to the plant or soil containing the microorganisms, can be characterized as a biofertilizer. This definition separates biofertilizer from organic fertilizer containing organic matter.

1.2. Microbial Functions Newly Recommended as Biofertilizer

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth. These bacteria are collectively known as plant growth promoting rhizobacteria (PGPR).

Some PGPR appear to promote growth by acting as both biofertilizer and biopesticides. The search for PGPR and investigation of their modes of actions are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers.

Modes of PGPR action include fixing N_2 , increasing the availability of nutrients in the rhizosphere, positively influencing root growth and morphology, and promoting other beneficial plant-microbe symbiosis. The combination of these modes of actions in PGPR is also addressed, as well as the challenges facing the more widespread utilization of PGPR as biofertilizers (Vessey, 2003. Plant and soil).

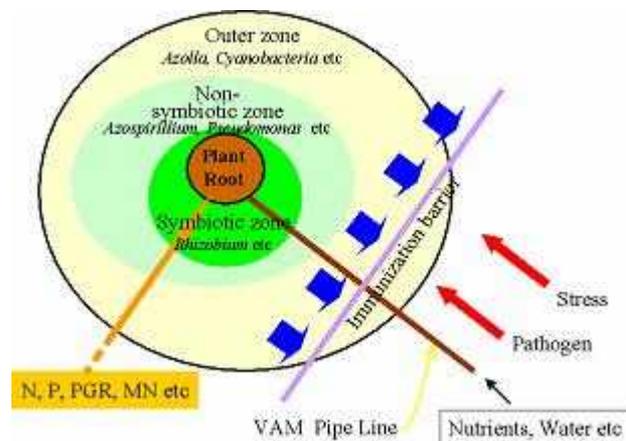


Fig. 1: Integrated microbial actions in soils.

There are two types of materials for agriculture, namely fertilizer or pesticide. It can be said that fertilizer is food, and pesticide is medicine for plants in conventional agriculture. On the other hand, biofertilizer and/or biopesticide are referred to each of them respectively in sustainable or environmentally friendly system (Fig. 1).

We have been interested that microorganisms mainly nitrogen fixer, phosphate solubilizer, and mycorrhizae are main sources for biofertilizer. As functional foods, restoratives and/or adjuvant are sometimes needed for human health care; plant growth promoting rhizobacteria may be one of interchangeable substances for crops.

There are several limitations to the use of biofertilizer for agricultural system. Primarily, efficacy is not reliable for most biofertilizer. This is because the mechanism of action of the biofertilizer in promoting growth is not well understood. However, research into biofertilizer is increasing, attempting to deal with these issues.

Research needs also to be conducted determining if and how variations in soil type, managements practices, and weather effect on biofertilizer efficacy. Furthermore, there is a block in biofertilizer development. It is difficult to test inoculant in field as routine experiments, as shown in Figure 2.

