Aquatic Resources Management

ARM 103 1.0 Aquatic Microbiology and Water Quality

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AQS 103 10 Aquatic Microbiology and Water Quality

Microbiological characteristics

Clean water is not a suitable substrate for the growth of microorganisms. The microbial growth in water can be aggravated when water is contaminated with organic matter as it provides food for the microbes. Waste water usually contains a high level of microorganisms and these microbes can find their way into surface and ground water. Therefore care should be taken to make drinking water free of pathogenic microbes.

Microbiological analysis is used to determine he diversity and density of the microbes available in natural and waste waters. Such analysis includes determining the total number of microbes which indicate the source of pollution and the microbiological characteristics of the microorganisms.

Types of microorganisms that can be found in natural and waste waters.

Type	Status
Bacteria	Common
Viruses	Rare
Yeasts	Rare
Moulds	Rare
Algae	Common
Protozoa	Common

Microorganism

Bacteria are the most important microorganism in water microbiology. Analysis of water for viruses is time consuming and often troubles some and complicated. Therefore determinations of viruses are not carried out in the usual microbiological analysis. Yeasts and moulds are not very common in surface water and if present they only play a subordinate role in surface water microbiology. They are frequently detected together with bacteria when analysis is carried out with culture media.

Algae can also be considered as a group of microbes living in water. However their diversity and density can easily be detected under light microscope.

General requirement for Microbiological work

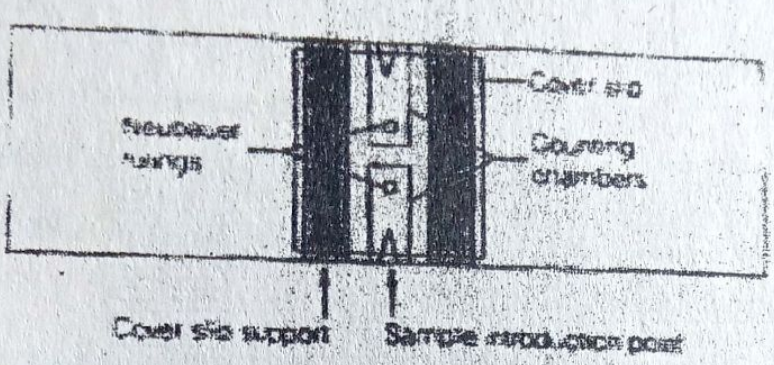
Analysis of pathogenic microbes must be carried out only by experts having appropriate special knowledge, observing the necessary precautions. Analysis of non pathogenic microbes also required trained personnel and facilities that are dedicated for microbiological works.

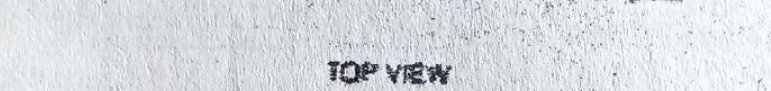
Steps to be taken for microbiological work

1. Glass ware and equipment must be carefully cleaned and sterilized each time before they are used.
2. All apparatus must be mechanically cleaned using cleaning agents rinsing first with clean tap water, them with 1% HCl acid and finally with distilled or demineralized water.
3. Glassware and equipment must be autoclaved at 120°C for 30 minutes before the cleaning process to avoid infection in the process of washing and rinsing.
4. After cleaning the apparatus and glass ware must be first dried, then sterilized for 2 hours at 180°C -200°C in a hot air sterilizer.
5. Culture media and culture solution must be sterilized with a super heated steam in an autoclave at 121°C for 20-30 minutes.

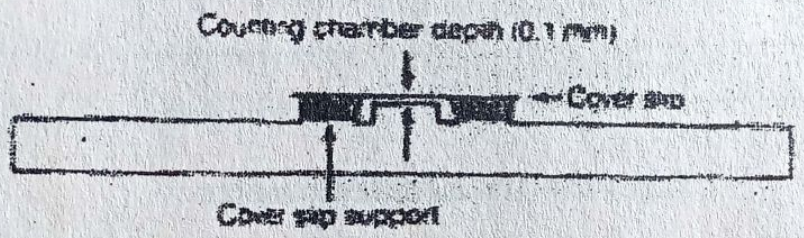
Direct method of counting microorganisms

