

# **LEGUME INOCULANT TECHNOLOGY AND QUALITY CONTROL PROCEDURES**

## **Workshop Manual**

IITA-Ibadan; NIGERIA

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## PART 1- BACKGROUND

### 1. Legume inoculant technology and Quality Control (QC) procedures

One of the purposes in evaluating rhizobia is to allow us to identify and select strains that are a more vigorously-nodulating and provide maximum nitrogen fixing benefit to individual varieties of a given legume species as well as being adapted to particular geographic, soil and environmental conditions in which the legume is grown. However, we have also learnt that the most effective nodulating rhizobium is not always present in the soil in sufficient numbers to ensure maximum productivity. Therefore a very successful and effective practice has been the inoculation of legumes with elite or selected strains.

The production of legume inoculants has been a very successful practice for over 50 years, but its manufacture is not a straight forward task as it requires skills in aseptic handling of rhizobia, an understanding of the conditions under which rhizobia grow, development of suitable carrier materials and the implementation of quality control systems to ensure a efficient production process and product integrity. Using high quality inoculants that contain high numbers of efficacious rhizobial cells increases the prospects of establishing the inoculant strain, particularly in a soil environment containing competitive rhizobia, thereby improving the potential for maximum nitrogen fixation.

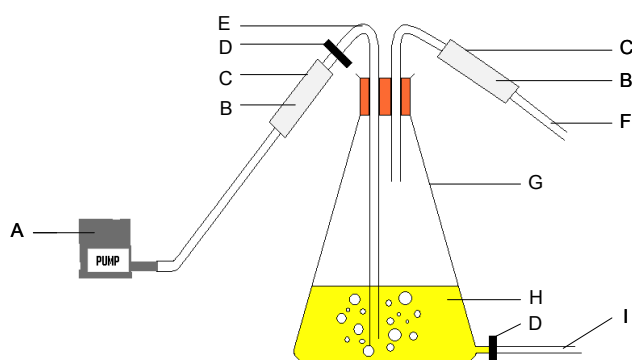
The main steps in Legume inoculant production are:

- Fermentation technologies
- Inoculant formulation
- Carrier selection
- Quality control (QC) processes

#### 1.1. Fermentation technologies

Once the top performing strains have been selected, these are then grown in large batch cultures in liquid medium. This process can be achieved at various scales: small (less than 20L media culture) or large (up to 10,000L). Glass fermentors as the ones you will be assembling at this workshop are commonly used for small production of broth cultures. A diagram is presented in **Fig 1** where a simple 5L Erlenmeyer flask can be fitted with air-lines connected with filters (either cotton filled filters or readily commercial available hepa-filters) to prevent the entry of contaminants. This system can be easily set-up and sterilized in an autoclave. Large fermentors systems are generally equipped with mechanical paddles to stir the medium and have a built-in system that allows the fermentor and the media to be sterilized *in situ*.

The inoculation of a small or large scale fermentation system is done by growing a starter culture in swirl flasks which generally is approximately 1% of the total volume of broth to be prepared. Higher inoculum levels of up to 10% can be used in particular if a small scale or glass fermentor is used. The higher the inoculum used the less time it will take to reach maximum growth in the fermentor however it could be impractical if a large fermentor is used.



**Figure 1** A: Aquarium pump, B: non-absorbent cotton wool, C: plastic syringe barrel, D: clamp, E: silicon and glass tubing air inlet, F: silicon and glass tubing air outlet, G: glass Erlenmeyer flask, H: broth culture, I: broth sampling tube (Somasegaran and Hoben, 1985)

## 1.2. Inoculant formulation

Inoculants may be prepared in various forms: solid carriers, liquid, granular and freeze-dried (**Fig. 2**). Solid carriers are the most common and widely used being peat the most popular carrier. Peat or solid carrier products are usually used for seed inoculation and applied directly to the seed. Its advantage is a longer shelf life compared with other formulations as long as sterile carriers have been used, but requires refrigeration during storage. Liquid inoculants are promoted as being easier to handle and can be applied directly to the soil. However, in many instances these have limited shelf life and like peat carriers, these also require cool temperature during storage, which increase handling and end-user costs and hence preclude their use in most developing countries.

Granular formulations are in the form of peat granule and mineral or clay based products and are mostly suited to large scale farming systems where the granules can be handled by the same machinery used to apply seeds and fertilizers. Granular formulations can be placed directly in the furrow and its major advantage is the ability to control inoculation and its rate of application as required (top dressing) and furthermore, it does not require refrigeration during storage. Freeze-dried formulations have a very high cell number content and it can be stored for long time, however the product would require the cells to be resuspended in liquid before its application as per liquid formulations and it requires costly equipment for its preparation.



