MICROBIAL CULTURE PREPARATION

Training Module

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PREFACE

This module is adapted from an industrial training conducted by Department of Biotechnology, Indonesia International Institute for Life Sciences (i3L), Jakarta, Indonesia. The training was aimed for culture preparation for soy sauce fermentation using *Aspergillus oryzae* and *Bacillus subtilis*. However, the protocols detailed in this module can be easily adapted for work using other microorganisms.

The whole module can be delivered as a three-day training (suggested schedule is provided). Only BSL1 microbiology lab with standard equipments are required. Moreover, there are no specific requirements for participants except familiarity with basic microbiology concepts. Some experience working in a lab environment will be beneficial.

We hope this module can be useful for anyone who wish to learn basic techniques in microbial culture preparation and as a foundation to learn more advanced methods in food and industrial microbiology.

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Laboratory Protocol

Session 1 – Media Preparation

Overview:

In this session, participants will learn how to prepare media (both agar and broth) and other required solutions such as saline solution for dilutions and glycerol stock solution for preservation.

Material:

Nutrient agar (NA), Potato Dextrose Agar (PDA), Phosphate Buffer Solution (PBS), type III water, ethanol 70%, sodium chloride (NaCl), 80% glycerol solution.

Equipment:

Petri dish, inoculation loop, beakers, bunsen burner, syringe, Erlenmeyer flasks 500 mL, Schott bottle, autoclave, weighing balance, spoon, hotplate, magnetic stirrer.

Procedure:

1.1. PBS preparation

1. As per the instruction from the manufacturer, put one PBS tablet in 200 ml of type III water in the Schott bottle.
2. Wait for it to dissolve completely.
3. Prior to autoclave, loosen the Schott bottle's cap. Autoclave at 121°C for 15 minutes.

1.2. Saline solution 0.9% (w/v) preparation

1. Dilute 0.9 grams of sodium chloride in 100 ml type III water.
2. Sterilize in the autoclave for 15 minutes at 121°C.

1.3. Glycerol stock solution 50% (v/v) preparation

1. Take 31.25 mL of 80% glycerol solution using a syringe and pour it in 50 mL type III water.
2. Sterilize in the autoclave for 15 minutes at 121°C.

1.4. Media preparation

The media used for this lab session are PDA for *A. oryzae* and NA for *B. subtilis*. The preparation step followed is general in nature and could be adapted to other types of growth media.

1. Prepare 100 mL media by diluting agar/broth powder in 100 mL of type III water in the Erlenmeyer flask. See the instructions in the packaging for the exact weight of the media.
2. Heat (medium to boil) to dissolve the media in hotplate prior to autoclaving, to ensure the agar is distributed uniformly throughout the medium, and stir the medium during boiling. Then, close the Erlenmeyer flask with aluminium foil.
3. Sterilize the media using autoclave for 15 minutes at 121°C.

4. For agar media, wait for the autoclaved media to be warm enough before pouring into Petri dishes. Avoid cooling down the media completely, as the agar will solidify in the Erlenmeyer flask before pouring it into the Petri dishes.
5. In aseptic condition, pour the warm media onto Petri dishes and wait to cool **(Figure 1)**.

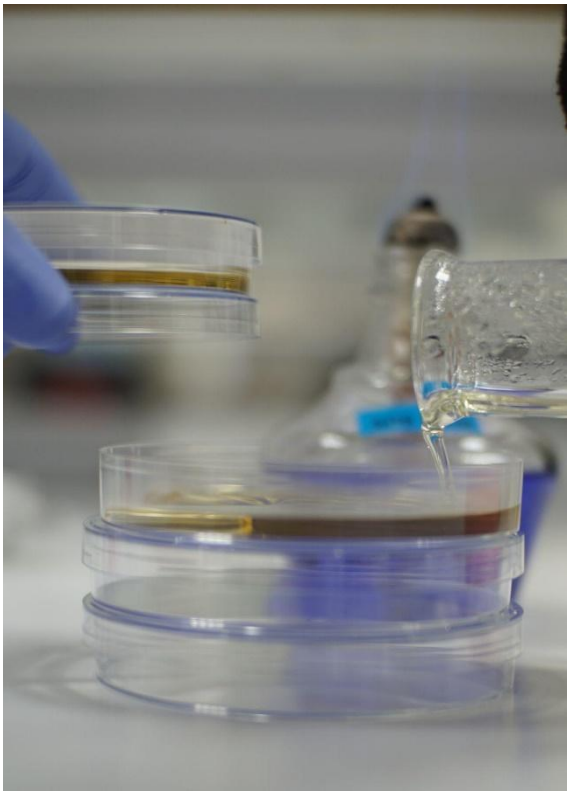


Figure 1

6. Put the Petri dishes on the bench upside down for 1 day, to make sure that the plates are not contaminated.
7. For broth media, wait for the autoclaved media to cool down completely and leave it on the bench until further use.

