KARPAGAM ACADEMY OF HIGHER EDUCATION

(Established Under Section 3 of UGC Act 1956)

Coimbatore – 641 021.

(For the candidates admitted from 2017 onwards)

DEPARTMENT OF MICROBIOLOGY

SUBJECT NAME: BACTERIOLOGY PRACTICAL **SUB.CODE:18MBU112**

SEMESTER: I **CLASS: I B.Sc (MB)**

SYLLABUS

Instruction Hours / week: L: 0 T: 0 P: 3 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

EXPERIMENTS

1. Preparation of different media: synthetic media BG-11, Complex media - Nutrient agar, McConkey agar, EMB agar.
2. Micrometry.
3. Motility by hanging drop method.
4. Simple staining
5. Negative staining
6. Gram's staining
7. Acid fast staining – demonstration permanent slide only.
8. Capsule staining
9. Endospore staining.
10. Isolation of pure cultures of bacteria by streaking method - Quadrant, Continuous and T-streaking.
11. Preservation of bacterial cultures by various techniques - Agar slants and deeps - Mineral Oil, Glycerol stocks
12. Estimation of Colony Forming Unit (CFU) count by spread plate method/pour plate method.

SUGGESTED READINGS

1. Pelczar Jr, M.J., Chan, ECS., and Krieg, N.R. (2004). Microbiology. 5th edition. Tata McGraw Hill.
2. Willey, J.M., Sherwood, L.M., and Woolverton, C.J. (2013). Prescott's Microbiology. 9th edition. McGraw Hill Higher Education.
3. Madigan, M.T., and Martinko, J.M. (2014). Brock Biology of Micro-organisms. 14th edition. Parker J. Prentice Hall International, Inc.
4. Tortora, G.J., Funke, B.R., and Case, C.L. (2008). Microbiology: An Introduction. 9th edition. Pearson Education.
5. Black, J.G. (2008). Microbiology: Principles and Explorations. 7th edition. Prentice Hall
6. Stanier, R.Y., Ingraham, J.L., Wheelis, M.L., and Painter, P.R. (2005). General Microbiology. 5th edition. McMillan.
7. Atlas, R.M. (1997). Principles of Microbiology. 2nd edition. WM.T.Brown Publishers.
8. Cappucino, J., and Sherman, N. (2010). Microbiology: A Laboratory Manual.9th edition Pearson Education Limited
9. Srivastava, S., and Srivastava, P.S. (2003). Understanding Bacteria. Kluwer Academic Publishers, Dordrecht.

Experiment No – 1 PREPARATION OF DIFFERENT CULTURE MEDIA

Aim: To prepare the given media for bacterial culture.

Introduction

Several types of media are available to grow bacteria. No matter what microbe a person is trying to culture, microbes (like all other organisms) require nutrients and energy for growth.

Media for bacteria differ by

1. Chemical composition

a.) defined (synthetic) media contains known chemical constituents to permit knowing exactly what is being metabolized

b.) complex media has some unknown chemical constituents, which is sometimes necessary since the exact requirements are unknown, restricting the proper preparation of a defined medium; often complex media contains digested proteins (peptones) isolated from meat, gelatin, or soy to serve as a carbon, nitrogen, & energy source for the microbe.

2. Physical nature

a.) liquid (broth); often prepared in Erlenmeyer flasks or test tubes

b.) semisolid; contains a lower amount of agar (see below) than solid media

c.) solid; great for bacterial isolation from a mixture of organisms, this type of media requires agar, which has been extracted from red algae.

Agar has useful properties such as melting at 90°C. Once it has melted it can be held in suspension at temperatures around 45°C (cool to the touch). This allows pouring media into Petri dishes and letting the media cool and solidify. Perhaps two other qualities that are important are the inability of microbes to utilize the carbohydrate as an energy source and the flexibility that it offers in regards to the wide range of temperatures at which it can be incubated.

3.) Function

a.) general-purpose media (supportive media)- includes tryptic soy agar/broth (TSA/TSB) and can support a wide range of microbes

b.) selective media- favors growth of one microbe over another microbe; includes eosin methylene blue (EMB), which inhibits Gram positive organisms

c.) differential media- differentiates among various groups of microbes; includes phenol red broth (PRB), which (after addition of specific sugars) differentiates among microbes that can ferment particular sugars.

d.) enriched media- supportive media with added nutrients; blood agar is a great example of enriched media

Materials Required

• BG-11, Nutrient agar, Mac Conkey agar, EMB agar other microbiological media (dry powder).

• Clean 500 ml Erlenmeyer flask

• Deionized (DI) water (H_2O)

• Aluminum foil and autoclave (indicator) tape

• Autoclave

Procedure

• Properly and accurately weigh the necessary amount of media. Add water until the total volume (media + water) equals the target amount of media. Gently mix by swirling.

• Cover the non sterile media with aluminum foil and a small piece of autoclave tape. Place in the autoclave to sterilize. The instructor will provide proper instruction on how to operate the autoclave.

• When the media is finished (the autoclave is finished) remove it using the orange autoclave gloves. Place the media on the bench top and allow it to cool until it is comfortable enough touch (~45 °C) with bare hands.

