FNCA Guideline for

Biofertilizer Quality Assurance and Control

Vol. 1 Quantification of beneficial microbes in biofertilizer

Biofertilizer Project

Forum for Nuclear Cooperation in Asia (FNCA)

March 2014

Table of Content

Foreword by Dr. Sueo Machi, the FNCA Coordinator of Japan ----- i
Editors and contributors ----- ii
Acknowledgements ----- iii

1. Introduction ----- 1
2. Biofertilizer sample dilution technique for microbial count ----- 3
3. Quantification of microbes in biofertilizer by the plate counting technique ----- 5
4. Quantification of rhizobia by using the most probable number (MPN) technique ----- 7
5. Quantification of nitrogen-fixing bacteria in aerobic species ----- 12
6. Quantification of microaerophilic nitrogen-fixing bacteria
   by using the Most Probable Number (MPN) technique ----- 14
7. Quantification of cyanobacteria or blue-green bacteria ----- 18
8. Quantification of arbuscular mycorrhizal spores with a stereo microscope ----- 21
9. Quantification of phosphate-solubilizing microbes ----- 24
10. Quantification of potassium -solubilizing microbes ----- 26
References ----- 28

Foreword

Forum of Nuclear Cooperation in Asia (FNCA) was established in 2000 to promote application of nuclear energy for sustainable development of society by the cooperation of 12 Asian countries with partnership.

Ministers of Member Countries in charge of nuclear energy meet annually to exchange views on the FNCA cooperation and nuclear energy policy.

Ten FNCA projects are being implemented in the areas of agriculture, human health, industry, nuclear/radiation safety, human resource development and nuclear security/safeguards in order to conduct R/D of common interest, sharing experience and exchanging information. For nuclear power development the annual study panel is organized for sharing experience with new comer countries of nuclear power.

Security of food supply for rapidly increasing population is the most serious challenge of our society. In this context nuclear technology can provide unique ways, such as mutation breeding to develop better varieties, sterile insect technique to control insect pests, and food irradiation for reduction of postharvest loss and safety.

Biofertilizer is one interesting technology to increase crop yield by using microbes not depending excess application of chemical fertilizer, which degrades soil environment and polluting ground water.

Therefore, the project of biofertilizer has been implemented to promote the production and application of biofertilizer by using the nuclear technology to improve quality of biofertilizer and efficiency of production.

In fact, though this project, member countries have proved that better quality and long shelf life biofertilizer can be produced by using radiation sterilized carriers instead of conventional steam autoclaved carrier. Radiation sterilization has been used for commercial production of biofertilizer in some FNCA countries by the successful collaboration between nuclear institutes and biofertilizer producer.

This book of Guideline for Biofertilizer Quality Assurance and Control is published by the excellent collaboration and effort of members of the FNCA project in participating countries. The book is following the first guideline book on production of biofertilizer edited by Prof. Ohyam, Niigata University the former Project Leader of Japan, and focusing on the quality assurance and control of biofertilizer.

I am confident that the guideline would be very useful for biofertilizer producer and researcher, and hope it enhances extension of biofertilizer to farmers in order to promote sustainable agriculture.

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Acknowledgments

This manual was edited based on "Manual of Bio-Fertilizer Analysis Methodology (ISBN 978-974-436-694-8)", which was published by Department of Agriculture in Thailand. We appreciate their acceptance to use it and kind cooperation. All photos without source in this guideline were cited from Thai manual.

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1. Introduction

By rising world's population and drying up petroleum resources in the future, agriculture is expected to be not relied on chemical fertilizer only. It is defined that biofertilizer is a substance which contains living microrganisms, when applied to seed, plant surfaces, or soil, the microorganisms colonize the rhizosphere (around and surface of the roots of the plants) or the interior of the plant and promote growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003), and it is distinguished from organic fertilizer, compost, and green manure etc. Compost inoculated with biofertilizer, which is called bio-organic fertilizer, is not included in this manual. However, this manual can be applied for microbial count in bio-organic fertilizer.

Biofertilizer provides necessary nutrients to the plants by using the function of microbes. In a case of rhizobium, it can fix atmospheric nitrogen (N_2) and converts it to ammonia. Ammonium (NH_4^+) is oxidized to nitrate (NO_3^-) to make them available to plant. In this case, biofertilizer creates and provides the nutrient to the plants directly, but most of microbes in biofertilizer help and promote nutrient absorption of plants from the soil and applied fertilizers. In a case of *Azospirillum*, which is the nitrogen-fixing bacterium living at the rhizospher, it is thought that plant growth promotion effect by biofertilizer is come from its ability to produce phytohormone to stimulate root growth for their effective nutrient absorption in a soil and not because of its ability of nitrogen fixation. Therefore, it needs available nutrients in a soil to activate this function. Also phosphrous-solubilizing microbes, which solubilizes phosphoric acid to help the absorption by the plants, only works when there is enough source of phosphoric acid in a soil.

The advantages to use biofertilizer are summarized as follows: 1) It enables efficient use of chemical fertilizer and it contributes to reduce the usage of chemical fertilizer. 2) Farmers can save on the cost of chemical fertilizer. 3) It contributes to reduce environmental pollution such as high concentration of NO_3 in groundwater. 4) Chemical fertilizer production needs a lot of energy especially for the nitrogen fertilizer production with Haber–Bosch process to convert atmospheric nitrogen to ammonia industrially. Application of chemical nitrogen fertilizer produces N_2O, which is known as one of main greenhouse gases beside CH_4 and CO_2. Therefore, use of biofertilizers lead to reduce greenhouse effect gas and develop low-carbon agriculture by reducing the usage of chemical fertilizer.

We cannot evaluate just by looking chemical fertilizer, whether it includes enough amount of active integrant. So quality check of chemical fertilizer is usually conducted by government organization to avoid poor quality fertilizer being utilized by the farmers. Active integrant of biofertilizer is beneficial microbes in it. Microbes in biofertilizer decrease themselves for their death, so important

condition is to include and keep a sufficient number of microbes under guarantee. Therefore registration and quality check of biofertilizer has to be assumed by government agencies as well as chemical fertilizer. The aim of this manual is to be a reference for the governmental organization or quasi-governmental organization which conducts the quality check of biofertilizer. And this manual can be also a standard method for microbial count in biofertilizer when biofertilizer companies register their products and sell them to the public market.

In this manual, basic common techniques are explained in chapter 2 to 3, and each method for quantification of microbes is drew up in chapter 4 to 9. Further basic experimental methods such as aseptic procedure have to be referred to other text for experimental method of microbes. We really hope this manual will be utilized actively to deliver more effective biofertilizers to the farmers, and it will enhance the reliability of biofertilizers.