Laboratory Protocol

Session 2 - Culture Transfer & Culture Morphology Characterization

Overview:

In this session, participants will learn how to transfer and revive stock culture with two culture transfer methods. Participants will also learn how to determine the cultural characteristics of microorganisms as an aid in identifying the culture using the gram staining technique. **Note:** culture will be prepared and serially diluted by the lab assistant beforehand.

Material:

A. oryzae, B. subtilis, C. etchellsii, T. halophilus, E.coli, crystal violet, Lugol's solution, safranin, acetone-alcohol solution, type III water, phosphate-buffered saline (PBS), ethanol 70%.

Equipment:

Microscope slide, microscope, immersion oil, micropipette, micropipette tips, Kimtech wipes, microcentrifuge tubes, inoculation loop, L-shape spreader, beaker, bunsen burner, petri dish.

Procedure:

2.1. Culture transfer - streak plate:

Note: this method is suitable for glycerol stock culture revival.

1. Burn the loop with a Bunsen burner **(Figure 2a)** and wait for it to cool down completely before continuing. Take a loopful of culture from the glycerol stock or culture from agar and place it on the agar surface in Area 1 **(Figure 2b)**. Burn the loop again.

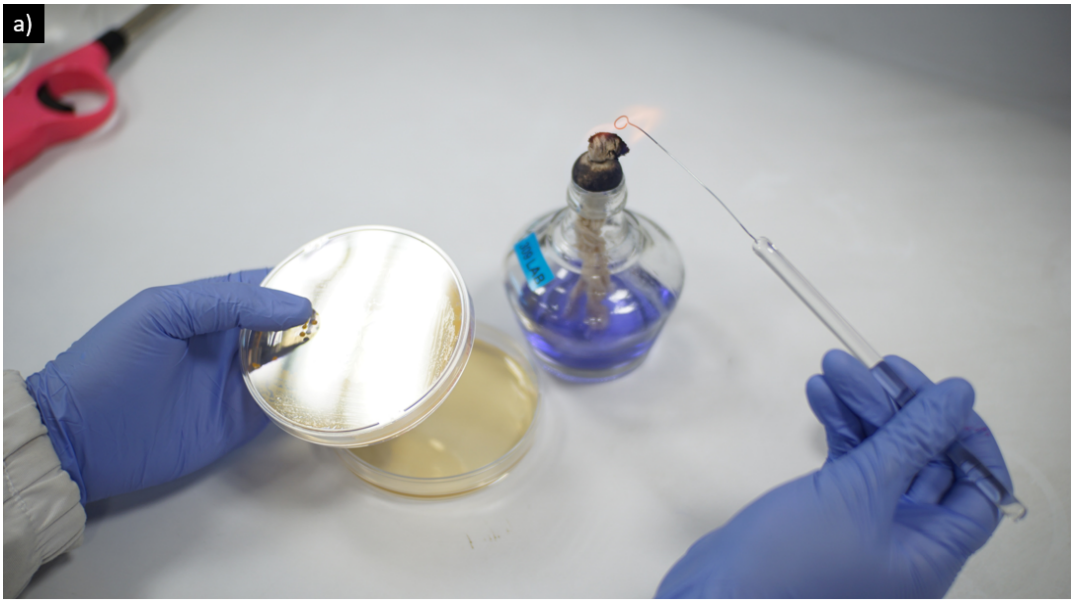


Figure 2a

Figure 2b

2. After the loop cooled down, place the loop where the culture has been placed and drag it rapidly several times across the surface of Area 1 **(Figure 2c)**. Reflame the loop and wait for it to cool down completely.

Figure 2c

3. Turn the petri dish in 90◦ and put the loop in the corner of the streaked culture in Area 1 and drag it rapidly several times across the surface of Area 2 **(Figure 2d)**. The loop should not make contact with Area 1 again.