• Carefully pour the media into Petri dishes, allowing the lid to partially cover the media. When the media has solidified, cover the Petri dish.

Observations and Result

Experiment No – 2 **MICROMETRY**

Aim: To measure the size of the given microbial culture using Micrometry scale method.

Principle

Micrometry is a microscopic procedure used to measure the size of the microorganisms. It uses two types of scales namely Ocular scale and Stage scale. With an ocular micrometer properly installed in the eyepiece of your microscope, it is a simple matter to measure the size of microorganisms that are seen in the microscopic field. An **ocular micrometer** consists of a circular disk of glass that has graduations engraved on its upper surface. The distance between the lines of an ocular micrometer is an arbitrary value that has meaning only if the ocular micrometer is calibrated for the objective that is being used.

A **stage micrometer**, also known as an *objective micrometer*, has lines scribed on it that are exactly 0.01 mm (10 µm) apart. To calibrate the ocular micrometer for a given objective, it is necessary to superimpose the two scales and determine how many of the ocular graduations coincide with one graduation on the scale of the stage micrometer. As shown in the figure 2.1 two scales appear when they are properly aligned in the microscopic field. In this case, seven ocular divisions match up with one stage micrometer division of 0.01 mm to give an ocular value of 0.01/7, or 0.00143 mm. Since there are 1000 micrometers in 1 millimeter, these divisions are 1.43 µm apart. With this information known, the stage micrometer is replaced with a slide of organisms to be measured.

To determine the size of an organism, then, it is a simple matter to count the graduations and multiply this number by the known distance between the graduations. When calibrating the objectives of a microscope, proceed as follows.

Materials Required

ocular micrometer or eyepiece that contains a micrometer disk, stage micrometer.

Procedure

1. If eyepieces are available that contain ocular micrometers, replace the eyepiece in your microscope with one of them. If it is necessary to insert an ocular micrometer in your eyepiece, find out from your instructor whether it is to be inserted below the bottom lens or placed between the two lenses within the eyepiece. In either case, great care must be taken to avoid dropping the eyepiece or reassembling the lenses incorrectly.

Only with your instructor's prior approval shall eyepieces be disassembled. Be sure that the graduations are on the upper surface of the glass disk.

2. Place the stage micrometer on the stage and center it exactly over the light source.

3. With the low-power (10X) objective in position, bring the graduations of the stage micrometer into focus, *using the coarse adjustment knob. Reduce the lighting.*

Note: If the microscope has an automatic stop, do not use it as you normally would for regular microscope slides. The stage micrometer slide is too thick to allow it to function properly.

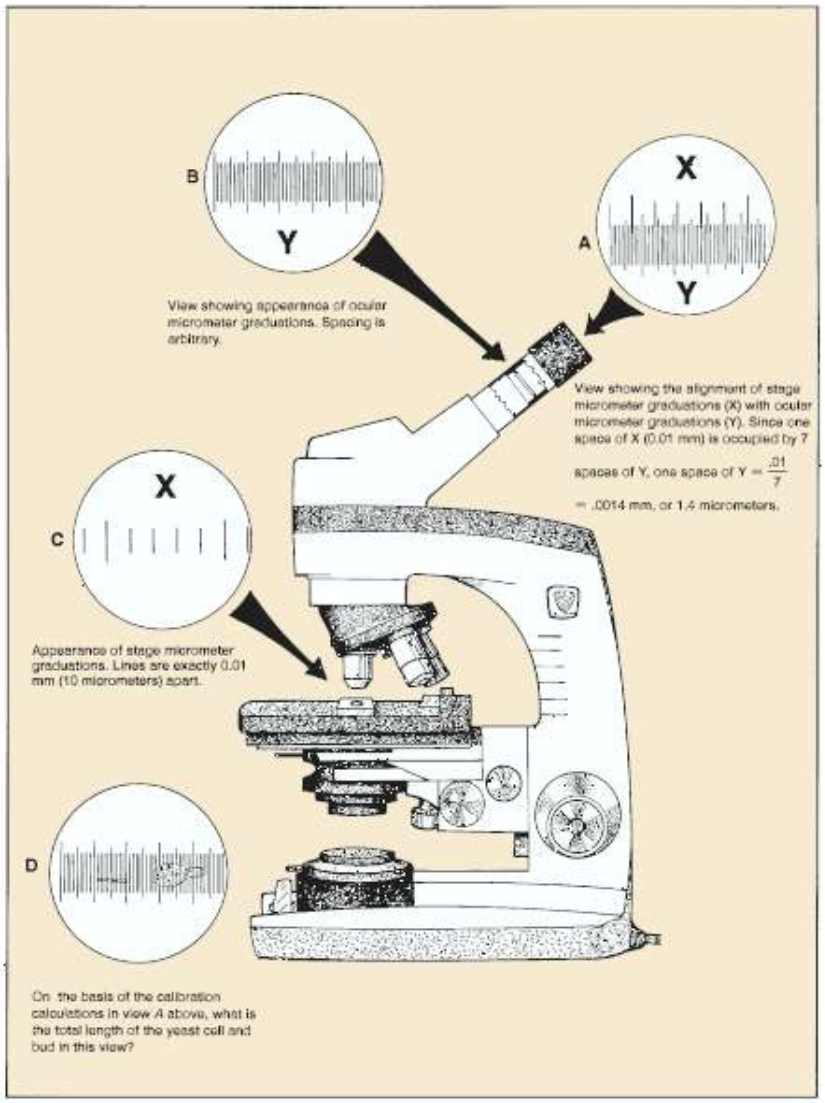

Figure 2.1 Micrometry scale measurement.