2. Biofertilizer sample dilution technique for microbial count

1. Principle

In order to count the living microbes in biofertilizer product by different methods, it is required that dilution of the biofertilizer is carried out to a level where the microbes can be counted correctly and accurately as specified by each methodology and the biofertilizer sample must be distributed thoroughly and homogeneously in the diluent as much as possible. The quantity of biofertilizer sample to be used for each analysis depends on the homogeneous characteristic of the sample used. In general, the sample should not be less than 10 g.

2. Equipment

Autoclave
Electric balance
Erlenmeyer or bottle
Hot-air oven
Laminar air flow cabinet
Mess cylinder
Pipette (1 ml)
Shaker
Test tubes
Vortex mixer

3. Materials and chemicals

1) Biofertilizer sample

2) 0.85% NaCl solution (Physiological solution)

NaCl	8.5 g
Distilled water	1 L

4. Procedures

1) Diluent preparation: Put 90 ml of 0.85% NaCl solution in erlenmeyer or bottle and put 9 ml of 0.85% NaCl solution in test tube according to different required dilution ratio. Then sterilize by autoclave at 121 °C for 20 minutes.

2) Weigh 10 g of biofertilizer sample or pipette 10 ml of liquid biofertilizer and put in the 90 ml physiological solution, then shake the sample at 180-200 rpm for 30 minutes (10^{-1} dilution).

3) Using the sterile pipette, transfer 1 ml to the test tube with 9 ml diluent (10^{-2} dilution). Vortex it and transfer 1.0 ml to other test tube with 9 ml diluent (10^{-3} dilution). Repeat this procedure and make serial dilution until 10^{-5} dilution or higher depend on the population of microbes in the

biofertilizer. Use a new sterile pipette for every dilution prepared.

4) Count the microbe number contained in diluted biofertilizer by different methods.

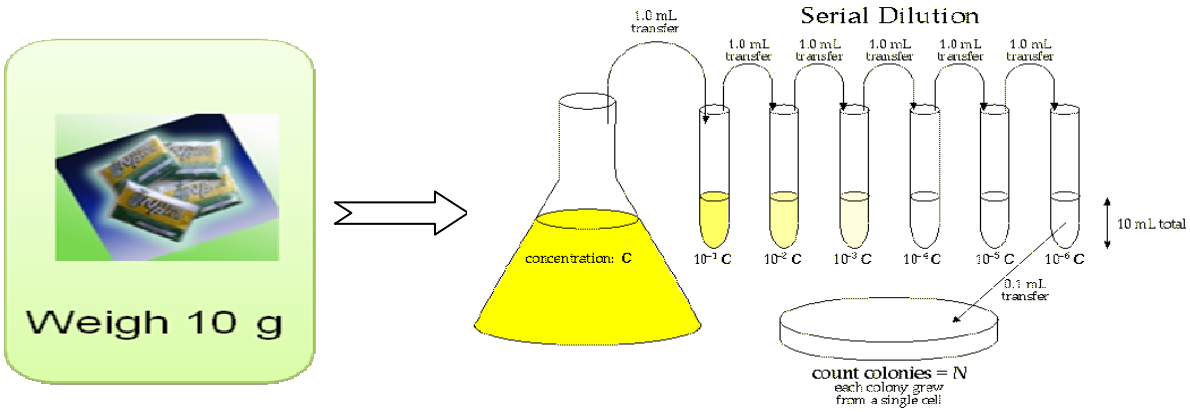


Figure1. Illustration of biofertilizer sample dilution technique for microbial count

(Source: Dr. Pham Van Toan)

3. Quantification of microbes in biofertilizer by the plate counting technique

1. Principle

The plate counting is a methodology to analyze the number of all viable cells of microbes that can increase and form a colony on the agar medium, with a hypothesis that one cell or a group of cell located next to the other will increase the number and pile up as a colony. The count will be most accurate when 1) the sample has the appropriate dilution, which means there are 30-300 colonies per one agar plate when the microbes are growing on the agar, 2) there is a proper distribution of the microbes in the sample and minimum group forming, 3) the medium is appropriate to the microbes, and 4) temperature and other environmental conditions are appropriate to the growth of such microbes.

2. Equipment

Incubator
Laminar air flow cabinet
Petri dish
Pipette (0.1 ml)
Triangle–end spreader stick

3. Materials and chemicals

1) Agar plates with appropriate medium according to type of micrbes
2) Samples of biofertilizer in different degree of dilution prepared according to the chapter 2
3) 70% ethanol

4. Procedures

1) Choose the appropriate ranges of diluted biofertilizer and put 0.1 ml aliquots on the agar plate in safety laminar air flow cabinet.
2) Put the triangle–end of the spreader stick in 70% ethanol and flame this part to sterile. Use the spreader to spread the inoculum equally on the agar medium. Use 3 plates for each degree of dilution and wait until the surface is dry.
3) Incubate plates upside down at appropriate temperature for suitable period to see the colonies.

5. Calculation

1) Count the number of colonies of microbes on the plate that has 30 - 300 colonies.
2) Sum of the amount of microbes from all 3 plates. Divide the sum by 3, in order to get an average number.

3) Multiply the obtained average number with the reciprocal of the dilution and again multiply with 10 (cultured amount of 0.1 ml per plate).

4) Record the obtained value as number of all viable cells of microbes per 1 g of biofertilizer (fresh weight).

Example

a. At the dilution degree of 10^4, the numbers of colonies counted from the 3 plates are 35, 40, and 45, respectively.

b. The sum of counted microorganisms is 35 + 40 + 45 = 120 colonies.

c. The average counted colonies is 120/3 = 40

d. Multiply the average value with the reciprocal of the dilution and 10. Then, adjust the number to be at the closest value. $40 \times 10^4 \times 10 = 40 \times 10^5 = 4.0 \times 10^6$

e. The reported number of all viable cells of microbes is 4.0×10^6 cells or cfu (colony forming unit) per 1 g of biofertilizer.