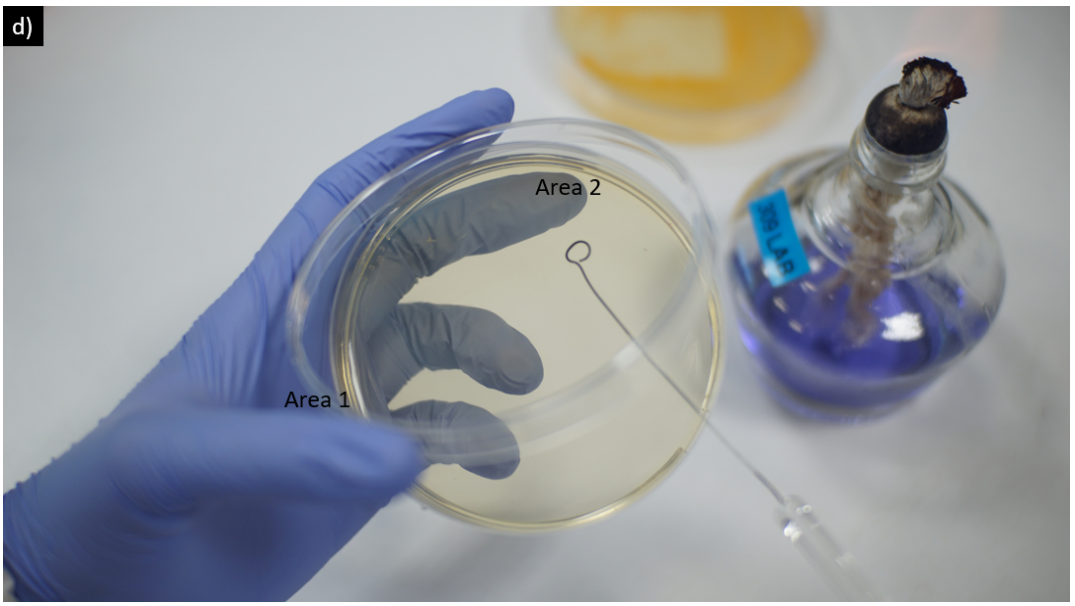


Figure 2d

4. Burn and cool the loop. Turn the petri dish in 90◦ again. Streak Area 3 with the same method as Area 2 **(Figure 2e).**

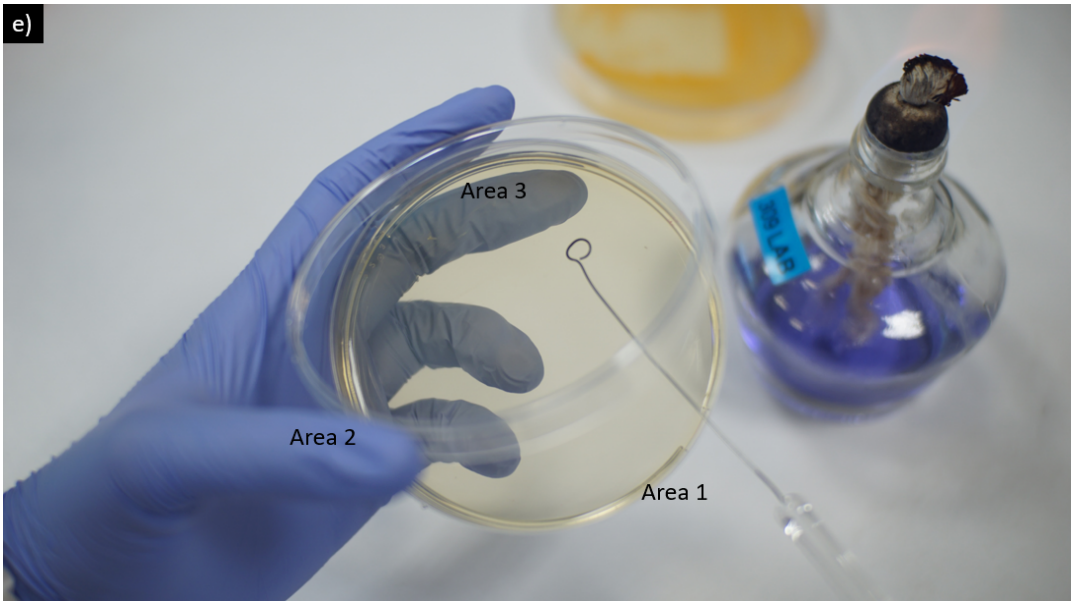


Figure 2e

5. For the last step, turn the petri dish in 90◦, and without burning the loop, put the loop in the corner of the streaked culture in Area 3. Drag and make a wider streak across Area 4 **(Figure 2f)**. Never let the loop touch any of the previously streaked areas.