**Figure 2** A: Solid carriers. B: Liquid formulations. C: Granular formulations: peat granules (left), clay granules (middle) and attapulgite clay (right). D: freeze-dried formulations.

### **1.3. Carrier selection and preparation**

The carrier is the medium that supports the live cells. A good inoculant carrier must support growth of rhizobia cells or maintain the desired populations over a required period of time, display high water holding capacity, non-toxic to the inoculant strain, neutral pH, provide rapid release of rhizobia cells upon use, be in abundant supply and environmentally safe. The most popular and widely used carrier is peat due to its protective properties and ability to support and maintain high levels of rhizobia cells over time when no other contaminating microorganism are present. The rhizobia cells in the peat are also conditioned into its new environment and are therefore better suited to tolerate stresses such as desiccation. However, peat is not widely available and in some countries, is under heavy government regulation. Alternative carriers have therefore been used with comparable results to peat; examples of those are: compost, coconut coir dust, filter mud, bagasse among others. In this masterclass you will use coconut coir dust, a by-product from the processing of coconut husk for coir fibre.

Carriers should be sterilized either by autoclaving or gamma irradiating before injecting with broth as the resident microflora in the carrier can restrict the growth of rhizobia through competition for resources or production of antibiotics. This can therefore reduce the final numbers of rhizobia cells and hence the quality of the product. If a non-sterile carrier is used, then a high rate of broth will need to be applied and also keep in mind that its shelf life will greatly be reduced in many cases to just one month.

### **1.4. Moisture potential**

The moisture potential of the carrier is important as it affects microbial growth and survival. Moisture potential for optimum rhizobial growth and survival is between 3.8 –log<sub>10</sub> Pa and 4.3 – log<sub>10</sub> Pa. Particle size, organic matter and clay content of peat play a role in water holding capacity and water potential. Peats with low organic matter content have been amended with materials such as coconut coir dust and fish pond filter mud to increase moisture holding capacity. The addition of two or more materials with different moisture potentials will result in final moisture potential that is an average of the mixed materials. It is desirable to increase the water holding capacity of carriers so that larger amounts of broth culture (and hence more cells) can be introduced before incubation and so that moisture potential is optimum for growth. Moisture potential can be difficult to determine and growth of microorganisms should be determined at a range of moisture contents to determine the optimum. Some inoculant products are maintained in dry formulations which have the advantage of greater temperature tolerance and lower requirement for cool storage.

#### **1.4.1. Determination of optimum moisture content in solid-based carriers**

Moisture content should be adjusted for optimum sterilization. A small amount of moisture is required for the production of steam within the carrier. If a carrier is too dry sterilization will not be complete. The efficacy of sterilisation should be measured by injecting broth without bacteria and measuring growth of contaminants over time for one month. Suspend carrier in sterile water, dilute and spread onto the surface of glucose peptone media. Record the dilutions at which growth occurs.

**Table 1:** Examples of treatments for measuring optimum moisture content for microbial inoculants

Moisture content (%)	Liquid added (mL)	*Volume of broth (mL)	Volume of sterile water (mL)
40	29.5	29.5	0
50	52.5	29.5	23
60	87.5	29.5	58

\*Calculations based on 70 g dry peat after adjusting to 20% moisture content for sterilisation

To determine viability of rhizobia cells in the carrier, the number of viable cells per g of carrier should also be measured at several time intervals to indicate the relative growth and survival. Rhizobial strains should be clearly differentiated from other organisms using techniques described later in this chapter.

#### 1.4.2. Calculating the amount of liquid to add to peat or carrier mixture

The equation below is used to calculate moisture content of 70 g dry peat. The same equation can be used for any quantity of peat but, as peat will invariably already contain some moisture, the mass of dry peat must first be calculated.

$$\frac{x}{70+x} = \frac{y}{100}$$

##### Example calculation of liquid added to peat to obtain specific moisture content

How much liquid (eg. broth) is added to 150 g peat with 20% moisture to get a final moisture content of 35%? Mass of dry peat

$$\frac{20}{100} \times 150 \text{ g} = 30 \text{ g}$$

$$150 \text{ g} - 30 \text{ g} = 120 \text{ g}$$

Moisture to add to dry peat

$$\frac{x}{120+x} = \frac{35}{100}$$

$$x = 0.35(120+x)$$

$$x = 42 + 0.35x$$

$$x - 0.35x = 42$$

$$0.65x = 42$$

$$x = 64.6$$

Therefore, 64.6 g moisture should be added to 120 g dry peat to achieve 35%. If peat already has 30 g moisture then 64.6 g – 30 g = 34.6 g should be added to 150 g peat.

To adjust 70 g of dry peat to 20% for sterilisation 17.5 mL of water should be added.