4. Rotate the eyepiece until the graduations of the ocular micrometer lie parallel to the lines of the stage micrometer.

5. If the low-power objective is the objective to be calibrated, proceed to step 8.

6. If the high-dry objective is to be calibrated, swing it into position and proceed to step 8.

7. If the oil immersion lens is to be calibrated, place a drop of immersion oil on the stage micrometer, swing the oil immersion lens into position, and bring the lines into focus; then, proceed to the next step.

8. Move the stage micrometer laterally until the lines at one end coincide. Then look for another line on the ocular micrometer that coincides *exactly* with one on the stage micrometer. Occasionally one stage micrometer division will include an even number of ocular divisions, as shown in illustration A. In most instances, however, several stage graduations will be involved. In this case, divide the number of stage micrometer divisions by the number of ocular divisions that coincide. The figure you get will be that part of a stage micrometer division that is seen in an ocular division. This value must then be multiplied by 0.01 mm to get the amount of each ocular division.

Example: 3 divisions of the stage micrometer line up with 20 divisions of the ocular micro-meter.

Each ocular division = 0.0015 mm

= 1.5 μm

9. Replace the stage micrometer with slides of organisms to be measured.

Observations and Result

Experiment No – 3 MOTILITY BY HANGING DROP METHOD

Aim: To determine the motility of the given bacterial culture using hanging drop method.

Principle

If it is necessary to study viable organisms on a microscope slide for a longer period of time than is possible with a wet mount, one can resort to a hanging drop slide. As shown in figure 3.1, organisms are observed in a drop that is suspended under a cover glass in a concave depression slide. Since the drop lies within an enclosed glass chamber, drying out occurs very slowly.

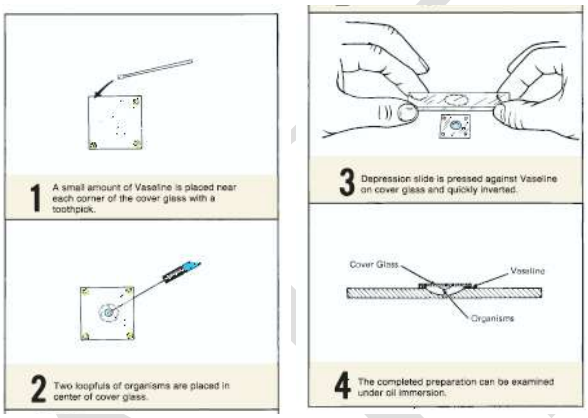

Figure 31. Preparation of hanging drop slide

Materials Required

cover glasses, depression slide, nutrient broth cultures of *Micrococcus luteus*, and *Proteus vulgaris* (young cultures), inoculating loop, Bunsen burner.

Hanging Drop Slides By referring to figure 3.1 prepare hanging drop slides of each organism. Be sure to use clean cover glasses and label each slide with a china marking pencil. When placing loopfuls of organisms on the cover glass, be sure to flame the loop between applications. Once the slide is placed on the microscope stage, do as follows:

1. Examine the slide first with the low-power objective. If your microscope is equipped with an automatic stop, avoid using the stop; instead, use the coarse adjustment knob for bringing the image into focus. The greater thickness of the depression slide prevents one from being able to focus at the stop point.

2. Once the image is visible under low power, swing the high-dry objective into position and readjust the lighting. Since most bacteria are drawn to the edge of the drop by surface tension, **focus near the edge of the drop.**

3. If your microscope has phase-contrast optics, switch to high-dry phase. Although a hanging drop does not provide the shallow field desired for phase-contrast, you may find that it works fairly well.

4. If you wish to use oil immersion, simply rotate the high-dry objective out of position, add immersion oil to the cover glass, and swing the oil immersion lens into position.

5. Avoid delay in using this setup. Water of condensation may develop to decrease clarity and the organisms become less motile with time.

Observations and Results

Experiment No – 4 SIMPLE STAINING

Aim: To stain the given bacterial culture by simple staining.

Introduction

Stains attach to something because of charge differences between the object and the stain. Different stains can appear as a different color because they contain different chromophore groups, which vary in the wavelength of light they absorb. In general there are two main stain types. Positively charged stains have a positive chromophore. The second type, negatively charged stains, has a chromophore that carries a negative charge. Positively charged stains are excellent in binding negatively charged structures such as bacterial cell walls and, if they can enter the cell, many macromolecular structures such as DNA and proteins.

Cationic (basic) stains have a positive charge associated with them while anionic (acidic) stains carry a negative charge. Examples of cationic stains include crystal violet, safranin, basic fuschin, & methylene blue. Examples of anionic stains include eosin, nigrosin, & congo red. Acid dyes are often used to stain the slide background, which leaves the microbe transparent. Thus, in the field of view the microbe will appear as clear dots against an opaque background. Stains require a short exposure time to their target followed by a brief, light rinse with deionized (DI) water. This removes any excess stain and allows better viewing of the cells that carry the stain.