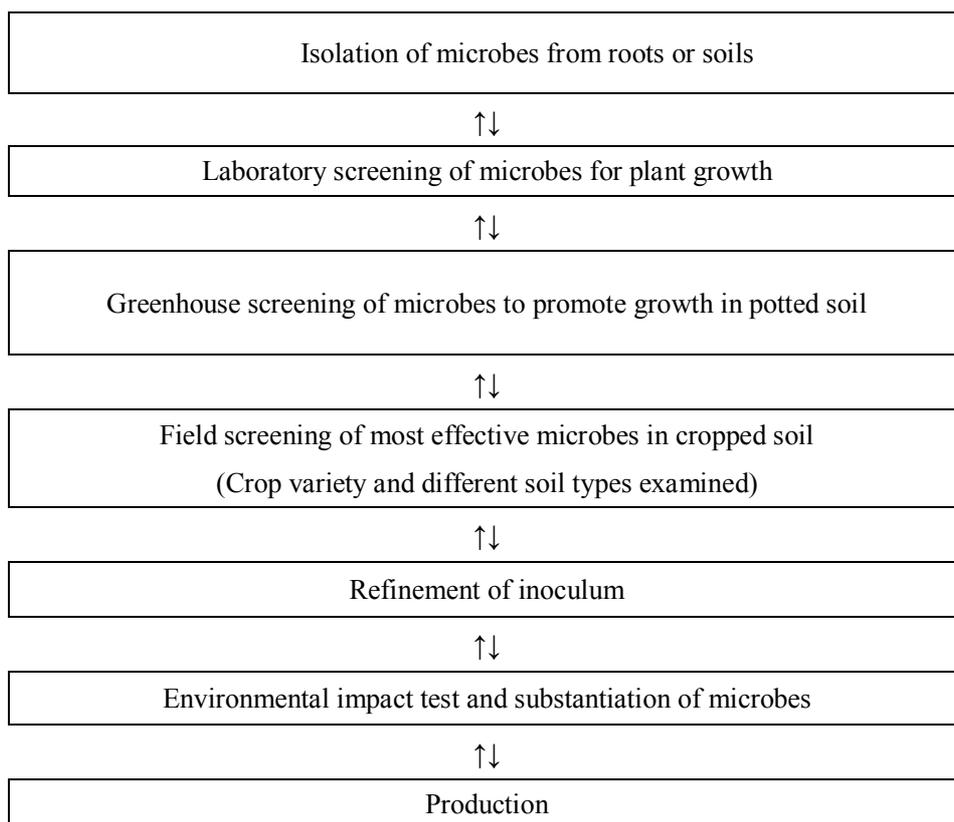


Fig. 2: Experimental process for biofertilizer.

1.3. Properties of Microbial Products

The microorganisms used for microbial products are bacteria of *Bacillus*, *Pseudomonas*, *Lactobacillus*, photosynthetic bacteria, nitrogen fixing bacteria, fungi of *Trichoderma* and yeast. Among the

microbes, the major species used for the inoculants is endospore-forming *Bacillus*. Usually, several species of microbes are used in microbial products with an available period of by- products of about 1~2 and/or 2~3 years.

Microbial products can be solid or liquid in form. Carriers used in solid type of microbial products are clay mineral, diatomaceous soil, and white carbon as mineral; rice, wheat bran, and discarded feed as organic matter. However, clay mineral and rice bran are most often used as carriers. Oftentimes, the effects of carriers and/or supplements are understood to represent the function of microbial products. It is important to seriously consider the control in the use of microbial products. In fact, farmers occasionally misunderstood this carrier effect as microbial action.

As manifested by producers, microbial products stimulate plant growth, decrease pest incidence, stimulate composting and ameliorate the soil. Among these functions, plant growth stimulation was often the main effect. However, there are instances when about 40% of the items are declared having multiple effects.

Quality of biofertilizer is one of the most important factors resulting in their success or failure and acceptance or rejection by end-user, the farmers. Basically, quality is meaning the number of selected microorganism in the active form per gram or milliliter biofertilizer. Quality standards are available only for *Rhizobium* in different countries. Specifications of biofertilizer are differ from country to country and maybe contain parameters like the microbial density at the time of manufacture, microbial density at the time of expiry, the expiry period, the permissible contamination, the pH, the moisture, the microbial strain, and the carrier. Quality has to be controlled at various stage of production (during mother culture stage, carrier selection, broth culture stage, mixing of broth and culture, packing and storage). Main parameters of biofertilizer in China are follows:

Forms	Liquid	Powder	Granular
Appearance	Without strange smell	Brown or black	Brown
Living target bacteria			
Fast-growing Rhizobium	$>0.5 \times 10^9/\text{ml}$	$>0.1 \times 10^9/\text{g}$	$>0.1 \times 10^9/\text{g}$
Slow-growing Rhizobium	$>1.0 \times 10^9/\text{ml}$	$>0.2 \times 10^9/\text{g}$	$>0.1 \times 10^9/\text{g}$
N fixation bacteria	$>0.5 \times 10^9/\text{ml}$	$>0.1 \times 10^9/\text{g}$	$>0.1 \times 10^9/\text{g}$
Si bacteria	$>1.0 \times 10^9/\text{ml}$	$>0.2 \times 10^9/\text{g}$	$>0.1 \times 10^9/\text{g}$
P bacteria			
Organic P	$>0.5 \times 10^9/\text{ml}$	$>0.1 \times 10^9/\text{g}$	$>0.1 \times 10^9/\text{g}$
Inorganic P	$>1.5 \times 10^9/\text{ml}$	$>0.3 \times 10^9/\text{g}$	$>0.2 \times 10^9/\text{g}$
Multi-strain bio-fertilizer	$>1.0 \times 10^9/\text{ml}$	$>0.2 \times 10^9/\text{g}$	$>0.1 \times 10^9/\text{g}$
Water content (%)		20-35	10
Size (ϕ mm)		0.18	4.5
Organic matter (%C)		>20	>20

pH	5.5-7.0	6.0-7.5	6.0-7.5
Non-target bacteria Contamination (%)	<5	<15	<20
Valid period	>6 months	>6 months	>6 months

1.4. 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 (fig. 3)

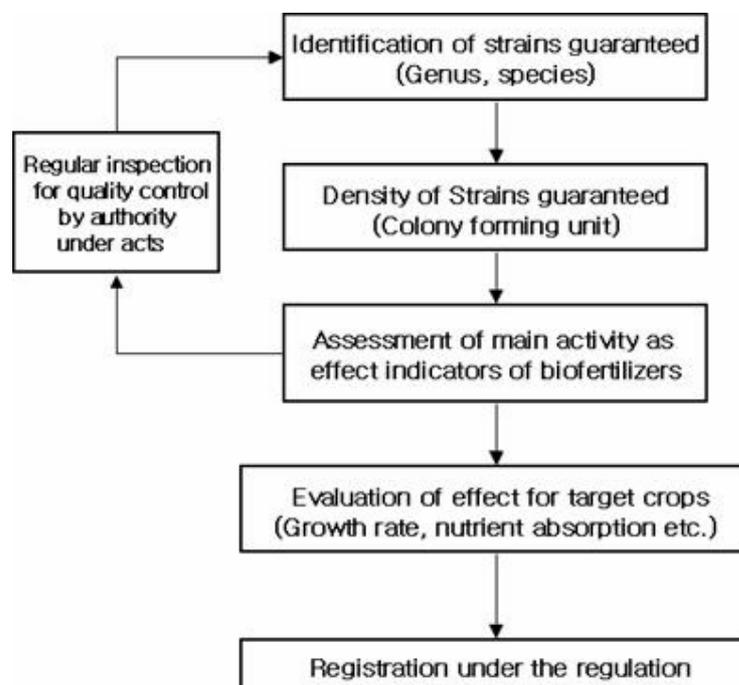


Fig. 3: Procedure of biofertilizer quality control.

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 °C. 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.

Gram stain

Solutions	Reaction and appearance of bacteria	
	Gram-positive	Gram-negative
I. Crystal violet (CV)	Cells stain violet	Cells stain violet
II. Iodine solution (I)	CV-I formed within cells; Cells remain violet	CV-I formed within cells; Cells remain violet
III. Alcohol	Cell walls dehydrated, Shrinkage of pores occurs, Permeability decreases, CV-I complex cannot pass Out of cells, cells remain Violet	Lipid extracted from cell walls, porosity increases, CV-I is removed from cell.
IV. Safranin	Cells not affected, remain violet	Cells take up this stain, become red.

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 $10^7 - 10^9$ 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 ($28 - 30^\circ\text{C}$) 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;

$$\text{no. of cells/ml} = \frac{\text{no. colonies} \times \text{dilution factor}}{\text{vol. of inoculum}}$$

$$\text{For example, no. of cells/ml} = \frac{32 \times 10^6}{0.1} = 32 \times 10^7$$

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 CaCO_3 . 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.

A Plant Infection Analysis using Most Probable Number Method (MPN)

This is an indirect method of assessing plant infection on nodulation. It is widely used when peat is not sterile. It takes more time than spread plate method (because we have to grow plants). We usually do MPN to compare the results with a spread plate method. Some laboratories conduct the MPN analysis and not by the spread plate method.