Where x is the amount of liquid added and y is the final percent moisture (eg. 50%).

## 1.5. Quality control (QC) processes

QC processes are a series of tests and monitoring systems that the manufacturer routinely implements during the inoculant production process as well as in the final product. Quality control must start with the rhizobia culture used to initiate the inoculant production and must include protocols to check for sterility of culture batch before it is used to inoculate the carriers. Some of the techniques that are implemented during inoculant production includes: Cell count, testing for contaminants, strain identity and inoculant efficacy.

A good quality inoculant is defined by their high level of rhizobia cells with over  $1 \times 10^9$  rhizobia cells per gram of inoculant for a solid carrier; able to demonstrate an effective nodulation and Nitrogen fixation with their target host; have none or minimal (less than a million) contaminating microbes and should also have an adequate shelf life. The importance of having such a high numbers of rhizobia cells is that these need to have the ability to compete against the already large but ineffective population of rhizobia cells in the soil and ensure a successful nodulation and nitrogen fixation response. Having high numbers will also maximize the ability of rhizobia cells to tolerate stress conditions such as desiccation, seed toxicity, soil pH among others. To ensure a high quality inoculant, manufacturing companies need therefore to go through strict QC programs applied on the entire production process. These programs include:

- monitoring of batch variability
- recovery of rhizobial cells from the carrier
- enumeration of inoculants
- identification of inoculants
- efficacy of inoculants
- enumeration of contaminants
- assessment of moisture content or relevant conditions that support life in the carrier
- assessment of product from different points in the supply chain

### 1.5.1. Cell counts

Cell counts are a very important part of the quality control process as it will determine the quality in terms of viable cells in the broth and in inoculated carriers. To count rhizobia cells, a sample of the broth or a suspension made from inoculant samples are serially diluted in saline solution (0.89% NaCl) and the appropriate dilutions spread onto growth agar media plates which are then incubated at 28°C for 2-3 (fast growers) to 6-7 days (slow growers). Colonies are then counted with the assumption that each rhizobia cell gives rise to a single colony. Miles and Misra or Drop plate method will be used during the exercises in this module. It is important that plates are checked daily in order to mark those colonies growing under the estimated time for the particular rhizobia strain in the sample. Cell counts are performed on the fermentation broth before it is used during carrier injection as this will give an estimate of cell numbers in the product. Cell counts are also necessary to be carried out in the final product as this will determine its quality.

### 1.5.2. Check for contaminants

During the fermentation process, it is essential that a sample of the culture broth is taken before the broth is used for carrier inoculation. The detection of contaminants can be achieved by Gram stain and by plating into glucose-peptone plates which does not favour the growth of rhizobia cells. Peat, liquid and freeze dried inoculants also needs to be tested (cell counts) and should not contain a high number of any organism other than the selected strains. Numbers of other organisms in the inoculants should ideally be absent, or at least 10-100 times lower in number than the selected rhizobial strain. Levels of non-rhizobial contaminants and moisture potential of moist peat –based inoculants are good indicators of potential shelf life and are checked routinely. If moisture potential and rhizobial numbers have been maintained and contaminant levels are low just prior to expiry, shelf lives can be extended for a further six months.

#### Gram stain:

Bacteria are classified as Gram-positive or Gram-negative bacteria due to their ability or inability of their cell membrane to retain the crystal violet stain. Gram stain is a rapid test where the shape of the bacteria can also be identified. Rhizobia are rod-shaped Gram-negative bacteria. This test is very useful to confirm that an organism is not a Rhizobium but can't confirm that it is Rhizobium. It allows an easy detection of Gram positive contaminants; however Gram negative contaminants will be harder to identify against rhizobia cells.

#### Plating on Nutrient agar or Glucose-Peptone plates:

Nutrient agar is a readily made rich media preferred by most contaminants and these will grow overnight when plates are incubated at 37oC whereas rhizobia cells will not grow at this temperature. This test is also a very simple but essential procedure that needs to be performed 24 hours prior using the culture broth for injecting inoculant bags. It involves plating 1ml of the fermentation broth into the Nutrient agar plate and incubating at 37oC overnight and any growth obtained on these plates will be contaminants. Plating 1ml of broth will allow detection of small levels of contamination in the broth. An alternative media commonly used to grow bacteria other than rhizobia is Glucose-peptone media. Glucose-peptone does not favour the growth of most rhizobia, but many contaminants grow easily and produce pH changes.

### 1.5.3. Strain identity

To ensure the correct strain has been used in the batch of inoculants, strain identity must be proven. Module 2 has presented with a series of methods such a RAPD or RFLP PCR that will facilitate the identification of rhizobial strains.