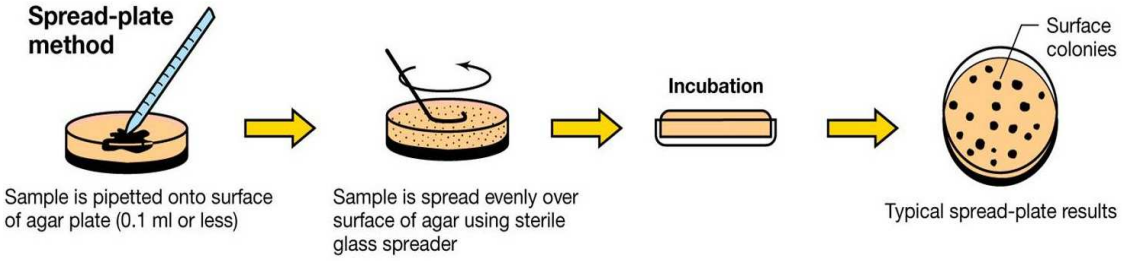


Figure 2. Illustration of stage of plate counting technique
(Source: Dr. Pham Van Toan)

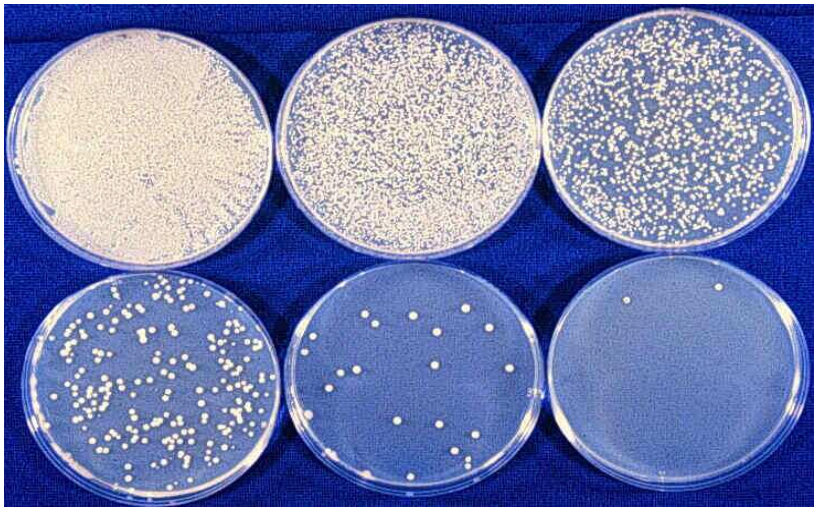


Figure 3. Illustration of microbial growth on the agar plate in different degree of dilution (Source: Dr. Pham Van Toan)

4. Quantification of rhizobia by using the Most Probable Number (MPN) technique

1. Principle

Counting of rhizobia, root nodule forming symbiotic nitrogen-fixing bacteria, in biofertilizer sample by using the plant infection method is a technique to count the amount of viable cells of rhizobia, which have a potential to infect plant and form root nodule. The process starts from the preparation of different degrees of biofertilizer dilutions. Put the prepared sample dilution into the growth pouch at the root of legume. Normally, siratro (*Macroptilium atropurpureum*) or other legumes that are of the same species of the one indicated in the rhizobia biofertilizer are being used widely. Count the number of the bag which the nodules are formed at the roots. Then look at the most probable number (MPN) chart in order to evaluate the amount of viable cells of rhizobia in the biofertilizer.

2. Equipment

Forceps

Growth light shelf with light bulbs for providing some light

Growth pouch made from thick and heat resistance plastic bag of size 16 cm x 18 cm

Hot-air oven

Laminar air flow cabinet

Petri dish

Pipette

Plastic tube

Rack for placing the growth pouches

3. Materials and chemicals

1) Nitrogen-free Nutrient Solution (Broughton and Dilworth, 1971)

Stock solutions

A:	$CaCl_2 \cdot 2H_2O$	294.1 g/l
B:	KH_2PO_4	136.1 g/l
C:	$FeC_6H_5O_7 \cdot 3H_2O$	6.7 g/l (feric-citrate)
	$MgSO_4 \cdot 7H_2O$	123.3 g/l
	K_2SO_4	87.0 g/l
	$MnSO_4 \cdot H_2O$	0.338 g/l
D:	H_3BO_3	0.247 g/l
	$ZnSO_4 \cdot 7H_2O$	0.288 g/l
	$CuSO_4 \cdot 5H_2O$	0.100 g/l
	$CoSO_4 \cdot 7H_2O$	0.056 g/l
	$NaMoO_2 \cdot 2H_2O$	0.048 g/l

Prepare four kinds of stock solutions. Add 0.5 ml of stock solution B, C and D in a 900 ml of sterilized distilled water. Once they become homogenous, pipette 0.5 ml of stock solution A and blend well. After that, adjust pH to 6.5 as well as adjusting the volume to 1,000 ml.

2) Seed of siratro or other legume

3) Concentrated H_2SO_4 is needed in case of seed of legume with hard seed coats instead of 4) and 5)

4) 95% ethanol

5) 5% H_2O_2 solution or 2.5% NaClO solution

6) Samples of biofertilizer in different degree of dilution prepared according to the chapter 1

4. Procedures

1) Preparation of the growth pouches: The growth pouches are made from thick plastic bag of size 16 cm x 18 cm with the folded edge straw paper inside. Put a plastic tube at one side of the growth pouch in order to put in 10 ml of nitrogen-free nutrients solution into the bag. Sterilize the prepared growth bag by autoclave at 121 °C for 20 minutes, and then place all prepared growth pouches on the rack made from stainless steel wire, cut into a rectangle on a piece of wood to wedge a gap between the wire frame for 1 cm.

2) Seed germination: Sterilize the seed of siratro or other legumes specified for rhizobia used for the biofertilizer production to disinfect their seed coat surfaces. Seed of legume with hard seed coats can be sterilized and softened by soaking in concentrated H_2SO_4 for 10 min, and then rinsed with sterilized distilled water 6 times. Soak the seed in sterilized distilled water and keep in the refrigerator at 4 °C for 3-4 hours. In case of seed of legumes with soft coat, such as soybean, rinse the seed in 95% ethanol for 10 second to remove waxy material. Seeds are sterilized by soaking in 5% H_2O_2 (hydrogen peroxide) solution or 2.5% NaClO (sodium hypochlorite) solution for 15 to 20 min, and rinsed with sterilized distilled water 6 times. Then soak the seed in sterilized distilled water and keep in the refrigerator at 4 °C for 3-4 hours. After that, place the seeds on sterilized cotton with moderate humidity in the petri dish with lid by evenly spreading the seeds. Then incubate the seeds in the plate at the temperature of approximately 28 °C. Leave the seeds until the root proliferation appears to be 0.5-1.0 cm long.

3) Planting of the seeds in the growth pouches: Place the sterilized growth pouches on the rack, and add 30 ml of sterilized nitrogen-free nutrients solution into each growth pouch. Make hole at the folded edge of the straw paper by using the sterilized forceps. Then use the sterilized forceps to grip the seed with root proliferation and insert the root into the hole of the growth pouch. Inoculate the biofertilizer solution with different degrees of dilution, starting from less to more, that means starting from 10^{-8} dilution to 10^{-1} dilution. To inoculate rhizobia contained in the biofertilizer onto the roots, the biofertilizer solution should be in contact with the root surface as much as possible by dropping 1 ml of diluted biofertilizer solution into each growth pouch of four replications in each degree of dilution. There must be one growth pouch to which rhizobia biofertilizer is not added. This growth pouch will be used as a control. After planting the seeds, place the growth pouches on the

light shelf that provides enough strength of light to the plant. The light must be at the distance of 15-17 cm from the top of the bags and provides the light for 12 hours a day at 25 °C. Add the nitrogen-free nutrients solution over time when needed for a period of 3 weeks.