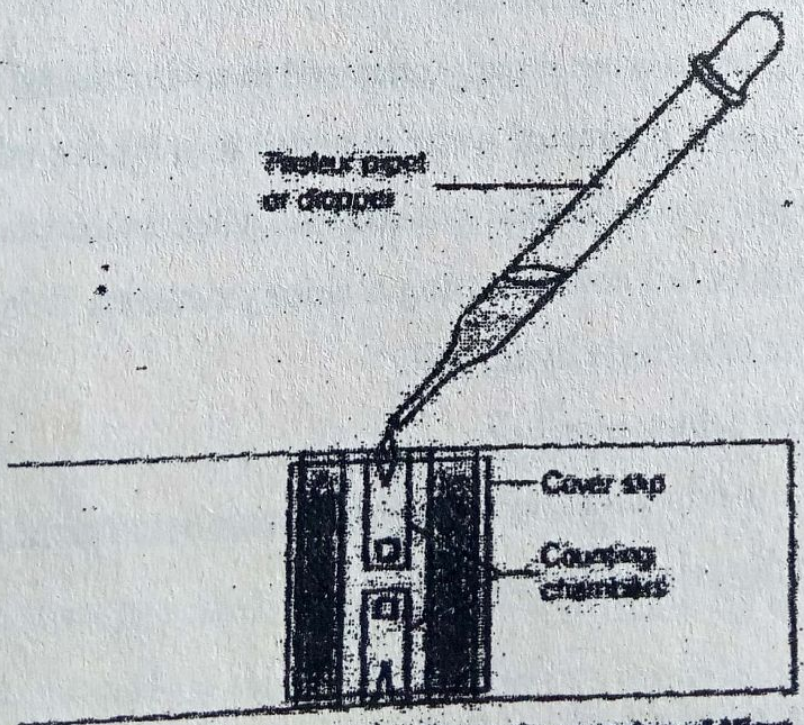
1. A counting chamber can be used to determine the number of bacteria in water.
2. Direct counting can also be performed by filtering a known volume of water through a grid membrane filter paper. The bacteria are dyed on the filter with appropriate dyes the filter paper is illuminated and the counting of microbes can be performed by using light microscope. This method certainly allows the low levels of bacteria when large quantities of water to be concentrated. However the accuracy of this method is very low and it is also impossible to differentiate live and dead bacteria.
3. Direct methods of counting bacteria are not employed in microbiological analysis of water.



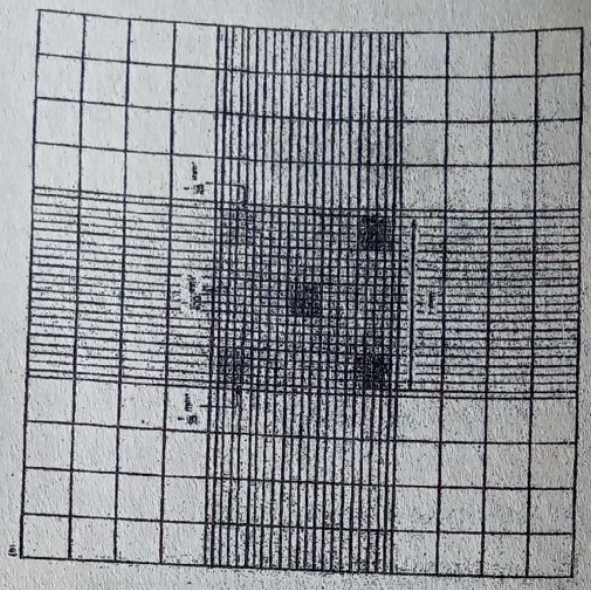


TOP VIEW





...12-2. Procedure for Dispensing a Suspension of Micro-...sms into a Hemocytometer Chamber



Top view of a hemocytometer

Indirect methods of counting microorganisms

Indirect methods of counting bacteria are usually practiced in microbiological analysis of water. In these methods a known volume of water is added to a sterile culture medium which is then incubated under recommended conditions. After incubation period, the colonies which have been formed are counted using a colony counter. It is assumed that each colony is a multiplication of a single living cell.

Standard maximum accepted colony number

Disinfected drinking water the maximum acceptable colony number should be 10 per 100 ml after the completion of the treatment processes.

Indicator microorganisms

Drinking water must not contain living pathogenic bacteria such as *Salmonella, Shigella* and other organisms capable of causing epidemics. The pathogenic bacteria are known as indicator microbes and can hardly be detected in normal cultures. The most important indicator microbes are the intestinal microbes and the presence of these bacteria in surface water is an indication of faecal contamination. The microbes generally indicative of faecal contamination in water are, *E. coli, faecal streptococci* and sulphite reducing anaerobic, spore forming organisms. The coli form bacteria and *Pseudomonas*

aeruginosa are used as indicator organisms to determine whether water is contaminated to a hygienically significant degree.