### 1.5.4. Efficacy of inoculants

It is essential that inoculants are assessed for the retention of their nodulation characteristics. Mutation during growth can occur, and in some instances this may compromise nodulation. Techniques such as the Most Probable Number (MPN) are effectively used not only to determine cell number in the inoculant but also that the nodulation capability of the strain has been maintained. MPN is also the most suitable technique to use to determine cell numbers on non-sterile inoculants



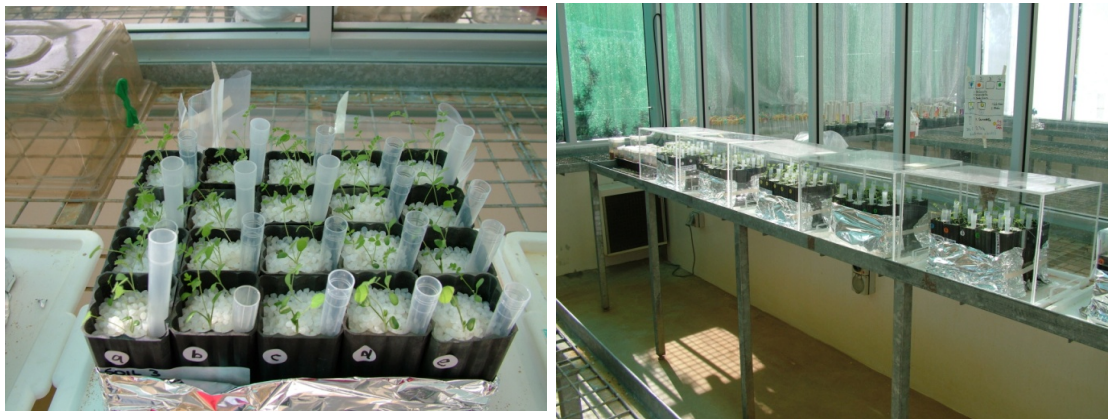
as the high number of contaminating organism in the product might impede rhizobia counts on plates due to their high rate of growth and/or antibiotic production.

### Most Probable Number (MPN) plant test

The most probable number method is an indirect way of counting rhizobia that involves inoculation of increasing dilutions of the bacterial culture or inoculants onto plants grown under aseptic conditions. Nodulation indicates that ineffective rhizobia were present in the inoculums; no nodulation that they were absent. For the method to give valid results all replicates of the last dilution should be negative (no nodules).

The MPN assays can be conducted in test tubes, plastic bags, pots of sand or Leonard jars or adaptations of these vessels, with one seedling growing per unit. Sow seedlings in chosen vessel three weeks before commencing the MPN allowing for extra seedlings to overcome mortality and for controls. The test must run for at least 25 to 30 days post inoculation.

The principal in the enumeration of bacteria by dilution is to serially dilute them to reduce the bacterial density to a number that can be accurately counted or estimated. Serial dilution can be applied to liquids, solids or soils. The method described here uses environmental soil samples. A ten-fold serial dilution is most often employed for dilution of soils or solids (such as a clay inoculant) a large mass (e.g. 10g) is usually selected, as quite often this will represent a sub-sample from a larger sample. Diluents can be sterile water or a more osmotically friendly solution of 1% (w/v) sucrose. The method provided is for small seeded legumes but if larger legumes are used, we recommend using larger planting pots. The 1 ml inoculant is placed across the roots of all seedlings, which are sown in pots containing steamed sand (**Fig 3**).



**Figure 3.** Example of MPN analysis

In this training you will then learn the basic techniques in the manufacture at a small scale of legume inoculants and perform QC tests.

## PART 2- EXPERIMENTS

### 2.1. Media and materials preparation.

Solutions:

- Saline solution: 1L NaCl 0.89%
- 1 L of YMA agar(pour plates after autoclave to make approximately 40 plates)
- 0.5L of Nutrient agar (NA)plates (pour plates after autoclave to make approximately 20 plates)

Materials:

- 1.5ml microcentrifuges (place in provided containers); Autoclave
- Blue and yellow tips in boxes; Autoclave
- 20 x 10ml glass pippettes (use canisters if available) otherwise wrap in aluminium foil and autoclave.
- 2 x 250ml glass beakers: cover with aluminium foil and autoclave.

#### Preparation of fermentation unit

For the set up of Fermentation unit following materials will be required:

- 5L flask
- Reagents to prepare 3L YMB
- In-let and out-let tubings
- 4 x hepa filters (if available)
- Aluminium foil

#### Method:

- i. Fill a 5L flasks with 3L of YMB.
- ii. Prepare the modified lids (which hold the inlet and outlet tubes with attached hepa filters as supplied) for autoclaving by wrapping each hepa filter with aluminium foil.
- iii. Fold the silicone hose between the lid and the hepa filter and hold using autoclave tape.