Materials Required

Safranin or crystal violet, Overnight bacterial cultures of *S. aureus* and *B. megaterium*, Glass slide, Nichrome loop.

Procedure

- Place a loop-full of bacteria from an overnight culture onto the center of a glass slide and prepare a smear.
- Heat fix cells by placing the slide on top of the Bunsen burner until it appears dry use a forceps to handle the slide.
- Add a drop of the stain to the smear and allow the same for 30 sec.
- Wash off the excess stain under tap water, blot dry and observed under microscope.

Observations and Results

Experiment No – 5 **NEGATIVE STAINING**

Aim: To determine the presence of capsule in the given bacterial culture by using negative staining.

Principle

Several types of stains can be performed on bacteria to determine their cellular makeup. The negative stain relies on using nigrosin, which leaves nonheat-fixed cells colorless against a dark background. There are several advantages to using a negative stain compared to a simple stain where cells are heat-fixed. One of the major advantages is that cells are more easily viewed in their "natural" state. This sometimes gives a better view of cell size and their morphological arrangement. For example, spirochetes will often lose their shape when heat fixed. Additionally, when cells are heat-fixed, they can be susceptible to lysing or damage, which leaves them difficult to view. For this reason, cells that are very delicate can be viewed much better using a negative stain. The acidic stain nigrosin is used to leave the cells transparent, since cells are surrounded by negative charges (following the old adage, "opposites attract"). In the negative stain, the acidic dye is repelled by the cellular charges and leaves the cell unstained.

Materials Required

Overnight cultures of *Serratia marcescens*,, Two glass slides, Nigrosin.

Methods:

- Place a drop of nigrosin to the left side of the slide.
- Place a loop-full of bacteria and mix with the nigrosin.
- Using the second slide, touch and drag the nigrosin across the first slide as shown in figure 5.1.
- Allow the slide to air dry and observe under microscope.

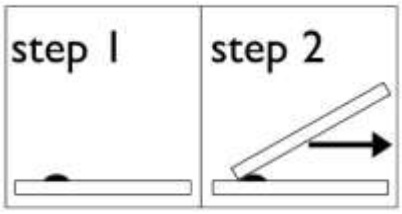

Figure 5.1 Negative staining of bacterial sample

Observations and Results

Experiment No – 6 **GRAM STAINING**

Aim: To differentiate the given bacterial culture into gram variants using gram staining.

Principle

One of the most important stains performed by both the fledgling microbiologist and professional microbiologist is the Gram stain. This stain is named after Hans Christian Gram who was the first to implement the technique. Although Hans did not know it at the time, the Gram stain allows differentiation between Gram negatives and Gram positives. This is largely due to the structure of the cell wall and the presence or absence of an outer membrane that occurs in Gram negative bacteria. After heat-fixing a loop-full of overnight culture, the cells are ready to be stained. First a primary stain, called crystal violet, is used as a primary stain. This cationic stain will adhere to all organisms, since cells carry an overall negative charge. After rinsing, a mordant is applied called Gram's iodine, which promotes retention of the primary stain.The second rinse is performed with EtOH. The EtOH functions to do several things one of which is to shrink the pores in the peptidoglycan layer. This traps the crystal violet-iodine complex in the Gram-positive cell. The larger pores and thinner peptidoglycan in Gram-negative organisms is not changed to such an extent compared to the Gram-positive organisms. However, the EtOH also removes the outer membrane of the Gram-negative organisms. In essence, the EtOH rinse effectively leaves the Gram-negative cells colorless. While the Gram positives are stained purple, a counter stain is applied to stain the colorless Gram negatives. These will appear reddish in color.

Materials Required

Overnight cultures of *S. aureus*, *E. coli*, and an unknown, Crystal violet, Gram's iodine, Safranin, distilled water, 95 % Ethanol.

Procedure.

• On three different areas of the glass slide place *S. aureus*, the unknown, and *E. coli*. Do not add too many cells.

• Heat fix cells to glass slide.

• Add enough crystal violet to cover the specimens and wait ~1 minute.

• Wash with distilled water.

• Add Gram's Iodine and wait ~1 minute.

• Add a few drops of 95 % Ethanol and tilt the slide onto the collection pan. Rinse.

• Counterstain with safranin for ~1 minute, rinse, and blot dry.

Observations and Results

Experiment – 7 ACID-FAST STAINING (Ziehl-Nelssen Stain)

Aim: To identify the acid fast bacilli in the given sample using acid fast staining.

Principle

While there are definitive Gram-positive and Gram-negative bacteria, there are some bacteria that do not give good Gram stain results. These bacteria are sometimes *acid-fast bacteria*. This is primarily due to the presence of additional materials, called mycolic acids, in their cell walls that disrupt the expected function (and results) of the Gram stain reagents. To properly identify acid-fast bacteria, a special staining procedure is used.