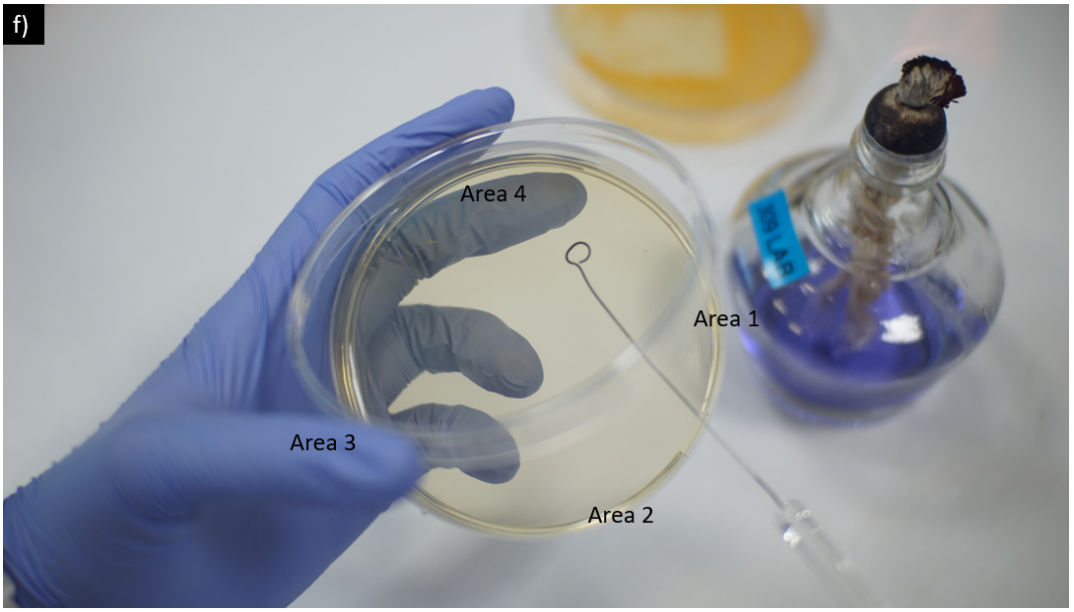


Figure 2f

6. Seal the petri dish with parafilm (optional) and incubate in an inverted position **(Figure 2g).**

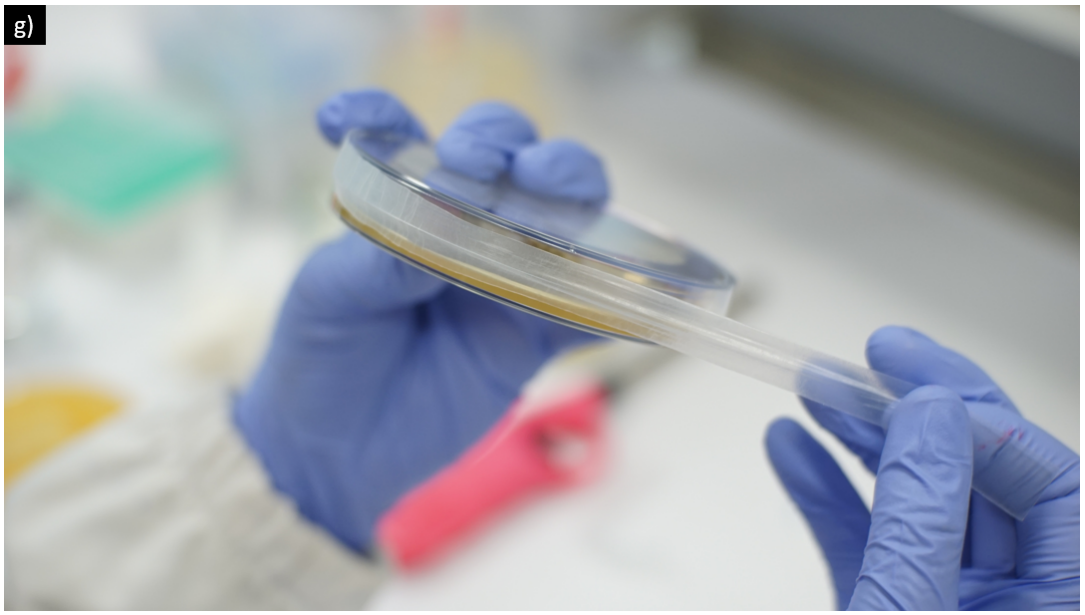


Figure 2g

2.2. Culture transfer - spread plate:

1. Prepare a beaker glass filled with ethanol and immerse the L-shaped spreader. Label all the Petri dishes with each dilution factor before continuing.
2. In a sterile condition, take 100 µL from one dilution using a sterile pipette **(Figure 3a)** and pour it on top of the agar **(Figure 3b)**.