**NOTE:** folding the silicone hose will prevent broth media going into the filters during the autoclaving process.

- iv. Place modified lid into flask.
- v. Autoclave Fermentor flasks
- vi. Let unit cool

### 2.2. Inoculation of Starter Cultures

Materials that are required to be prepared before the start of this exercise are:

- Pre-streaked plates with the fast growing strain.
- Two sterilized 30ml YMB in 100ml flasks.

Materials require for this session:

- Laminar flow
- Inoculating loop
- 2 x 30ml YMB flasks

Method:

- i. Select two volunteers among yourselves to inoculate one 30ml media flask each
- ii. In laminar flow, inoculate the flask with a loopfull of the strain.

**NOTE:** when inoculating the flasks make sure that you do not touch the mouth of the flask

- iii. Place in shaker @200rpm for two days

These starter cultures will be used to inoculate the fermentation units.

### 2.3. Inoculation of Fermentation unit

Working in two groups, each group will take care of one fermentation unit. Label units as **A** and **B** (group A and group B):

**Method:**

- i. In laminar flow, remove bung from starter culture and flame mouth of flask.
- ii. Carefully lift the lid from the fermentor unit
- iii. Pour contents of 30ml of starter culture into flask.
- iv. Remember to label your fermentation unit with strain, date.
- v. Place fermentation unit on bench and connect the air inlet hose to an aquarium pump.

**NOTE:** Ideally the fermentation unit should be placed in a 28°C room, however if not available, room temperature is fine (around 25°C)

- vi. Turn aquarium pump on and check that air flows freely through both filters and broth is bubbling gently and agitating the medium at the same time.
- vii. Leave broth for approximately 36-48 hours for fast grower.

### 2.4. Quality control

Materials:

- Sterile 10ml glass pipettes
- Pipette pump/fillers
- 2 x 30ml yellow lid McCartney's or sampling containers (sterile)
- Automatic pipettes: P1000 and P200
- Blue and yellow tip boxes
- Glass slides
- Gram stain kit
- Nutrient agar plates
- Microscopes

The day before the fermentation culture broth is ready to be used to inject the coir dust inoculant bags, the culture needs to be checked for purity:

**Method:**

Group A and group B:

- i. After 24h incubation, disconnect your fermentation unit from air supply and take the unit into laminar flow
- ii. Lift lid carefully.
- iii. Remove aseptically approximately 10ml of culture using a sterile 10ml glass pipette and place in an empty sterile McCartney.
- iv. Use the 10ml samples for the tests below

The samples collected from each fermentation unit will be enough for all participants to perform the tests individually:

**2.4.1. Gram stain**

- i. Take 20  $\mu$ l from sample collected above onto a microscope slide
- ii. Let drop dry then flame to heat fix cells onto slide

Perform Gram stain as per standard procedure (see Appendix A)

**2.4.2. Check for contaminants on Nutrient agar (NA) plates:**

NA media will be used as it is a ready-made general purpose media. An alternative media commonly used to grow bacteria other than rhizobia is Glucose-peptone media. Glucose-peptone does not favour the growth of most rhizobia, but many contaminants grow easily and produce pH changes.

- i. Label one NA plate with your name, date and fermentation unit
- ii. Take 1ml from sample collected above and place it on Nutrient agar plate
- iii. Tilt plate around gently to ensure sample is spread on plate and let dry
- iv. Incubate at 37°C oven overnight (do not turn plates upside down)
- v. Check for growth of contaminants the next day.

**2.5. Carrier injection**

For this exercise, you will be provided with sterilized inoculant carrier bags. After 2 days of growth of strain in the fermentation unit and no contaminants are growing on the NA plates during the overnight test; the broth is then ready for inoculant preparation.

Materials:

- Sterile 10ml glass pipettes
- 2 x 30ml yellow lid McCartney's or sampling containers (sterile)
- Automatic pipettes: P1000 and P200
- Blue and yellow tip boxes



- Glass slides
- Microscopes
- 2 x 250ml sterilized glass beakers
- 20ml Syringes
- 18G needles
- Labels
- Ethanol 70%
- Cotton
- parafilm

**Method:****Group A and Group B:**

- i. Disconnect your fermentation unit from air supply and take the unit into laminar flow
- ii. Lift lid carefully.
- iii. Carefully, pour approximately 200ml of culture into a sterile 250ml glass beaker. The samples collected from each fermentation unit will be enough for all participants to inject their own carrier bag:
- iv. In laminar flow, sterilize a small area in a corner of the carrier bag with 70% ethanol.
- v. Fit a 20ml sterilized syringe with a 18 gauge needle
- vi. Withdraw appropriate volume of broth culture and insert needle carefully in the sterilized corner of the bag.