First, a loop-full of bacteria is placed on the slide and heat fixed. The slide is then placed over a heat source and carbol fuschin is added. A small piece of paper towel is placed over the specimen as well. The heat helps drive the carbol fuschin into the cell while the paper towel helps retain moist conditions. The slide is removed, rinsed, and decolorized using acid-alcohol. Methylene blue is used as a counter stain. After the carbol fuschin step, all bacteria are purplish in color. After the acid-alcohol rinse, only acid-fast bacteria will retain the carbol fuschin. Non acid-fast bacteria will be colorless. However, this change after the methylene blue is added. *Mycobacterium* is a genus that has acid-fast members. Some *Mycobacterium* species that cause serious disease in humans include *M. leprae* and *M. tuberculosis*. A final word of caution should be given when viewing acid-fast specimens. Similar to the Gram-positive or negative species, some acid-fast species show inconsistent stain results.

Materials Required

Overnight cultures of *E. coli*, *M. smegmatis*, Steam bath, Carbol fuschin, Acid-alcohol, Methylene blue.

Procedure

• Heat fix *E. coli and Mycobacterium* cells to respective glass slide.

• Place the slide over a boiling water bath

• After 4-5 minutes, remove the slide

• Rinse with acid-alcohol until the slide remains light pink

• Rinse with water for a few seconds

• Use methylene blue to counter stain and wait about 2 minutes

• Rinse and blot dry

Observations and Results

Experiment No – 7 CAPSULE STAINING

Aim: To determine the presence of capsule in the given bacterial sample.

Principle

Some microbes produce an outer, sticky matrix consisting primarily polysaccharide. Bacterial capsules confer an advantage in attachment to surfaces and evading immune systems. For microbiologists wanting to view the capsule under a compound light microscope, a simple stain cannot be used since much of the time stains will not adhere to the capsule due to its chemical nature. Additionally, some stains will shrink away from the bacterial cells when drying, giving the false impression that a capsule is present. To properly stain a cell with a capsule, two stains are used. The first stain, crystal violet, targets the cell. However, the capsule itself is not stained due to its nonionic nature. A 20% copper sulfate solution can then be used to rinse the crystal violet off and will leave the bacteria looking purple with a faint but noticeable halo. Alternatively, a smear can be made using a loop full of bacteria and small amount of India ink. The smear is air dried and rinsed. Next, crystal violet is added and allowed to sit for about one minute. This stains the cells purple. The slide can be rinsed and blotted with bibulous paper.

Materials Required

Crystal violet, India ink, overnight cultures of *Klebsiella pneumoniae* and *Micrococcus luteus.*

Procedure

• Put a drop on India ink on one side of the slide

• Using sterile technique, place a loop full of bacteria with the India ink and smear

• Air dry for 30 seconds

• Add crystal violet and wait ~1 minute

• Rinse and blot dry using bibulous paper

Observations and Result

Experiment No – 8 ENDOSPORE STAINING (Shaeffer-Futton Stain)

Aim: To determine the presence of endospore in the given bacterial sample using endospore staining.

Principle

Bacteria are ubiquitous; however, conditions that are conducive for rapid growth are not always present. Under harsh conditions, such as extreme temperatures, some bacteria can produce a spore within their cells, aptly called an endospore. The endospore is a survival mechanism that can persist under extreme conditions. Several interesting studies have been done to determine the endospore wall composition. Equally interesting are the different phases of endospore development. The vegetative cell expresses distinct sets of genes to produce the endospore in a process called sporogenesis. Upon completion of the endospore the vegetative cell can die, leaving a free spore (Figure 8.1.). Free spores are extremely hardy and can survive boiling and intense radiation. When conditions conducive for growth return, the spore will germinate (germination) and a new vegetative cell results. The vegetative cell can undergo binary fission and result in several more bacteria. If harsh growing conditions return, an endospore is generated again. The cycle can continue over and over.

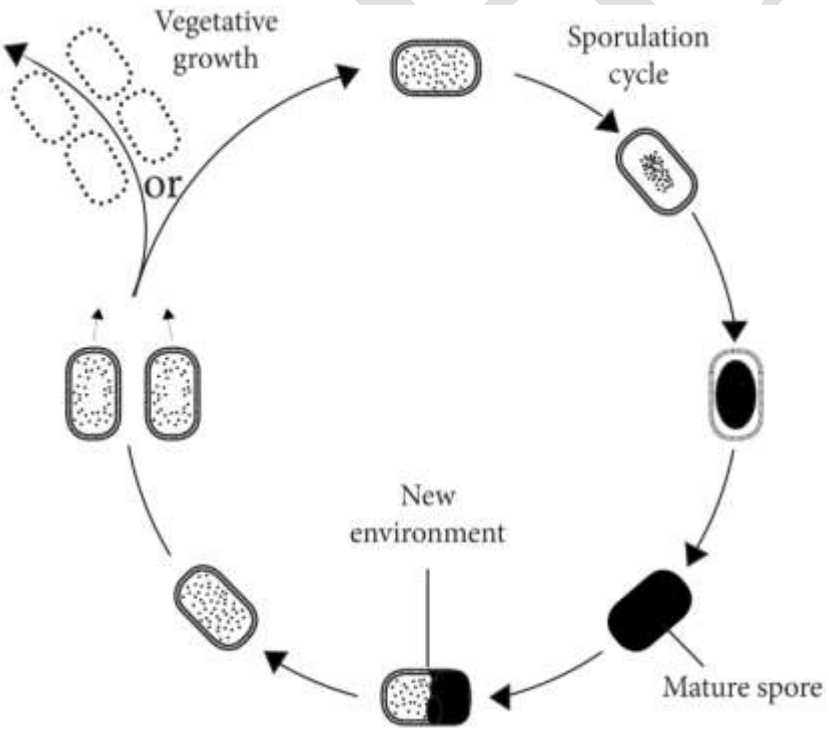

Figure 8.1. Sporulation cycle. Endospores exposed to environmental conditions conducive for growth lead to upregulation of genes responsible for metabolic pathways and vegetative growth (Willey *et al.,* 2011).