Figure 3a

Figure 3b

3. Burn the L-shaped spreader using a Bunsen burner **(Figure 3c)** and wait for it to cool down completely.

Figure 3c

4. Spead the dilution until all area is covered **(Figure 3d)**. After that, incubate the media in an inverted position.

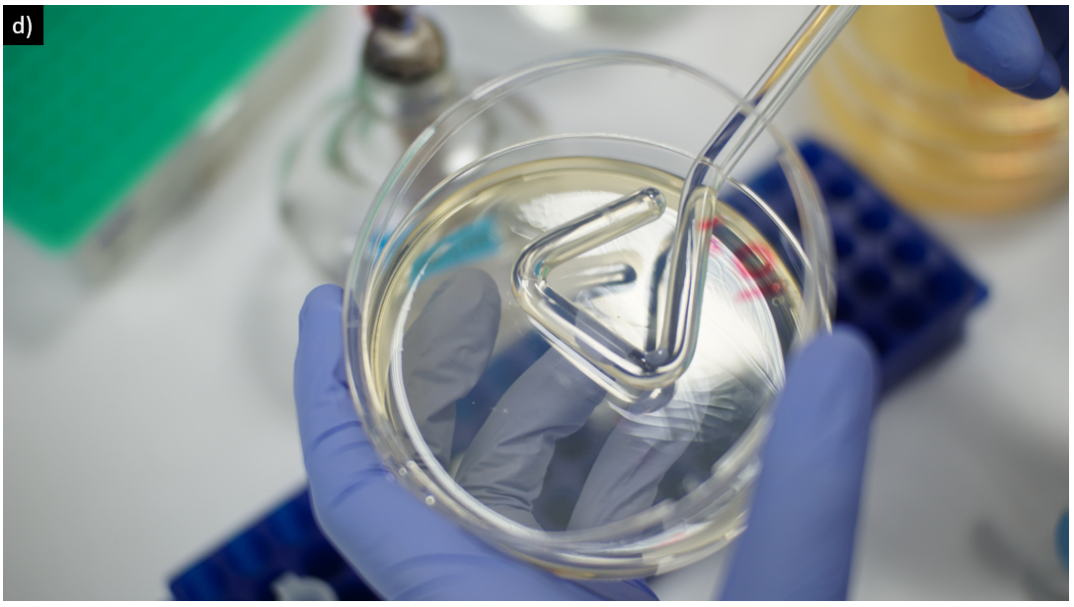


Figure 3d

2.3. Gram staining:

1. Add a drop of water to the glass slide. In a sterile condition, take a colony from the agar with a loop and smear the loop with the water.
2. Fix the bacteria by moving the slide 3 times through the blue part of a flame.
3. Cover the smear with crystal violet and leave it for 1 minute, tap off on tissue paper.
4. Add 1 drop of Lugol's solution on the smear for at least 1 minute, tap off on tissue paper.

5. Decolorize with 1 drop of acetone-alcohol solution on the slide for 1-2 seconds. Do not let the smear stay too long under the acetone- alcohol-solution. Immediately rinse with water and tap the slide gently with tissue.
6. Cover the smear with Safranin for about 30 seconds.
7. Wash the smear in a beaker glass filled with tap water.
8. Blot it dry.
9. Examine the slide under the microscope.

2.4. Spore and mycelium visualization

1. Add a drop of water to the glass slide. In a sterile condition, collect the spores from the agar with a loop and smear the loop with the water.
2. Fix it by moving the slide 3 times through the blue part of a flame.
3. Examine the slide under the microscope.

Laboratory Protocol

Session 3 - Growth Curve, Spore Harvesting & Counting, Glycerol Stock Preparation

Overview:

In this session, participants will learn how to measure the OD, examine microbial growth curves and process the data, and also conduct serial dilution and plate count. Participants will also learn how to prepare koji starter culture from spore harvesting to cryopreservation.