5. Calculation

After 3 weeks, count the number of the growth pouches in which root nodules were observed, and look at the MPN table (Table 1) to derive the m value. Then calculate the amount of rhizobia per 1 g of biofertilizer as follow:

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

X = Amount of rhizobia per 1 g of biofertilizer

m = Number obtained from the MPN table

d = Lowest dilution degree of the biofertilizer solution added to the bean (10^1)

v = Biofertilizer solution volume applied to plant (1 ml)

For example, prepare the diluent of the biofertilizer sample at 8 degree of tenfold dilution, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} (s = 8), apply 1 ml of each degree of diluted biofertilizer to each plant of 4 replications (n = 4). There are 20 growth pouches in which root nodules were observed. When we look at the MPN table with this figure, we will see that the m value is equal to 1.7×10^4 (m = 1.7×10^4), then calculate the amount of rhizobia as follow:

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

$$X = \frac{1.7 \times 10^4 \times 10^1}{1}$$

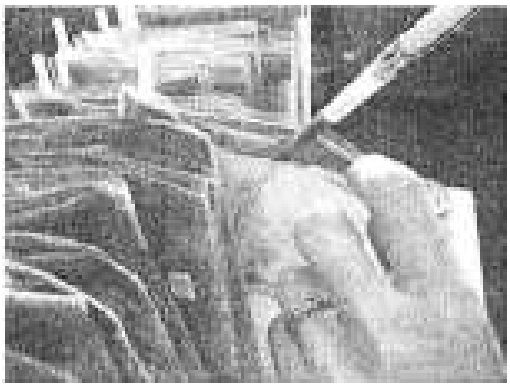
Therefore, the amount of rhizobia is 1.7×10^5 cells per 1 g of biofertilizer.

Table 1 : MPN table exhibited the amount of rhizobia evaluated by plant infection method (m)

Number of growth pouches in which root nodules were observed	Dilution Degree (s)	
Repeated Times (n = 4)	s = 10	
40	> 7 x 10^8	
39		
38	6.9	
37	3.4	
36	1.8	
35	1.0	
34	5.9 x 10^7	
33	3.1	s = 8
32	1.7	> 7 x 10^8
31	1.0	
30	5.8 x 10^6	6.9
29	3.1	3.4
28	1.7	1.8
27	1.0	1.0
26	5.8 x 10^5	5.9 x 10^5
25	3.1	3.1
24	1.7	1.7
23	1.0	1.0
22	5.8 x 10^4	5.8 x 10^4
21	3.1	3.1
20	1.7	1.7
19	1.0	1.0
18	5.8 x 10^3	5.8 x 10^3
17	3.1	3.1
16	1.7	1.7
15	1.0	1.0
14	5.8 x 10^2	5.8 x 10^2
13	3.1	3.1
12	1.7	1.7
11	1.0	1.0
10	5.8 x 10^1	5.8 x 10^1
9	3.1	3.1
8	1.7	1.7
7	1.0	1.0
6	5.8 x 1	5.8 x 1
5	3.1	3.1
4	1.7	1.7
3	1.0	1.0
2	0.6	0.6
1	<0.6	<0.6
0		

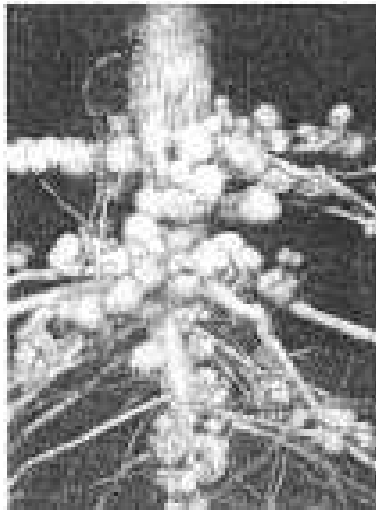
Source Somasegaran and Hoben (1994)

(A)



(B)

(C)



(D)

Figure 3. Illustration of quantification of rhizobia by the MPN tequnique.

(A) Rhizobia biofertilizer dilution

(B) Rhizobia inoculation to the legume root

(C) Cultivation of the inoculated legumes in the growth pouches

(D) The root nodules developed by rhizobi

5. Quantification of nitrogen-fixing bacteria in aerobic species

1. Principle

The quantification of nitrogen-fixing bacteria in aerobic species such as *Azotobacter*, *Bijerinckia* and other species in PGPR (plant growth promoting rhizobacteria) biofertilizer sample is done by the method for counting viable cells that grow and become the colonies on the agar media. The nutrition in the solid medium does not contain nitrogen. The bacteria that can grow in this nutrient are those with nitrogen fixation capabilities under normal atmospheric condition. Normally, the nutrition requirement varies in each species.

2. Equipment

Autoclave
Electric balance
Hot-air oven
Incubator
Laminar air flow cabinet
Petri dish
Pipettes (0.1 and 1 ml)
Shaker
Triangle–end spreader stick
Vortex mixer

3. Materials and chemicals

1) Media

LG medium for *Azotobacter* (Döbereiner, 1980)

To 1,000 ml of distilled water add followings and sterilize by autoclave 115 °C for 15 min.

Glucose	10.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
KH_2PO_4	0.15 g
K_2HPO_4	0.05 g
$CaCl_2$	0.02 g
$FeCl_3 \cdot 6\ H_2O$	0.01 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.002 g
Agar	15g

Adjust the pH to 6.8 with 1N NaOH.

NB medium for *Beijerinckia* (Döbereiner, 1980)

To 1,000 ml of distilled water add followings and sterilize by autoclave 115 °C for 15 min.

Glucose	20.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2$	0.02 g
$Na_2MoO_4 \cdot 2\ H_2O$	0.02 g
$FeCl_3 \cdot 6\ H_2O$	0.01 g
Agar	15g

Adjust the pH to 5 with 1N NaOH.

2) Sample of biofertilizer in different degree of dilution prepared according to the chapter 2.

4. Procedures

1) Prepare the N-free media as in the ingredient to be left in petri dish overnight.

2) The diluted biofertilizer was dropped and spread on each selective medium.