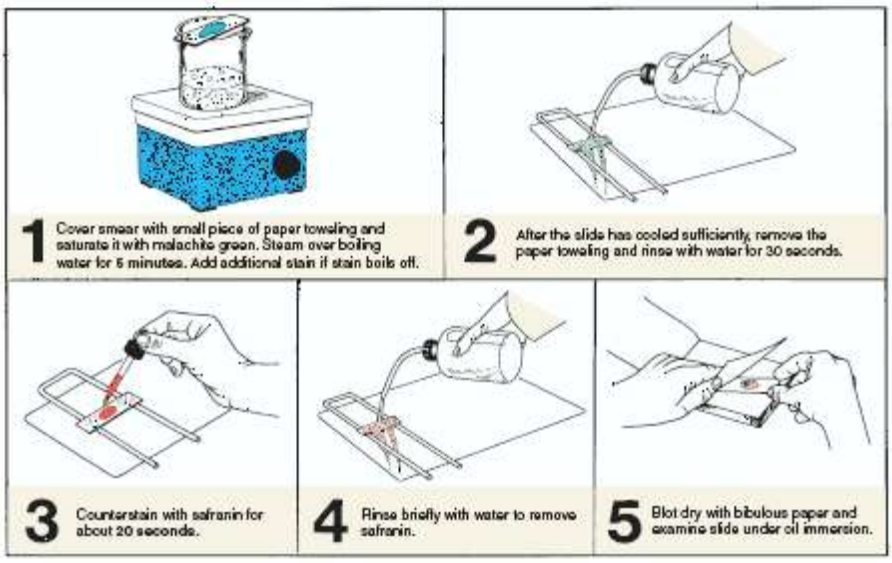

Figure 8.2 Endospore staining procedure

Two genera of bacteria that have medical importance are *Clostridium* and *Bacillus*. *Clostridium tetani* produces an endotoxin that causes tetanus. *C. perfringens* and *C. defficile* can cause gas gangrene and pseudomembranous colitis, respectively.

Materials Required

Overnight culture of *Bacillus megaterium*, Hot water bath, Malachite green, Safranin

Procedure

• Place a loop full of *B. megaterium* on a glass slide.

• Heat-fix the specimen.

• Place the slide over the boiling water bath.

• Cover the specimen with a small piece of paper towel.

• Apply malachite green.

• Allow the heat to carry the malachite green into the endospore for 6-7 minutes. Be sure to reapply malachite green if it the towel becomes dry.

• Rinse the slide with water and apply safranin as the counterstain.

• After 1 or 2 minutes, rinse and blot dry.

Observations and Result

Experiment – 10 ISOLATION OF PURE CULTURE OF BACTERIA BY STREAKING METHOD

Aim

To aseptically culture and isolate bacterial culture using streak plate method.

Principle

Bacteria are ubiquitous and can be found growing on almost any substrate available. Although only ~1% of bacteria are able to be grown in a laboratorysetting, several species of bacteria can easily be cultured. An important component of culturing bacteria involves isolating a single species from a mixed culture, where several species might be present. Agar plates with general purpose media offer a great way to sample bacteria from the environment. Care must be taken to provide as direct method as possible when transferring a swab sample to media for culturing so as not to introduce bacteria from unwanted sources. For example, a person needs a sterile (wet) swab to sample a (dry) surface. Exposure time of air to that of the inside of the Petri dish should also be minimized. When maintaining cell lines, microbiologists often work in a laminar flow hood.

This apparatus is designed to minimize the introduction of contamination into a pure culture or to assist in isolating one species from a mixture of species. It relies on a high efficiency particulate air (HEPA) filter and airflow. If the goal is to obtain a pure culture, a streak plate is made. Streaking for isolation often uses agar plates or slant tubes. Agar plates are a great way for isolating bacteria but they are also often used for calculating the number of bacteria per volume of substance. Slant tubes have the benefit of minimizing workspace and can be stored longer than Petri plates, since they often minimize the rate of dehydration.