Note: the media will be pre-made and the bacteria will be grown beforehand by an assistant.

Material:

B. subtilis, nutrient agar (NA), type III water, phosphate-buffered saline (PBS), ethanol 70%, blank and cultured broth, potato dextrose agar (PDA) slants, falcon tubes, trypan blue dye, glycerol stock solution

Equipment:

Micropipette, micropipette tips, microcentrifuge tubes, cuvette, UV-Vis spectrophotometer, L-shaped spreader, bunsen burner, beaker, colony counter, haemocytometer

Procedure:

3.1. OD Measurement

1. Prepare blank (sterile broth) and cultured broth (prepared by the assistant)
2. In aseptic condition and using a micropipette with attached tips, transfer 3 mL of the blank into the 1st cuvette. Discard the tips and take a new one. Transfer 3 mL of cultured broth into the 2nd cuvette. Discard the tips.
3. Follow the instruction on the UV-Vis spectrophotometer manual guidebook on how to operate the machine and software.
4. Measure the blank and make the absorbance 0 by clicking Auto Zero in the software.
5. With the wavelength of 600 nm, measure the cultured broth.

3.2. Serial Dilution

1. Prepare 6 microcentrifuge tubes containing 900 µL of PBS in each microcentrifuge tube.
2. Take 100 µL of cultured broth **(Figure 4a)** and transfer it to one microcentrifuge tube containing 900 µL of PBS **(Figure 4b)**. This is called the 10^{-1} dilution