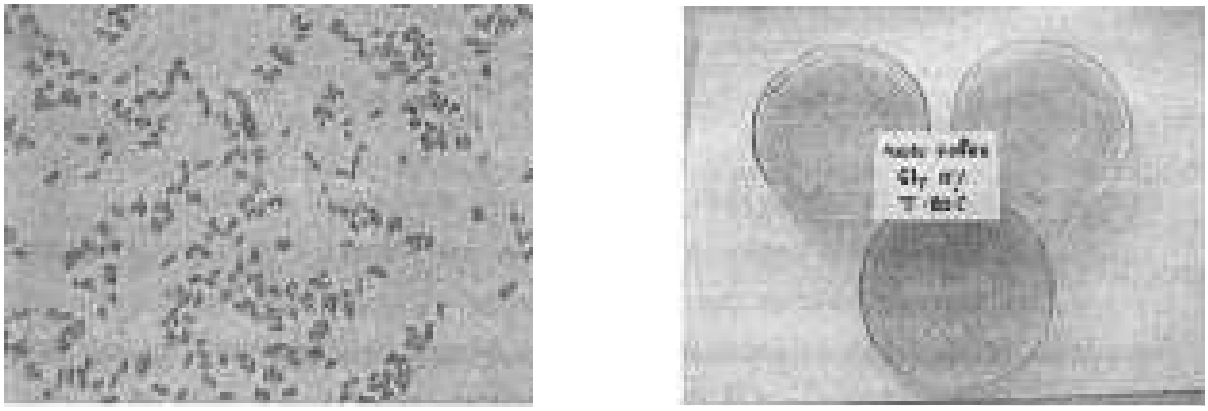
3) Incubate *Azotobacter* at 30 °C for 3 to 5 days, for *Beijerinckia* at 30 °C for up to 1-3 weeks.

4) Colony morphology of *Azotobacter* shows a creamy mucilaginous appearance, while *Beijerinckia* produce raised mucilaginous viscous colonies.

5. Calculation

1) Count and calculate the nitrogen-fixing bacteria by plate counting method according to the calculation of chapter 3.

2) Report the results as the total amount of living *Azotobacter* or *Beijerinckia* per g of biofertilizer.



(A) (B)

Figure 4. The enumeration of aerobic nitrogen-fixing bacteria in aerobic species by the plate counting method.

(A, B) The cell and colony characteristic of *Azotobacter* spp. upon the surface of the solid media after incubation at 30 °C for 3-4 days.

6. Quantification of microaerophilic nitrogen-fixing bacteria by using the Most Probable Number (MPN) technique

1. Principle

The number of microaerophilic species of nitrogen-fixing bacteria in PGPR (plant growth promoting rhizobacteria) biofertilizer sample can be counted by their ability to grow in semi-solid medium without nitrogen. Technically, one cell or a group of cells living nearby will multiply in the agar medium and can be estimated using the most probable number (MPN) technique. The MPN is the method to estimate the most probable number of microbes existing in the sample of biofertilizer with the most accurately statistical estimation when 1) the sample has appropriate dilution series, 2) there are particular nutrients for each genus or species, and 3) the incubation temperature and the other environmental factors are suitable for the growth of each genus or species.

2. Equipment

Autoclave
Electric balance
Erlenmeyer bottle
Incubator
Laminar air flow cabinet
Microwave
Hot-air oven
Pipettes (0.1 ml and 5 ml)
Test tube

3. Materials and Chemicals

1) Nitrogen free semi-solid medium (Nfb) for *Azospirillum* (Döbereiner, 1980)

To 1,000 ml of distilled water add followings and sterilize by autoclave 121 °C for 15 min.

DL-Malic acid	5.0 g
KOH	4.5 g
K_2HPO_4	0.4 g
$MgSO_4 \cdot 7H_2O$	0.2 g
KH_2PO_4	0.1 g
NaCl	0.1 g
$CaCl_2$	0.02 g
$FeCl_3 \cdot 6\ H_2O$	0.01 g
$Na_2Mo_4 \cdot 2H_2O$	0.002 g
Bromthymol blue (0.5% in ethanol)	2 ml
Biotin	10 mg

Difco agar 1.75 g (high quality and no nitrogen content)

2) Sample of biofertilizer in different degree of dilution prepared according to the chapter 2

4. Procedures

1) Prepare the nitrogen-free semi-solid agar medium according to the ingredients in the Nfb medium and divide them into 20 ml test tubes with caps, at 5 ml per test tube. Then sterilize test tubes by autoclave at 121 °C for 15 min.

2) Drop 0.1 ml of the diluted samples in the test tube filled with agar medium. Use 5 burettes per level of dilution.

3) Keep the test tubes in an incubator at 30 °C for 3 days.

4) Keep record of the result of each dilution level that the pellicle appears underneath the surface as positive result. In case of "*Azospirillum*-like" microbes, they produce the pellicle; whitish ring circle, approximately 0.3 mm from the semi-solid agar surface and tune color of surface medium from green to blue after 48 hour of incubation.

5) Use the result for estimation of MPN as in Table.2.

5. Calculation

1) The assumption in biofertilizer analysis is utilized with the tenfold serial dilution, which are 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Five test tubes were filled with 0.1 ml diluent for each dilution level.

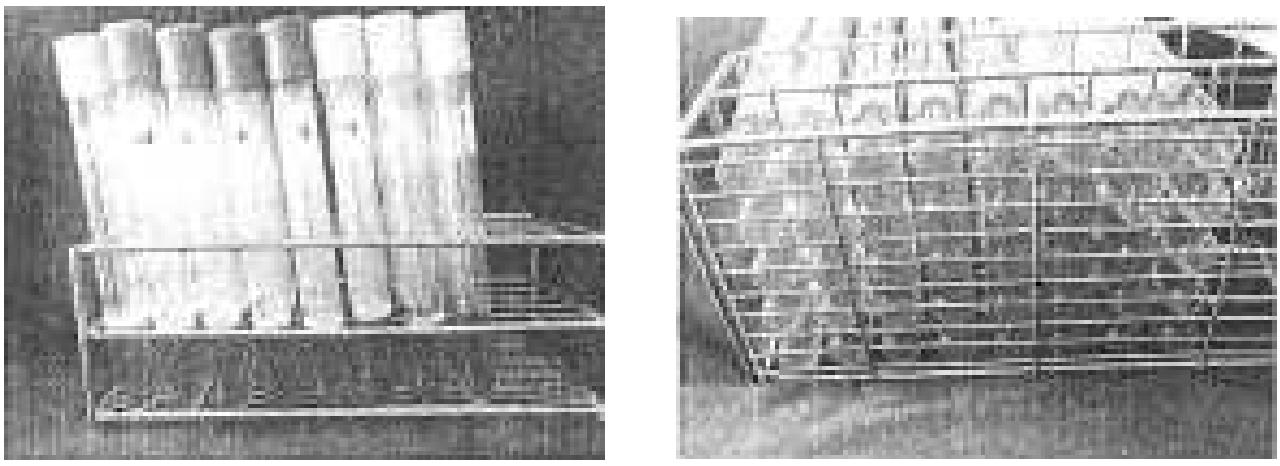
2) The data of the test tube with positive results as 10^{-1}= 5, 10^{-2} = 5, 10^{-3}= 5, 10^{-4} = 3, 10^{-5}= 1 in the experiment are p^1=5, p^2=3, p^3=1. The numbers 5-3-1 are used for open table.

