SOPs: MICROBIOLOGY PRACTICAL EXERCISES FOR UNDERGRADUATE STUDENTS

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Experiment No. 1: Autoclaving.

Background: Autoclaves use pressurized steam to destroy microorganisms, and are the most dependable systems available for the decontamination of laboratory waste and the sterilization of laboratory glassware, media, and reagents. An autoclave is a pressure chamber used to sterilize equipment and supplies by subjecting them to high pressure saturated steam at 121 °C for around 15–20 minutes depending on the size of the load and the contents. It works on the principle of heat under pressure as steam can penetrate cells easily and can destroy spores efficiently.

Preparation and Loading of Materials

1. Fill liquid containers only half full.
2. Loosen caps or use vented closures.
3. Always put bags of biological waste into pans to catch spills.
4. Position biohazard bags on their sides, with the bag neck taped loosely.
5. Leave space between items to allow steam circulation.
6. Household dishpans melt in the autoclave. Use autoclavable polypropylene or Stainless steel pans.

Cycle Selection

Use liquid cycle (slow exhaust) when autoclaving liquids, to prevent contents from boiling over.

Select fast exhaust cycle for glassware.

Use fast exhaust and dry cycle for wrapped items.

Time Selection

Take into account the size of the articles to be autoclaved. A 2-liter flask containing 1 liter of liquid takes longer to sterilize than four 500 mL flasks each containing 250 mL of liquid.

Material with a high insulating capacity (animal bedding, high sided polypropylene containers) increases the time needed for the load to reach sterilizing temperatures. Autoclave bags containing biological waste should be autoclaved for 50 minutes to assure decontamination.

Removing the Load

1. Check that the chamber pressure is zero.
2. Wear lab coat, eye protection, heat insulating gloves, and closed-toe shoes.
3. Stand behind door when opening it.
4. Slowly open door only a crack. Beware of rush of steam.
5. After the slow exhaust cycle, open autoclave door and allow liquids to cool for 20 minutes before removing.

Precautions:

1. When unloading an autoclave, wear heat resistant gloves, eye protection, and lab coat.
2. To prevent steam burns, make sure that the autoclave pressure is near zero before opening the door.
3. Allow steam to escape gradually by slowly cracking open the autoclave door. Allow load to cool for 10 minutes before removing.
4. Do not autoclave sealed containers or full bottles with narrow necks as they may explode.
5. Do not autoclave materials containing solvents, volatile or corrosive chemicals (such as phenol, chloroform, bleach, etc.), or radioactive materials

Experiment No. 2: Measurement of bacterial size.

Background: The diameter of a cell can be easily measured using an ocular micrometer which has graduation in arbitrary units. This arbitrary graduation of the ocular micrometer is calibrated using a stage micrometer by superimposing the two scales.

Material and Reagent Requirements: Light microscope, ocular and stage micrometer, slide having stained lactobacillus.

Methodology:

1. The ocular micrometer is placed on the circular shelf inside the eyepiece in such a way that the graduations sketched on the ocular, is visible when an observation is made using the microscope.

2. Place the stage micrometer on the stage of a microscope and focus the graduations using low power objectives. The graduations on stage micrometer are spaced 0.01mm (10μm) apart.

3. Superimpose the two scales and record the number of ocular division coinciding exactly with the number of divisions of the stage micrometer. The calibration factor or the least count of ocular micrometer is calculated as follows :

If 13 ocular divisions coincide with 2 divisions (2X10μm=20μm) of stage micrometer

Then 1 ocular division = 20μm/13 divisions
= 1.54μm

4. Now remove the stage micrometer from the stage and place the slide having cell preparation under low power magnification. Position the cell being observed in such a

way that the ocular micrometer is able to measure the diameter of a cell or the length/diameter of a cell component in arbitrary units. Calculate the size as shown below:

If the diameter of a cell is occupying 5 divisions of ocular, the diameter of the cell will be: 5 divisions X 1.54µm = 7.7µm

5. Similarly for high power objective the ocular micrometer calibration has to be done again following the same procedure and then cell diameter is can be measured focusing the cell in high magnification.

Precautions:

1. Calibrate the micrometers properly.
2. Bacteria should be stained properly to measure the size.

Experiment No. 3: One Step Growth of Bacteriophage.

Background: Viruses that attack bacteria are called bacteriophages or simply phages. Phages, like other viruses, cannot exist without a suitable host.

To replicate, a virus should induce its host to synthesize components that are necessary for the assembly of new virus particles. The virus accomplishes this process by first attaching to the host (adsorption) and then injecting its nucleic acid into the cell (injection or penetration). The viral DNA can stay free in the cell and be replicated as such, or it can be incorporated into the host chromosome and be replicated simultaneously with it. Viral proteins are next synthesized with the host's machinery under the direction of viral DNA and the new virus particles are assembled mechanically.

These particles can find their way out of the cell or lyse the cell and be released into the medium, ready to infect new cells.