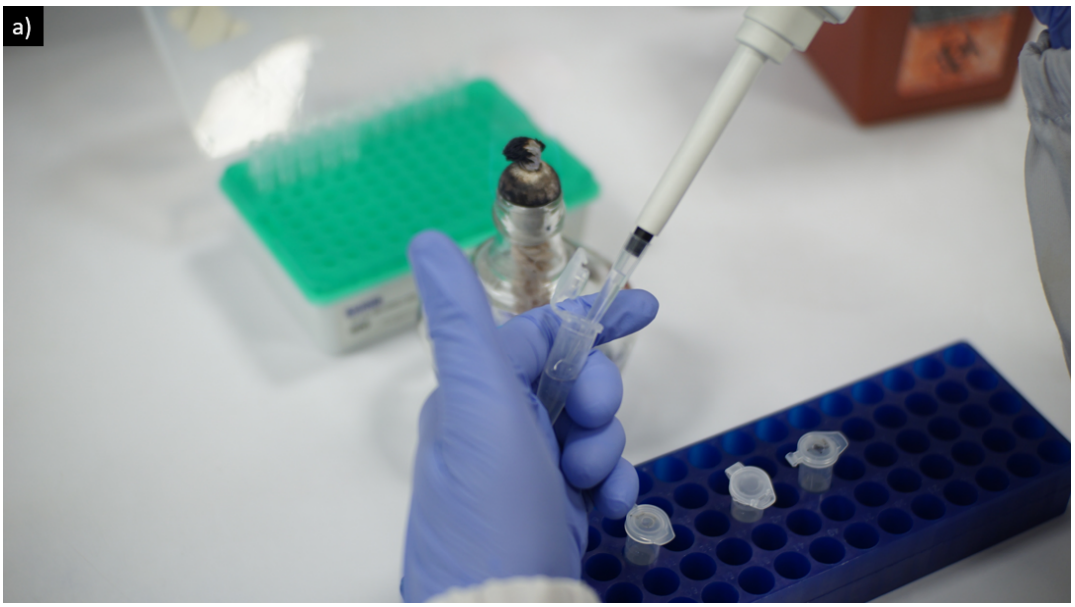


Figure 4a

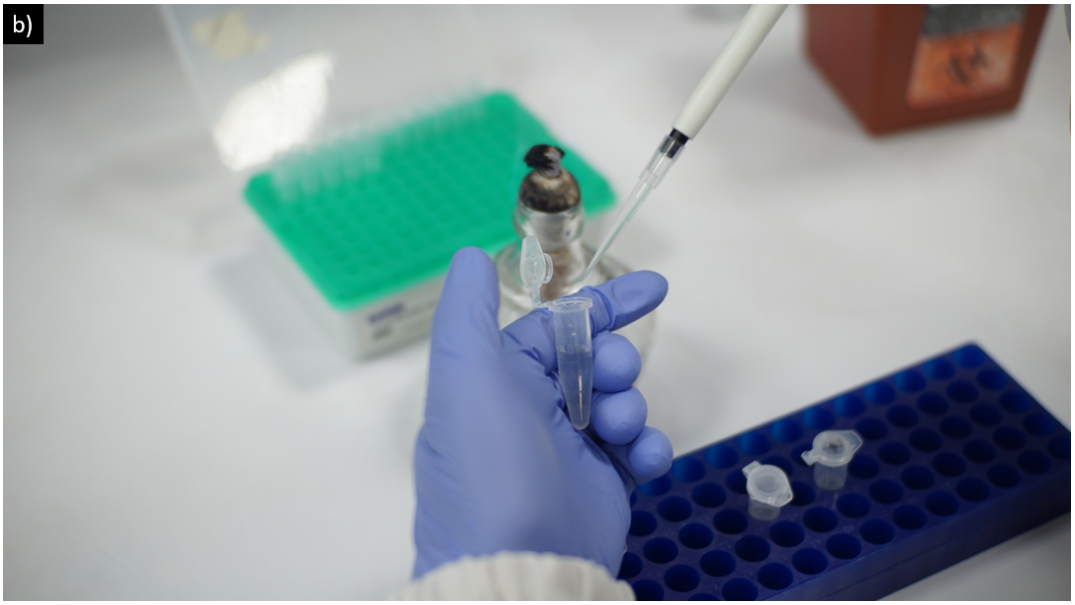


Figure 4b

3. From the 10^{-1} dilution, take 100 µL and transfer to a new microcentrifuge tube containing 900 µLof PBS **(Figure 4b)**. This is called the 10^{-2} dilution

Figure 4c

4. Repeat the steps several times until 10^{-6} dilution.

3.3. Microbial enumeration - Spread plate method

1. Label agar media, one agar media per dilution factor. For each dilution factor, take 100 µL and transfer to one agar media and spread using a spreader. Repeat for every dilution (see *Culture transfer - spread plate* section)
2. Incubate agar media in 37C incubator. After the incubation period, count the number of colonies in each agar media. Count agar media which contains between 30-300 colonies. Less than that is marked Too Few to Count (TFTC), or if more than 300 colonies, Too Numerous to Count (TNTC).
3. To determine CFU/mL in the spread plate method, use the equation below

$$\text{CFU/mL} = \frac{No\ of\ colonies\ x\ dilution\ factor}{Volume\ of\ culture\ plate}$$

3.4. Subculturing of *A. oryzae*

Note: the media will be prepared by the assistant prior to the lab session.

1. Prepare fresh PDA slants in flat glass bottles or PDA in petri dishes. To subculture, cut a small block of agar containing mycelium with green spores using a sterile spatula or inoculation loop (a) and place it on the 2 PDA slants or petri dish (b)