3) Each result was multiplied by the second dilution, which equals 1.1 x 10^4 x 10 (0.1 ml for inoculation), equals 1.1 x 10^5 or 110,000 cells per g of biofertilizer.

Table 2 MPN table for the tenfold diluted sample when 5 test tubes are used for each degree of dilution (Cochran, 1950)

		MPN Value of the 3rd degree of dilution (p3)					
P1	P2	0	1	2	3	4	5
0	0	-	0.018	0.036	0.054	0.072	0.09
0	1	0.018	0.036	0.055	0.073	0.091	0.11
0	2	0.037	0.055	0.074	0.092	0.11	0.13
0	3	0.056	0.074	0.093	0.11	0.13	0.15
0	4	0.075	0.094	0.11	0.13	0.15	0.17
0	5	0.094	0.11	0.13	0.15	0.17	0.19
1	0	0.02	0.04	0.06	0.08	0.10	0.12
1	1	0.04	0.061	0.081	0.10	0.12	0.14
1	2	0.061	0.082	0.10	0.12	0.15	0.17
1	3	0.083	0.10	0.13	0.15	0.17	0.19
1	4	0.11	0.13	0.15	0.17	0.19	0.22
1	5	0.13	0.15	0.17	0.19	0.22	0.24
2	0	0.045	0.068	0.091	0.12	0.14	0.16
2	1	0.68	0.092	0.12	0.14	0.17	0.19
2	2	0.093	0.12	0.14	0.17	0.19	0.22
2	3	0.12	0.14	0.17	0.20	0.22	0.25
2	4	0.15	0.17	0.20	0.23	0.25	0.28
2	5	0.17	0.20	0.23	0.26	0.29	0.32
3	0	0.078	0.11	0.13	0.16	0.20	0.23
3	1	0.11	0.14	0.17	0.20	0.23	0.27
3	2	0.14	0.17	0.20	0.24	0.27	0.31
3	3	0.17	0.21	0.24	0.28	0.31	0.35
3	4	0.21	0.24	0.28	0.32	0.36	0.40
3	5	0.25	0.26	0.32	0.37	0.41	0.45
4	0	0.13	0.17	0.21	0.25	0.30	0.36
4	1	0.17	0.21	0.26	0.31	0.36	0.42
4	2	0.22	0.26	0.32	0.38	0.44	0.50
4	3	0.17	0.21	0.24	0.28	0.31	0.35
4	4	0.34	0.40	0.47	0.54	0.62	0.69
4	5	0.41	0.48	0.56	0.64	0.72	0.81
5	0	0.23	0.31	0.43	0.58	0.76	0.95
5	1	0.33	0.46	0.64	0.84	1.1	1.3
5	2	0.49	0.7	0.95	1.2	1.5	1.8
5	3	0.79	1.1	1.4	1.8	2.1	2.5
5	4	1.3	1.70	2.2	2.8	3.5	4.3
5	5	2.4	3.5	5.4	9.2	16	-

Source - Alexander, M. 1982



(A) (B)

Figure 5. Illustration of quantification of microaerophilic nitrogen-fixing bacteria by using MPN technique.

(A) Pattern of the growth of bacteria in *Azospirillum*-like bacteria, after 48 hours incubated

(B) Growth of bacteria in *Azospirillum*-like bacteria from left to right, from 10^{-1} - 10^{-8} = 5-5-5-5-5-5-5-1, then compare the last 3 digit with the MPN Table, and calculate the amount of all bacteria.

7. Quantification of cyanobacteria or blue-green bacteria

1. Principle

Cyanobacteria are a kind of low class organism in the group of bacteria, has an ability to fix nitrogen gas (N_2) from the air. The same method for quantification of microbes in biofertilizer by counting viable cells of microbes (plate counting method) can be applied to cyanobacteria as well.

2. Equipment

Autoclave
Electric balance
Hot-air oven
Laminar air flow cabinet
Lamp
Light shelf
Micropipette
Petri dish
Shaker
Triangle–end spreader stick
Vortex mixer

3. Materials and Chemicals

1) BG-11 Medium for cyanobacteria (Allen, 1955)

Solution A: Nitrogen-free nutrient for then cyanobacteria consists of

Distilled water	999 ml
$MgSO_4$	0.037 g
Na_2CO_2	0.02 g
$CaCl_2 \cdot 2H_2O$	0.02 g
Anhydrous citric acid (Citric acid)	6 mg
$FeNH_4$ citrate	6 mg
Na_2EDTA	1 mg
K_2HPO_4	0.038 g
Difco agar	12 g

Stock solution B: Stock solution of micronutrient that consists of following components:

Distilled water	1,000 ml
H_3BO_3	2.8 g
$MnSO_4 \cdot H_2O$	1.56 g
MoO_3	0.15 g

$ZnSO_4 \cdot 7H_2O$	0.08 g
$CuSO_4 \cdot 5H_2O$	0.08 g
$K_2Cr_2(SO_4)_4 \cdot 24H_2O$	0.1 g
$NiSO_4 \cdot 6H_2O$	0.045 g
$Co(No_3)_2 \cdot 6H_2O$	0.05 g
$Na_2WO_4 \cdot 2H_2O$	0.018 g
TiO_2	0.017 g
NH_4VO_3	0.02 g

Mix 1ml of Stock solution B to Solution A and adjust pH to 7.8. Sterilize by autoclave 121 °C for 15 min.

2) Samples of biofertilizer in different degree of dilution prepared according to the chapter 2

4. Procedures

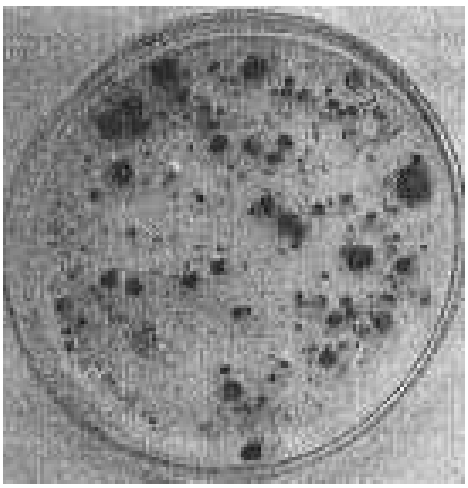
1) Place nitrogen-free medium on the petri dish.