**NOTE:**

- Volume of injection varies according to size of inoculant bags. This will have to be determined prior injection.
  - When inserting the needle into the bag, care should be taken as to avoid piercing the opposite side of the bag.
- vii. Inject broth into the bag
  - viii. Seal the hole with a label
  - ix. Gently massage the bag until the inoculum has been absorbed by the peat.
  - x. Remember to label your bags with strain, date and your name
  - xi. Place bags in incubator at 28°C.

**2.6. Quality Control****2.6.1. Cell counts: Miles & Misra drop plate method**

Using the same culture sample obtained for injecting your bags, you can now proceed to determine the quality of the broth used. All participants to perform the exercise below

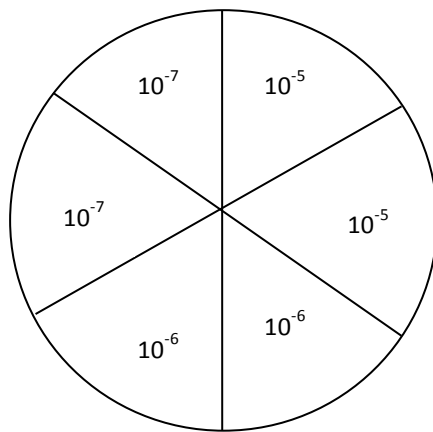
Materials:

- YMA plates
- Saline solution
- 1.5 sterile microcentrifuges

- Sterile Blue and yellow tip boxes
- Automatic pipettes: p1000, P100 and P20
- Parafilm

**Method:**

- In laminar flow, prepare 7 seven 1.5ml eppendorfs and label tubes with dilution values:  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ .
- Add 0.9 ml of sterile saline to each tube
- Perform the serial dilution (1/10) of the fermentation broth sample by firstly adding 0.1 ml of the inoculum to the first tube labelled  $10^{-1}$
- Close lid of the tube and vortex for few seconds
- Take 0.1 ml from  $10^{-1}$  tube and add to the  $10^{-2}$  tube and repeat step iv.
- Continue as per step v until you reach dilution  $10^{-7}$
- Radially mark a YMA plate into six equal sectors (like a pie) on the outside bottom of the plate (Remember to label your plates with your name and date)
- Label two sectors for each dilution:  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$



- Place 20 ul of the  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  dilution onto their respective sections on the plate.
- Wait until the drops have been absorbed before placing plates in the incubator.
- Seal the plates with Parafilm and incubate plates at  $28^{\circ}\text{C}$  for 2 days.

NOTE: Your plates will be ready on Day 8 for counting; however as you will be on a field trip, please indicate your trainer that your plates will be require to be store in the fridge on that day.

**Determination of cell counts from culture broth****Method:**

- Remove your plates from Day 6 from the fridge
- Visualize colonies under dissecting microscope for counting
- Count drops with 5-50 colonies
- Calculate cell counts as per equation below

$$\text{CFU/ml} = \text{N}^{\circ} \text{ of colonies counted} \times \frac{1000}{\text{Aliquot plated}} \times \frac{1}{\text{Dilution factor}}$$

## 2.6.2. Testing of inoculated carrier

### Materials:

- YMA plates
- Saline solution
- 1.5 sterile microcentrifuges
- Sterile Blue and yellow tip boxes
- Automatic pipettes: p1000, P100 and P20
- Sterile McCartney's
- Parafilm

### Method:

- i. Remove your inoculant bag from the 28°C incubator
- ii. Place bag in laminar flow
- iii. Sterilize a small area of the bag with 70% ethanol.
- iv. With an sterilized blade, make a small incision on the sterilized area of the bag
- v. Flame a spatula and let cool
- vi. With the sterilized spatula remove approximately 2-3 grams of inoculant.
- vii. Place sample in an empty sterile McCartney (or yellow lid 30ml container)
- viii. Seal bag with sticky tape
- ix. Prepare six 1.5ml sterile microcentrifuge tubes with 0.9ml of saline
- x. Label tubes  $10^{-2}$  to  $10^{-7}$
- xi. From the peat sample taken, weight 1g of inoculated carrier and place into sterile McCartney.
- xii. Add 9 ml of sterile saline (this is dilution  $10^{-1}$ )
- xiii. Shake for approximately 10 minutes
- xiv. Continue to serial dilute (1/10) your  $10^{-1}$  sample to  $10^{-7}$  as per shown on exercise 4.9 above
- xv. Radially mark a YMA plate into six equal sectors (like a pie) on the outside bottom of the plate (Remember to label your plates with your name and date)
- xvi. Label two sectors for each dilution:  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$
- xvii. Place 20 ul of each dilution onto their respective quadrants on the plates.
- xviii. Wait until the drops have been absorbed before placing then in the incubator.
- xix. Incubate plates at 28°C until colonies are observed (2 days)
- xx. Visualize colonies under dissecting microscope for counting
- xxi. Count drops with 5-50 colonies