If the number of phage particles was monitored during growth, a growth curve could be drawn which would be similar to that of the bacterial growth curve except in the last stage. The phage growth curve starts with a latent or eclipse period (similar to the bacterial lag phase). During this phase, the infection, adsorption, injection and syntheses

of new viral DNA and protein coat occur. The next phase is called the maturation or release stage (similar to the log phase in bacteria) when new phage particles are assembled and released. The cycle can then start over with the infection of new cells. In this manner, the shape of the curve would look step-wise and that is why the process is called "one-step phage growth curve".

Material and Reagent Requirements: Bacterial culture or host suspension,

Phage lysate, Phage base plates, Phage soft agar tubes, Phage broth (9.9 ml/tube), Sterile 1.0 ml pipettes Water bath, 37°C,Water bath, 50°C.

Methodology:

1. The bacterial culture or the host suspension that we will be using is *E. coli* B and our phage is T4. You will find the host suspension (2 x 108 cfu/ml) and the phage lysate (4 x 106 pfu/ml) in the 37°C water bath in the lab. Keep them at this temperature all the time. To the "Adsorption Tube" which already contains 0.1 ml of the phage lysate, add 0.9 ml of host culture, mix well, record the exact time and return the tubes to the 37°C water bath.

2. Obtain 2 large test tubes containing 9.9 ml of phage broth and label them 10-2 and 10-4 and place them at 37°C.
3. At precisely 10 min after starting the incubation of the Adsorption Tube, remove 0.1 ml of adsorption mixture and transfer to the tube marked 10-2. Mix thoroughly by vortexing.
4. Use another sterile pipet to transfer 0.1 ml from tube 10-2 to tube 10-4. Mix thoroughly by vortexing and place at 37°C.
5. At exactly 20 minutes into the experiment, add 0.1 ml from the 10-4 tube and 0.1 ml of the host culture to a soft agar tube and mix thoroughly by rolling the tube in the palms of your hands.
6. Pour the soft agar onto a base plate labeled "20 min" and rotate the plate to evenly spread the soft agar across the surface of the entire plate. Allow the agar to gel, invert the plate, and incubate at 37°C overnight.
7. Repeat steps 5 and 6 at precisely 25, 30, 40, 50, 60, 70 and 80 minutes into the experiment.
8 Your plates will be moved to a refrigerator after 24 hours of incubation at 37°C.

9. Calculate the multiplicity ratio in the Adsorption Tube. This is done by dividing the number of phage by the number of bacteria in the Adsorption Tube at the start of the experiment. This ratio provides an estimate of the number of phage available to infect each bacterium.

10. Obtain your plates. Count the number of plaques (pfu) per plate and complete the table in the "Results" section. Plates with a large number of plaques can be divided into 2 or 4 sections and only one section counted and then the result multiplied by 2 or 4 to get the total plaque number per plate.

Calculate the actual counts based for the number of plaques found on your plates multiplied by the dilution factor and the amount of inoculums used.

Incubation time (min)	No. of plaques observed on plate	Actual counts in Ads. Tube (pfu/ml)	Relative titers
20			
25			
30			
40			
50			
60			
70			
80			

PRECAUTATIONS:

Soft agar (molten) tubes are kept at the 50°C water bath. Remove only one tube at a time to use immediately. The soft agar solidifies in the tubes in a few minutes, if the tube is placed at room temperature. So, once again: do not remove any soft agar tube from 50°C bath until needed.

Experiment No4 : Microbial cells counting by serial dilution techniques.

Background:

Bacteria commonly are present in large amounts in the natural samples such as soil, water etc. Therefore, to get readily countable numbers of bacteria, we have to make a wide range of dilutions to get countable numbers. This is done by making serial 10-fold dilutions of the bacteria that cover the entire probable range of concentrations. Then we transfer 0.1 ml of each dilution to an agar plate, which in effect makes another 10-fold dilution, since the final unit is CFU/ml and we only streak 0.1 ml.

The number of viable cells in a culture can be determining by the number of **colony forming units CFU** with the **colony counting technique**. Between 20 and 200 CFU can be counted on a typical Petri plate. Microbial cultures of high density must be **diluted** before they are plated. A known volume [0.1ml] of these dilutions is plated onto suitable growth medium in the Petri dish. After the plates have been incubated up to a week, the average number of colonies on plates is determined.

Requirements:

Soil sample, distilled water, micropipettes, test tubes, nutrient agar, micro tips, petriplates etc.

Methodology:

1. Take 9ml of distilled water in 8 test tubes.
2. Weigh 1g of soil sample and add it to test tube containing 9ml of distilled water and mix it properly.
3. Take 1ml from stock and add it to next test tube containing 9ml distilled water.
4. Repeat the step for all the tubes and discard last 1ml. (dilutions from 10^{-1}. 10^{-2},10^{-3}……..10^{-7} are made)
5. Take 0.1ml from each test tube to inoculate petriplates containing solidified agar .
6. Spread inoculum on surface of agar with the help of spreader.
7. Incubate for 24hrs and count the number of colonies on petriplates.

Calculations:

Colony forming unit = No of Colonies X Dilution Factor

Precautions:

1. Make dilutions carefully.
2. Autoclave media properly before inoculation.
3. Inoculate media after solidification.