This method is based on the assumptions that:

1. If a viable rhizobia is inoculated on its specific host, nodules will develop on that roots.
2. Nodulation on that inoculated plant is a proof of the presence of infective rhizobia.
3. Absence of nodule is a proof of the absence of infective rhizobia.
4. Uninoculated plants are used as control, with absence of nodule.

Estimation of MPN

Plants within any given pouch are considered as a growth unit. Nodulation is recorded + for “nodulated growth unit” or – for absence of nodule. The actual number of nodules on each plant has no meaning on MPN count. If replications are in quadruplicated, the reading may be 4, 3, 2, 1 or 0 units. The highest dilution should show no nodulation. Refer to table, ten-fold dilutions (Table A.14.6 in Handbook for Rhizobia, Somasegaran and Hoben, 1994) the number of replications is indicated by “n” and “s” signifies the number of dilution steps.

The estimated number of rhizobia per g is calculated by the formula:

$$x = \frac{m \times d}{v}$$

m = number from MPN table A.14.6 (Vincent 1970)

d = lowest dilution (first unit)

v = volume of a aliquot inoculated

Contaminants have some effect on counting. In the presence of contaminants, count of MPN will give lower results than plate counts (R.J. Roughley 1967).

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

the 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 & Misra). 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 °C 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 inoculant.
5. Preferred counting range should be 10-30 colonies.

C. Computation

Example:

If the average number of colonies per drop is 30 at 10^{-5} dilution, the number of viable cells is:

$$1/0.03 \times 30 \times 10^{-5} = 1,000 \times 10^{-5} = 1 \times 10^{-8} \text{ ml}$$

ENRICHED NUTRIENT AGAR

Name of chemical	g/l
Peptone	10
NaCl	1
Beef Extract	5
Agar	18
Distilled water	1,000 ml

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 °C), 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.

References

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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 (Fig. 1).