2) Diluted biofertilizer was dropped and spread, to see the colonies according to the procedures of chapter 3. Seal the plates with para film and place them on lighted shelves with the light intensity of 7,000 lux at the temperature of 30 °C for 30 - 45 days. Once growth of cyanobacteria is apparent, count the colonies from plates that contain 30 - 300 colonies.

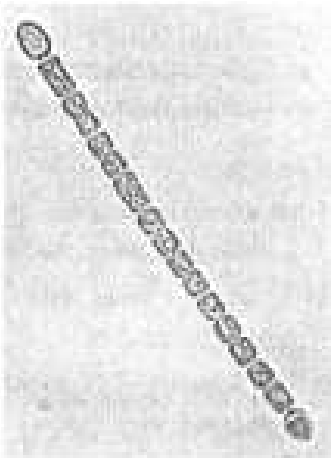
5. Calculation

1) Count and calculate the amount of cyanobacteria by plate counting method according to the calculation of chapter 3.

2) Report the results as the total amount of living cyanobacteria per g of biofertilizer.



(A)



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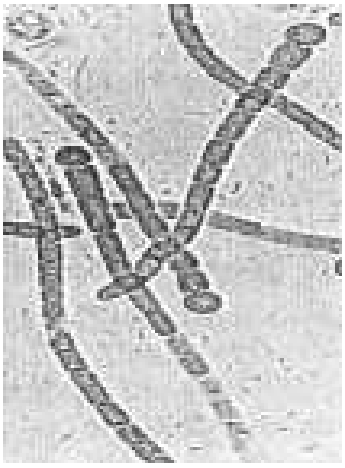

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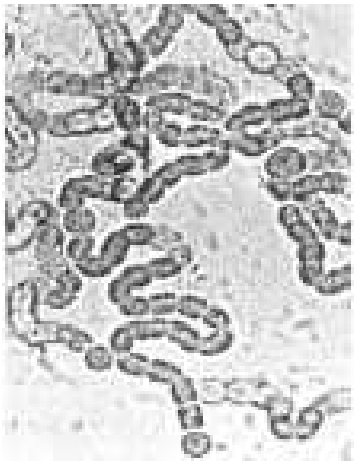


(D)

Figure 6. The characteristics of cyanobacteria

(A) Characteristics of cyanobacteria grown on BG-11 agar

(B) Characteristics of *Anabaena* cyanobacteria

(C) Characteristics of *Calothrix* cyanobacteria

(D) Characteristics of *Nostoc* cyanobacteria

8. Quantification of arbuscular mycorrhizal spores with a stereo microscope

1. Principle

To count the number of living spores in arbuscular mycorrhizal biofertilizer through wet sieving, centrifugation, and slide methods.

2. Equipment

Electric balance
Erlenmeyer flask
Glass slide (2 x 3 inch)
Hand counter
Hot plate stirrer
Paper cup (16-ounce)
Petri dish
Refrigerator
Scalpel
Sieving-bucket centrifuge with speed over 2,000 rpm and 50 ml tube with lid
Stainless-steel beaker (1 L)
Standard sieve (45 and 425 microns)
Stereo microscope

3. Materials and Chemicals

1) 50 % sugar solution: Prepare 50 % sugar solution by placing 500 g of sugar into a 2 L Erlenmeyer flask. Add 1 L of distilled water. Set it on a hot plate at 80 - 90 °C. Stir with hot-plate stirrer until the sugar dissolves into clear syrup. Leave it to cool, and then place it in a refrigerator at 7 - 10 °C.
2) 10 % KOH solution
3) Root dye solution: Prepare root dye solution by mixing 100 ml of lactic acid with 0.16 g of trypan blue dye.
4) 10 % NaClO solution
5) Sterilized mixture of soil and sand (1 : 1 ratio)
6) Corn seeds
7) Glycerol

4. Procedures

1) Prepare sample by placing 100 g of sample into 1 L stainless-steel beaker. Pour 400 ml of water in the beaker. Set it for at least 30 min.
2) Mix the prepared sample with sample solution by stirring in a single direction for 1 min. Leave it

for 2 - 3 min. Pour the solution through the 425-micron sieve and then through the 45-micron sieve set underneath. Repeat with the sediments left in the beaker by adding another 400 ml of water and stir in one direction for 1 min. Leave it again for 2 - 3 min and pour the solution through the 425-micron and 45-micron sieves. Then clean the 425-micron sieve with water and pour the water into a petri dish to count for live spores under a stereo microscope.

3) Place sediments from the 45-micron sieve into a 50 ml centrifuge tube and fill the remainder of the tube with water. Place in centrifuge and set spin speed at 2,000 rpm for 3 min causing deposits to appear. Pour out the excess water and replace with 50 % syrup until it is full. Use stirrer to blend deposit with syrup. Place in centrifuge again and spin for 1 min at 2,000 rpm causing deposits to appear. Pour the solution on top through the 45-micron sieve. Dump the deposits at the bottom of the centrifuge tube. Use water to clean the sediments on the 45-micron sieve for 3 - 4 times until water passing through the sieve becomes clear. Pour the sediments with water into petri dish to count the number of all spores under a stereo microscope.

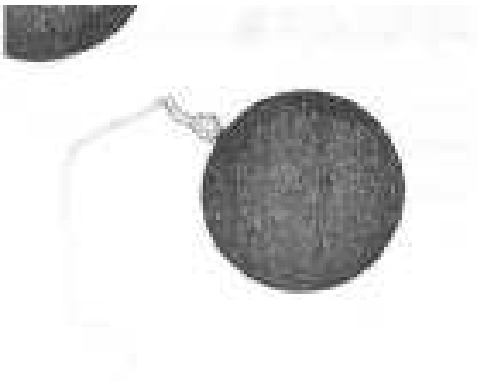
4) Bring all the spores to test in an epiphyte (corn) by putting 500 g of sterilized mixture of soil and sand in a 1:1 ratio into 10 paper cups. Grow the corn seeds by placing one into each cup. The corn seeds must be sterilized on the surface by immersing in 10 % NaClO solution for 3 - 5 min and cleaned with distilled water until free of NaClO solution. Grow the corns for 30 days and retrieve the corn roots before washing them. Boil them in 10 % KOH solution at 80 °C for 5 - 10 min or until the roots become transparent. Wash with water to clear them of KOH solution. Dry off some water and heat in root dye solution at no more than 80 °C for 5 min. Leave them to cool for a while or leave them overnight. Then, pour out the dye solution. Pick roots to cut into 1 cm pieces for a total of 100 pieces. Place them on slides and observe through microscope. Roots found with blue-dyed fibres, vesicles or arbuscules are indication of arbuscular mycorrhizal fungi colonization.