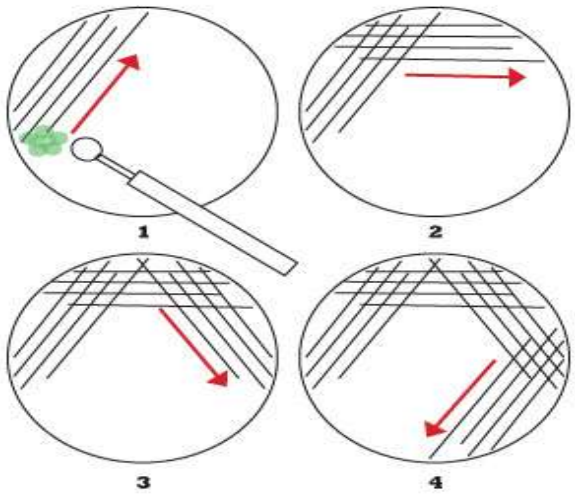

Figure 10.1. Quadrant streak method for isolating microbes (Cappuccino and Sherman, 2004).

Materials Required

Petri dishes with general purpose media (e.g. TSA), Sterile swab, Sterile water, Overnight culture in broth tube.

Procedure

- Swab something of your choice and streak the sample onto the media. If it is a dry surface, moisten the swab before streaking the media. It might help to slowly rotate the swab.
- Use a quadrant streak (Fig. 1) to obtain a pure culture of the bacteria in the test tube.
- Begin by first sterilizing a loop in the Bunsen burner. Remove the loop and let cool briefly. Remove a loop of bacteria from the test tube and place this sample on a small sector of the Petri dish.
- Transfer the bacteria aseptically, that is, without contaminating the original culture.
- Sterilize the loop again to kill everything. Streak the end of the previous sample on the plate in a manner that drags some of the microbes to a new sector of the Petri dish.
- Again, sterilize the loop and return to the plate to streak again. After performing the quadrant streak, incubate both plates at 37°C.

Observations and Results

Experiment No – 11 PRESERVATION OF BACTERIAL CULTURES BY VARIOUS TECHNIQUES - AGAR SLANTS AND DEEPS - MINERAL OIL, GLYCEROL STOCKS.

Aim: To preserve the given bacterial culture using agar slants, deeps, mineral oil and glycerol.

Principle

Preservation of bacterial cultures become indispensible owing to its long term storage and routine use. Use of mineral oil is one such method of preserving bacterial culture

Reagents/equipment

1. Sterile (autoclaved) 50% glycerol solution in Aqua dest. Note, glycerol is rather viscous, so pour the stock glycerol directly into a bottle and estimate the volume with your eye along the volume scale. Add Aqua dest. Prior to aliquoting the 50 % glycerol solution, add a magnetic bar and heat the solution on a magnetic stirrer. After heating, the solution can be easily pipetted and aliquoted into cryo tubes with screw caps. For example, aliquot 300 -l glycerol solution into 2 ml tubes.

Autoclave.

2. Freshly grown cells:

a. If clones, on LB medium with antibiotics (liquid or plates)

b. If strains, on appropriate medium (liquid or plates).

3. Other items: Trays, pipettes, sterile pipette tips, freezer.

Procedure

- Ensure that your cells have grown up well and are in exponential growth phase prior to harvest.

Options for harvest:

If on agar plates, scrape of biomass with a sterile inoculation loop and dissolve in a

sterile liquid medium (the same as used for the agar plates or similar). Vortex and pipette aseptically into sterile cryo vials with 50 % glycerol so that the end concentration reaches between 10-15 %. For example, for 2 ml cryotubes with 300.

50 % glycerol solution, add 700 -l liquid sample.

Vortex.

If in liquid solution, vortex carefully your sample, and then simply aliquot an appropriate amount to reach an end concentration of 10-15 %. For example, for 2 ml cryotubes with 300 -l 50 % glycerol solution add 700 -l liquid sample. Vortex Vortex and place at -80 °C (no need to freeze in liquid N2).

Observations and Result

Experiment – 12 ENUMERATION OF MICROBES BY SPREAD PLATE AND POUR PLATE METHOD

Aim: To enumerate the microbes in the given sample using spread plate and pour plate method.

Principle

Determining the number of microbes in a sample is important for several reasons. It is useful to know their numbers in river systems, healthy soil compared to contaminated soil, agricultural fields, areas in oceans, and food samples. In one gram of soil, it is estimated that 1 x 108- 1 x 109 bacteria are present and only ~1% of these can be cultured in a lab. Some estimates suggest that there are about 25,000 species of bacteria in one gram of soil. For soil samples, a dilution is made and the number of microbes present is estimated by working backwards mathematically. Although there are ways to determine the number of all microbial cells in a sample (direct count methods), this lab will focus on how to determine the number of living (viable) cells are in a sample.

The viable count (or plate count) method counts only cells that can grow when cultured. Two types of viable count methods are

1. pour plate method

2. spread plate method

Both of these methods require dilutions. It is based on the fact that individual cells will grow into colonies. Using this method, there is no way to confirm one cell gave rise to one colony since there is a chance that two cells could start close on the Petri dish, then merge and appear as one colony. Instead of bacterial cells, bacterial colonies are counted and the term colony forming unit (CFU) is used (Figure 12.1).

Samples with 30-300 colonies often give accurate counting results-outside of this range CFUs become increasingly difficult to count (if too many) or the numbers are too low to be reliable (if too few). The dilution technique begins with measuring a soil sample and adding water. Although the example below shows one gram of soil, more or less can be used as a starting point, but this will, of course, change the method to determine CFUs. For simplicity, we will use one gram of soil dissolved in 9 ml of water. To determine CFUs/ml multiply the number of colonies on the plate after incubation by the reciprocal of the dilution sample. Also, be sure to account for the amount of suspension plated.