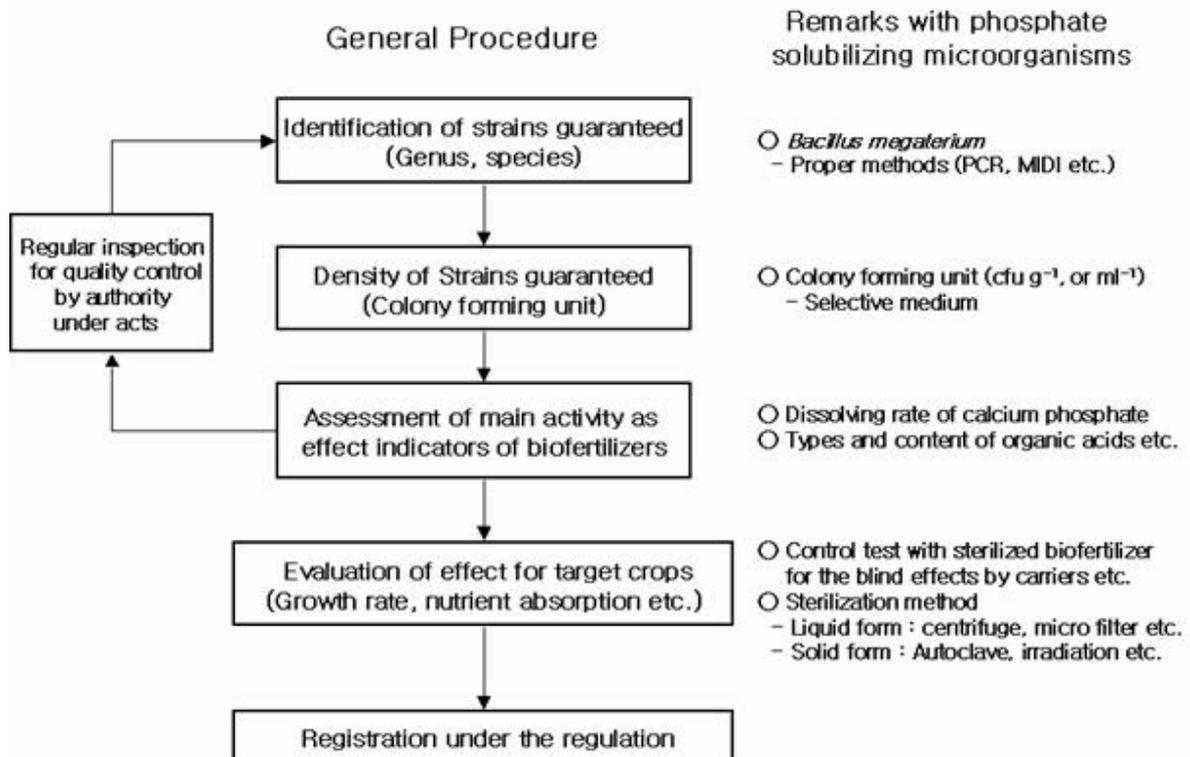


Fig. 1: General procedure for quality control of biofertilizers.

2.4.1. Inoculation on the Media



- Dilute inoculants using diluents
- Inoculate suitable diluted solution on selective agar medium
- Plate dilution method (Fig. 2)

Fig 2: Inoculation

2.4.2. Count of Colony Forming Units



Fig. 3: Colonies on agar medium

- Count colonies forming clear zones on agar plate (Fig. 3)
- Calculate colonies as per unit (g or ml)

[For example:

When average number of colonies is 45 on 10^{-5} series.

The total colony number is 45×10^5 / g dry matter.]

2.4.3. Analysis of Organic Acid



Fig. 4: Solubilization of medium solution

- Cultivation of isolate on solution medium contained insoluble phosphate
- Check the solubility on medium
 - Transparency or clearance (Fig. 4)
- Analysis of the organic acids in solution by the HPLC (Fig. 5)

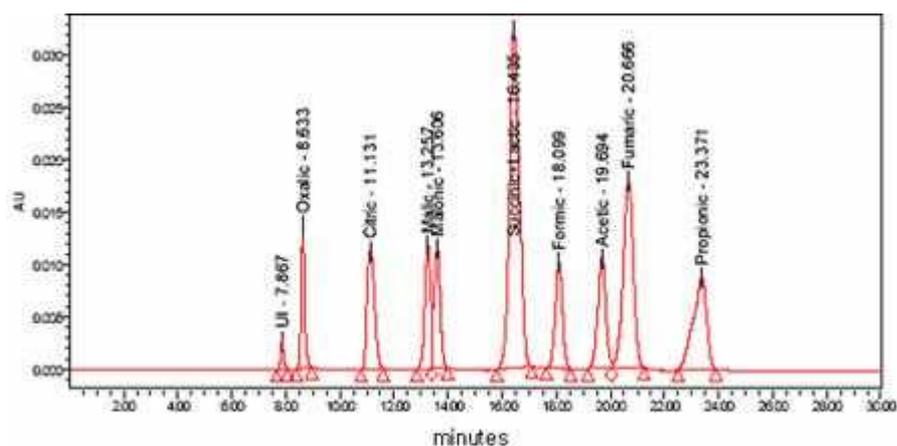


Fig. 5: Liquid chromatography of organic acids.

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.

2.4.5. Prospects

Phosphate solubilizing microorganisms play an important role in plant nutrition through the increase in P uptake by the plant, and also plant growth promoting microbes are an important contributor to biofertilization of agricultural crops. Apart from fertilization, microbial P-mobilization would be the only possible way to increase available phosphate for plant. Accordingly, great attention should be paid to studies and application of new combinations of phosphate solubilizing bacteria and other plant growth promoting rhizomicrobes for improved results as mentioned early.

Concerns about the possible health and environmental consequences of using increasing amounts of mineral fertilizers and chemical pesticides have led to strong interest in alternative strategies to ensure yields and protection of crops. Use of microbial inoculants for biofertilizer in agriculture represents an attractive environmentally friendly alternative. This new approach to farming often referred to as sustainable agriculture.