### Determination of cell counts from injected carrier

#### Method:

- i. On day 10, check your plates after 24h incubation to check for contaminants.
- ii. If no colonies are observed, put your plates back in the incubator at 28°C.
- iii. On day 11, remove your plates from the incubator and visualize colonies under dissecting microscope for counting.
- iv. Count drops with 5-50 colonies
- v. Calculate cell counts as per equation above
- vi. Express your results as **CFU/g**

### **2.6.3. Most Probable Number (MPN) plant test**

After your inoculant bags have been incubated for approximately 2 week, your inoculants should be tested regularly for efficacy. The Most Probable Number (MPN) plant test will not only confirm Rhizobium numbers in the product but would also confirm that the strain used for making the inoculant is effective on the specific legume that the product is intended to.

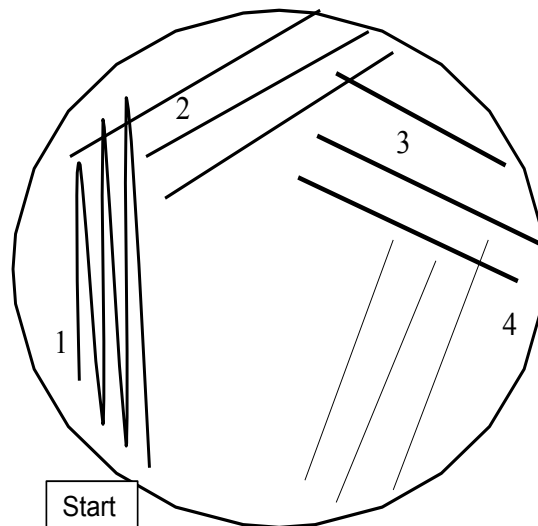


## PART 3 – APPENDICES

### Appendix A: Fundamental Microbiology Techniques

#### A.1. Streaking a plate

Streaking pattern for isolation of single colonies. The inoculating loop should be flamed when starting a new set of strokes, to ensure adequate dilution of the culture and development of well separated colonies.



#### A.2. Gram staining

Prepare laminar flow cabinet with flame, glass slides in 70% EtOH jar.

- I. Using forceps, remove a glass slide from the jar and flame to remove the ethanol. Label the name of the culture in pencil on the frosted section of the slide.
- II. Using a sterile loop, place a drop of sterile DI H<sub>2</sub>O on the slide surface.
- III. Using a sterile loop, transfer a very small amount of culture to the drop and mix to resuspend.
- IV. Allow the culture to dry on the slide and then pass the slide through the flame several times to fix it to the slide.
- V. When all slides are ready, take them to the sink for Gram staining
- VI. Place a drop of Crystal violet solution and stain for 60 seconds. Rinse with distilled water
- VII. Place a drop of Iodine solution and stain for 45 seconds. Rinse with distilled water
- VIII. Wash with Acetone: Ethanol (1:1) solution followed by distilled water
- IX. Place a drop of Safranin solution and stain for 20 seconds
- X. Rinse and blot dry slide
- XI. Visualise under microscope

### A.3. Culture storage at -80C

Rhizobia can be stored as glycerol stocks at -80°C.

80% (v/v) glycerol

Glycerol	80 ml
Deionised (DI) water	20 ml

Add DI water to glycerol. Stir until dissolved. Aliquot 5 ml into McCartneys & autoclave at 121°C for 20 min.

- i. In the laminar flow cabinet, aliquot 300 µl of sterile 80% (v/v) glycerol into a labelled cryovial.
- ii. Aliquot 1700 µl of sterile 0.89% (w/v) saline or sterile medium into the cryovial.
- iii. Add a large loopful of fresh plate culture to the cryovial.
- iv. Resuspend by vortexing and store at -80°C.

### A.4. Culture storage in ampoules (Julie to add)

Rhizobia can also be stored as lyophilised culture in glass ampoules.

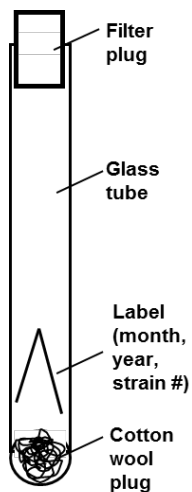
Peptone and sodium glutamate stocks:

10% Na glutamate                      10g in 100 ml DI H<sub>2</sub>O

10% peptone                              10g in 100 ml DI H<sub>2</sub>O

Aliquot 5 ml into McCartneys & autoclave at 121°C for 20 min. Store at -20°C.

Prepare the glass ampoule tubes:



Prepare each ampoule tube as demonstrated, with cotton wool at the bottom, printed label and filter plug.