Materials Required

Soil sample, Four test tubes with 9 ml of H2O, Plastic transfer pipette, T200 pipette, Four nutrient agar plates, sterile L-shaped glass spread rod (L-rod).

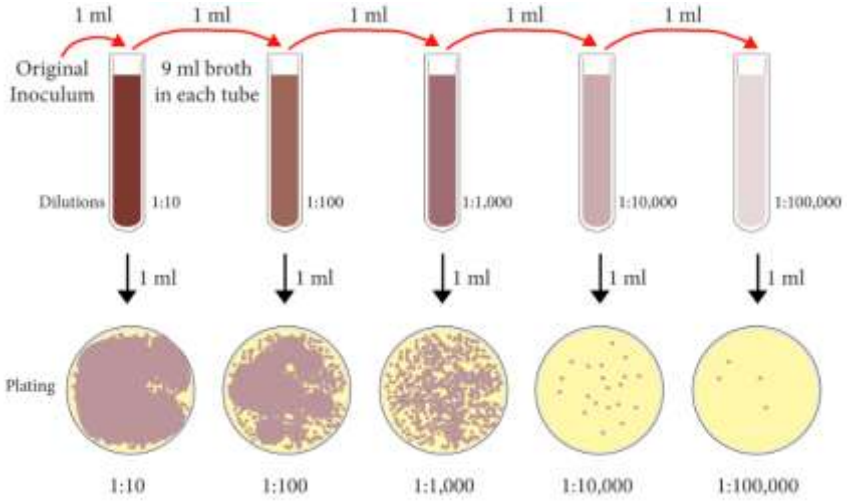

Figure 12.1. Serial dilution and plating of soil sample. In the above image the dilution uses water. However, dilutions can also use molten agar and is subsequently called the pour plate method.

Procedure

- Prepare test tubes for a serial dilution so that each test tube has 9 ml of water.
- Carefully weigh 1 gram of soil sample and place into a glass test tube.
- Cover with parafilm and shake vigorously for 1 minute. Be sure all soil is dissolved.
- Use 1 ml of the dissolved soil sample and transfer it to 9 ml of water. Continue the dilution, taking careful note of each dilution.
- Plate 0.1 ml of each of the last 3 dilutions onto nutrient agar plates and use the L-rod to spread the sample evenly.
- Similarly plate 1 ml of the sample from last 3 dilutions over suspended with nutrient agar under spread plate.

Observations and Results

Possible Viva Questions

1. Define Glycocalyx
2. Comment on endospore staining.
3. Discuss on the enumeration of microbes using colony count and turbidimetric method.
4. Write any two ecological significance of the archae bacteria.
5. What is meant by thermophiles?.
6. What are cyanobacteria?
7. Define halophile
8. What are methanogens?
9. What is meant by biological cycle?
10. How are archae bacteria classified? Explain.
11. Write a brief note on halophiles
12. Write in detail about the general characteristics about archae.
13. Write short notes on barophiles and pshyrophiles.
14. Explain in detail about cultivation of anaerobic bacteria.
15. Define culture media
16. Define growth.
17. Define generation time. How are bacterial growth classified?
18. Define simple and complex media.
19. Describe about turbidostat and chemostat.
20. Write short notes on factors affecting microbial growth.
21. Distinguish between moist and dry heat sterilization with apt examples.
22. Define protoplasts and sphaeroplasts.
23. Draw a neat diagram on the structure of flagella.
24. Distinguish between prokaryotes and eukaryotes.
25. Comment on the archae bacterial cell wall.
26. Discuss about the structure of the gram positive cell wall.
27. Describe the stages involved in the formation of endospore.
28. Discuss on the role of endospore in the bacterial sustainability.
29. Comment on the action of penicillin on the bacterial cell wall.
30. Discuss the structure and function of pili.
31. Write a brief note on the capsule stain.
32. Explain the principle behind the acid fast stain.
33. Write short notes on Gram stain.
34. Explain about the principle behind pure culture techniques.
35. Define Stain.
36. What is meant by mordant?
37. Distinguish between eubacteria and archae bacteria.
38. Write short notes on the concept of species, taxa, and strain.
39. What is meant by 16S rRNA gene sequencing.
40. Comment on the mechanism of gene sequencing and ribotyping.

41. Discuss in detail about the signature sequences.
42. Discuss in detail about reverse sequencing methods.
43. What is meant by evolutionary chronometers?.
44. Comment on acidic and basic dyes.
45. Write short notes on the use of chemical methods for controlling microbes.
46. Write short notes bacterial motility and cell count techniques.
47. Write short notes on calculation of specific growth rate.
48. Define taxonomy.
49. Define genus and species
50. Write any two points about principle of taxonomy.
51. What is meant by monophasic and polyphasic taxonomy.
52. Discuss on RAPD.
53. Write about the archae bacterial cell wall.
54. How are cyanobacteria cultured in lab?
55. Explain in detail about the role of phytoplankton in microbial kingdom.