5. Calculation

$$\text{Number of spores per g} = \frac{\text{Total of counted spores on 425 micron and 45 micron sieves}}{100}$$

(A)

(B)



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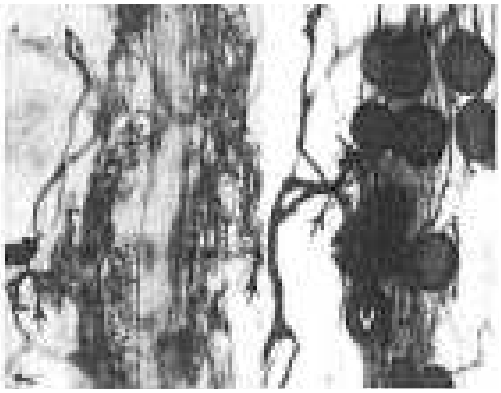


(D)

Figure 7. Illustration of quantification of arbuscular mycorrhizal spores.

(A) Screening sample mycorrhizal biofertilizers

(B) Spore counting

(C) Characteristics of arbuscular mycorrhizal spores

(D) Arbuscular mycorrhizal fungus entering plant root

9. Quantification of phosphate-solubilizing microbes

1. Principle

The quantification of phosphate-solubilizing microbe is the method of counting colonies with halo zone on the agar media containing insoluble phosphate by the plate counting technique. According to the target phosphate, different kinds of medium are selected.

2. Equipment

Autoclave
Electric balance
Incubator
Laminar air flow cabinet
Petri dish
Pipettes (0.1 ml)
Scale
Shaker
Stereo microscope
Triangle–end spreader stick
Vortex mixer

3. Materials and Chemicals

1) Pikovskaya medium for phosphate-solubilizing microbes (Pikovskaya, 1948)

Glucose	10 g
$Ca_3(PO_4)_2$	5 g
$(NH_4)_2SO_4$	0.5 g
Yeast extract	0.5 g
KCl	0.2 g
NaCl	0.2 g
$MgSO_4 \cdot 7H_2O$	0.1 g
$FeSO_4 \cdot 7H_2O$	0.002 g
$MnSO_4 \cdot H_2O$	0.002 g
Agar	15 g
Distilled water	1,000 ml

Dissolve the ingredients together and sterilize by autoclave at 121 °C for 15 min. Then pour in petri dish.

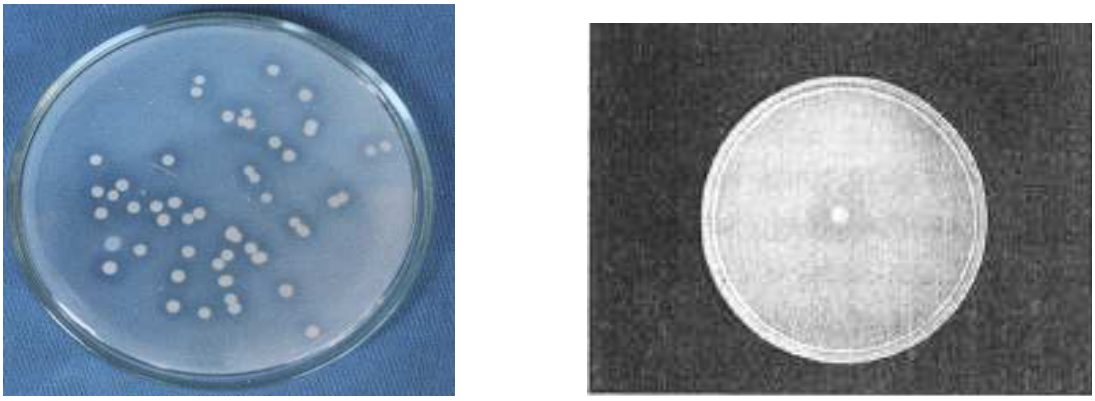
2) Samples of biofertilizer in different degree of dilution prepared according to the chapter 2

4. Procedures

1) Incubate plate at room temperature with appropriate medium, on which diluted biofertilizer was dropped, to see the colonies according to the procedures of the chapter 3.
2) Count the number of colonies with halo zone after 3-5 days of incubation.

5. Calculation

1) Calculate the number of phosphate-solubilizing microbes, which create the colonies with halo zone, according to the calculation of the chapter 3.
2) Report the results as the total amount of living phosphate-solubilizing microbes per g of biofertilizer.



(A) (B)

Figure 8. Illustraion of quantification of phosphate-solubilizing microbes.
(Source: Dr. Pham Van Toan)

(A) Characterization of colonies created by phosphate-solubilizing microbes on the agar containing insoluble phosphate

(B) Transparent rings created by phosphate-solubilizing microbes on the agar containing insoluble phosphate

10. Quantification of potassium-solubilizing microbes

1. Principle

The quantification of potassium-solubilizing microbes is the method of counting colonies on the agar media with insoluble source of potassium by the plate counting technique.

2. Equipment

Autoclave
Electric balance
Incubator
Lamina air flow cabinet
Petri dish
Pipettes (0.1 ml)
Scale
Shaker
Stereo microscope
Triangle–end spreader stick
Vortex mixer

3. Material and Chemicals

1) Aleksandrov medium for potassium-solubilizing microbes (Hu et al., 2006)

Distilled water	1,000 ml
Glucose	5.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCO_3$	0.1g
$FeCl_3$	0.005 g
Insoluble mica powder as potassium source or potassium aluminum silicate	2.0 g
Agar	20.0 g

Adjust pH to pH 7.0-7.2 and sterilize by autoclave at 121 ˚C for 15 min. The insoluble potassium sources in this medium can be replaced by some other insoluble ones, such as potash feldspar, soil minerals and glass powder.

Soil mineral preparation: Add 20% or 6 mol/L HCl to soil sample after removal of organic residues from soil. Amount of HCl is ten times of soil. Boil the soil solution for 30 minutes, and then filter the soil solution using Watman Filter Paper No.1. Rinse the soil with distilled water until no Cl^- reaction.

2) Samples of biofertilizer in different degree of dilution prepared according to the chapter 2.

4. Procedure

1) Drop diluted biofertilizer on plate to see the colonies according to the procedures of the chapter 3.

2) Count the number of colonies after 3, 5, 8 and 10 days of incubation at 30 ℃.

5. Calculation

1) Calculate the number of potassium-solubilizing microbes according to the calculation of the chapter 3.

2) Report the results as the total amount of living potassium-solubilizing microbes per g of biofertilizer.

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