For each strain, prepare 6 – 8 ampoule tubes.  
Autoclave in alfoil-covered racks.

Equipment required:

Sterile Pasteur pipettes with filters

Sterile forceps, Petri plates, probe

## Preparation of samples:

- i. In the laminar flow cabinet, thaw 1 bottle of peptone and 1 bottle of glutamate. Flame the necks of the bottles and pipette the glutamate into the peptone and mix.
- ii. Using sterile forceps, remove the filter plug from six ampoule tubes and place them in a sterile Petri dish.
- iii. Take a large loopful of fresh plate culture and place it in the peptone/glutamate mix. Resuspend by vortexing. Flame the neck of the bottle, and with the Pasteur pipette put 4-5 drops onto the cotton wool plug at the bottom of the ampoule tube.
- iv. Flame the tweezers and replace the filter plugs in the ampoule tubes.
- v. With the sterile probe, push the filter plug securely down to the level of the label.
- vi. Take the ampoule tube and using a blow torch, position the flame just above the filter plug. Rotate the tube until the glass melts and the ampoule is constricted.



## Lyophilisation of the samples

- i. Attach the constricted ampoule tubes to the vacuum pump manifold.
- ii. Operate the vacuum pump until a hard vacuum is obtained.
- iii. Using a blow torch, flame the ampoule tube at the constriction point until it melts, sealing the tube while still under vacuum.
- iv. The ampoules are now ready for long term storage.

## Appendix B: Growth media

### B.1. Glucose-Peptone Media

Add following in distilled water to prepare 1 L of Glucose Peptone media

Glucose	5 g
Peptone	10 g
Agar	15 g

Autoclave at 121°C for 20 min. 10ml of Bromocresol purple (1% in ethanol) is added to the melted agar before pouring into plates.

### B.2. ½ LA medium

Weigh out following ingredients and add into 800 mL of distilled water while stirring.

D-glucose	5 g
Mannitol	5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.8 g
NaCl	0.1 g
Yeast extract	1.25 g
CaCl <sub>2</sub> .2H <sub>2</sub> O	0.2 g
Agar	12 -15 g

To this add the four liquid stocks

<i>Liquid stocks</i>	
A. K <sub>2</sub> HPO <sub>4</sub> (0.87 g/L)	20 mL
B. KH <sub>2</sub> PO <sub>4</sub> (0.68 g/L)	20 mL
C. FeSO <sub>4</sub> .7H <sub>2</sub> O (0.5 g/L)	10 mL
D. Trace element solution	1 mL

Composition for 1 L Trace elements solution	
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> .10H <sub>2</sub> O	2.34 g
MnSO <sub>4</sub> .4H <sub>2</sub> O	2.03 g
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0.22 g
CuSO <sub>4</sub> .5H <sub>2</sub> O	0.08 g
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0.126 g

Adjust the pH to 6.8 using 0.1 M NaOH. Add agar if required, and make volume up to 1 L before autoclaving at 121°C for 20 min. Pour media when approximately 60 °C into plates in a laminar flow unit.

**NB** The concentration of P is low by normal standards and serves to reduce culture gumminess in genera such as *Sinorhizobium*. However, some rhizobial species such as that from *Hedysarum spinisissimum* require a 5 fold increase in P to grow on ½ LA. For assessment of growth at low pH, GOOD buffers should be added at 10 – 20mM.

### B.3. Yeast Mannitol Broth (YMB)

Dissolve following in distilled water to prepare 1 L of YMB

Mannitol	10 g
K <sub>2</sub> HPO <sub>4</sub>	0.5 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 g

NaCl 0.1 g

Yeast Extract 0.5 g

Adjust pH to 6.8 and autoclave at 121°C for 20 min

To prepare YEAST-MANNITOL AGAR (YMA) 15 g agar is added prior to autoclaving

## **Appendix C: Reagents and stocks**

### **C.1. Ethanol 70% (w/v)**

Add 70 ml of 100% ethanol (AR grade) to 30 ml of sterile deionised water

### **C.2. Hypochlorite solution (bleach) 4% (v/v)**

Dilute the 12% bleach stock to final 4% by adding 30 ml to 70 ml of sterile deionised water

Note. At the CRS, we also use 4.5% (v/v) bleach which is provided by the manufacturer at this concentration.

### **C.3. Normal saline (155 mM NaCl)**

Dissolve 0.89 g NaCl in deionised water to make up a total volume of 100 ml.

Autoclave at 121°C for 20 min and store at room temperature.

### **C.4. Sucrose 1% (w/v)**

Dissolve 10 g of sucrose in deionised water to make up a total volume of 1000 ml. Autoclave at 121°C for 20 min and store at room temperature.