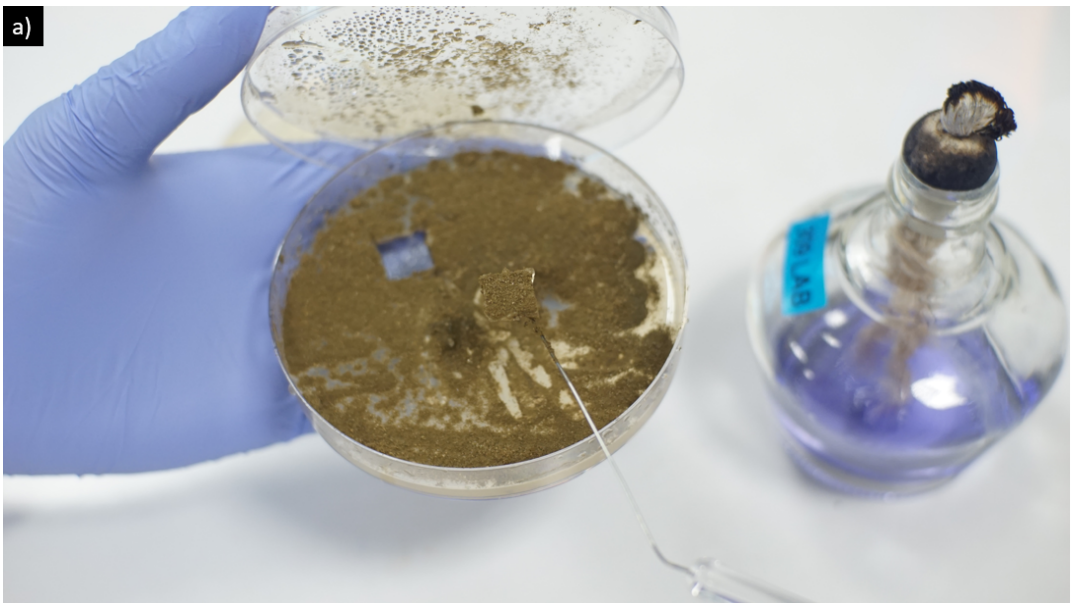


Figure 5a

Figure 5b

2. Incubate the PDA slants at 25ºC for 7 days or until the dark green spores become apparent. During the incubation, the caps should be loosely placed onto the bottles. Once the mycelium starts to grow on the agar slant, the bottles should be incubated upside down to avoid condensation dripping onto the cultures.

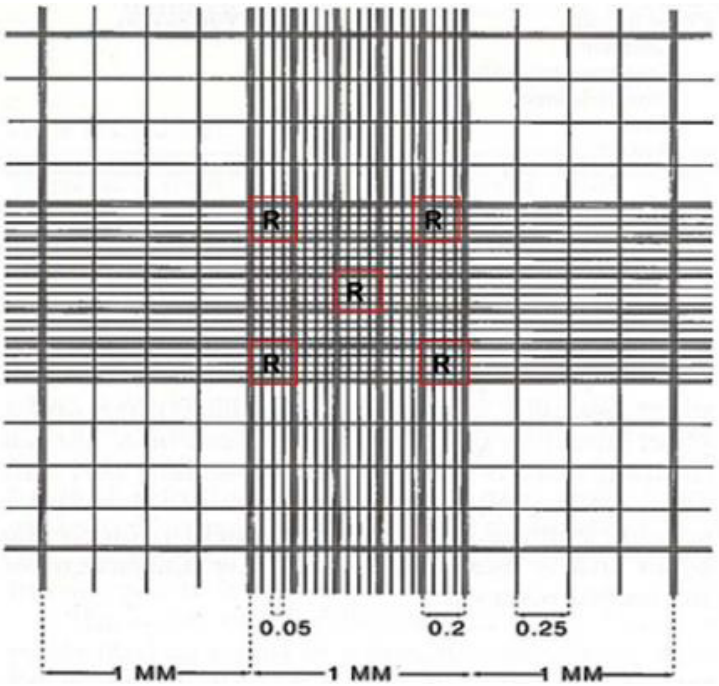
3.5. Spore harvesting

1. Prepare 50 mL of saline solution (PBS or 0.85% NaCl solution)
2. Under aseptic conditions, pour 50 mL of saline solution into the flat glass bottles containing the spores.

3. Close the flat bottle tightly with the cap and shake vigorously to dislodge the spores from the mycelium. The saline solution will turn dark green as more spores are dislodged. Collect the spore solution using a sterile container.

3.6. Spore counting

1. Prior to use, clean both haemocytometer and its coverslip gently with ethanol 70% and Kimtech wipes
2. Put the coverslip over the counting surface
3. Take 10 µL from the spore solution and transfer it into a sterile microcentrifuge tube
4. Take 10 µL of trypan blue dye and add it to the microcentrifuge tube containing the spore solution. Resuspend the mixture
5. Take 10 µL from the mixture and gently load the mixture to each chamber underneath the coverslip of the haemocytometer, allowing the chamber to fill by capillary action. Don't let the chamber be overfilled or underfilled
6. Observe the cells on the 5 marked areas under a microscope.



7. Count the cells with the given equation below

$$\text{Spores/mL} = \frac{n \, x \, 5 \, x \, 10 \, x \, 1000}{2}$$

n = Total cells in all 5 marked areas

8. Adjust the spores concentration by diluting with sterile PBS or 0.9% NaCl solution

Side note:

- Be very careful when cleaning the haemocytometer and the coverslip. If the haemocytometer is cleaned with too much friction, it could lead to damaging the counting surface. The

coverslip is very sensitive and also prone to break. Make sure to clean both the haemocytometer and its coverslip in one direction

- Avoid touching the haemocytometer and the coverslip without gloves

3.7. Culture preservation in glycerol solution

1. Take 500µL of glycerol stock solution and 500µL of broth inoculated with the culture into a microcentrifuge tube with the ratio of 1:1 (v:v)
2. Store the microcentrifuge tube in the cryo freezer