Since *E. coli* can live for a short time in water occurrence of these microbes in water indicates relatively fresh contamination.

Standard value: in drinking water *E. coli* number must be zero per 1 ml of water.

Coli form bacteria must not be detectable in drinking water.

Sampling

Generally containers used in the sampling of water for microbiological analysis are sterile, glass Stoppard bottles (250-200 ml) wrapped in aluminum foil. The sample should be taken from approximately 30cm below the surface. Any contamination due to handling during this stage should be avoided. After filing the sample bottle should be sealed immediately and the bottle neck should be protected with aluminums foil.

Transportation and Storage

Sample bottle should be transported in insulated boxes without exposing o direct sunlight. Samples should be processed immediately after arrival at the laboratory. However the period of storage (at 4°C) should not exceed 24 hours. If the time interval between sampling and investigation is long, the bacteriological tests have to be carried out *in situ*.

Methods of the Detection and enumeration of Microorganisms

As the number of indicator organisms in water may be very small direct inoculation on solid media is not practicable and other methods should be used by which large volumes can be examined and by which the number of microbes in 100 ml of the sample can be estimated .

1. **Pour plate method**
2. **Multiple test tube**
3. **Membrane filtration**

These techniques can be used to detect and enumerate each indicator organism; however the media and the incubation conditions differing according to the organisms sought.

Practical 1- Detection and enumeration of Microorganisms

A. Pour plate method

The usual method of counting heterotrophic bacteria in water is by pour plate method with yeast extract agar. Separate counts are made of those aerobic microorganisms which form visible colonies in the medium after 24 hours incubation at 37°C and of those which form colonies after 3 days at 20-22°C. The most usual application of the colony count is to detect change, especially sudden change in the microbial content of certain waters.

TOTAL COLONY COUNT Colonies 1 ml^{-1}

Application

The method is applicable to potable and surface water and effluents.

Apparatus

1. Incubator with a thermostat
2. Petri dishes (10 cm diameter)
3. Conical flasks
4. Thermometer (precision 0.1 °C)
5. Pipettes (bacteriological)
6. Gas burner (or spirit lamp)
7. Colony counter
8. Water bath
9. Autoclave

Nutrient Agar (pH = 7.0)

Nutrient broth	13g
Agar	20g

Sterilized distilled water 1L

Procedure

Preparation of samples

Remove the stopper or cap, sterile the mouth of the bottle with a flame, pour off some of the contents, replace the stopper or cap and again shake the bottle in order to distribute any organisms uniformly throughout the water.

Diluents (Ringer's Solution/ saline solution)

Ringer's Solution

Sodium chloride	2.250 g
Potassium chloride	0.105 g
Calcium chloride (anhydrous)	0.120 g
Sodium bicarbonate	0.050 g
Distilled water	1 L

Saline solution:

Sodium chloride	9.0 g
Distilled water	1 L

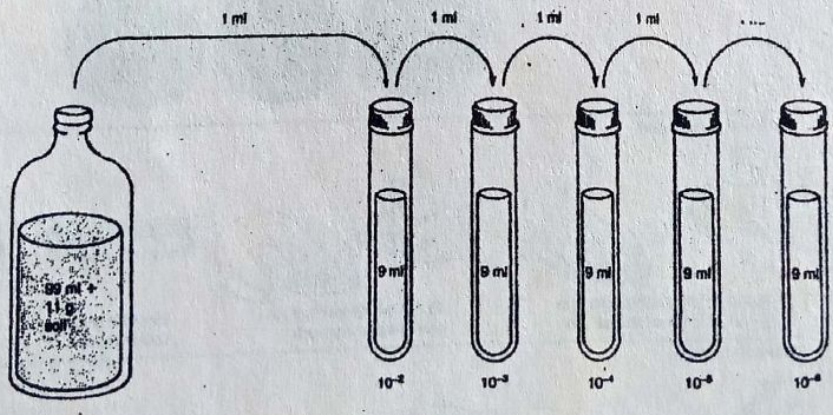
Sterilization: Autoclave at 121 °C for 15 minutes.

Making the dilution

Measure out 9 ml of the diluent into sterile dilution bottles or tubes.

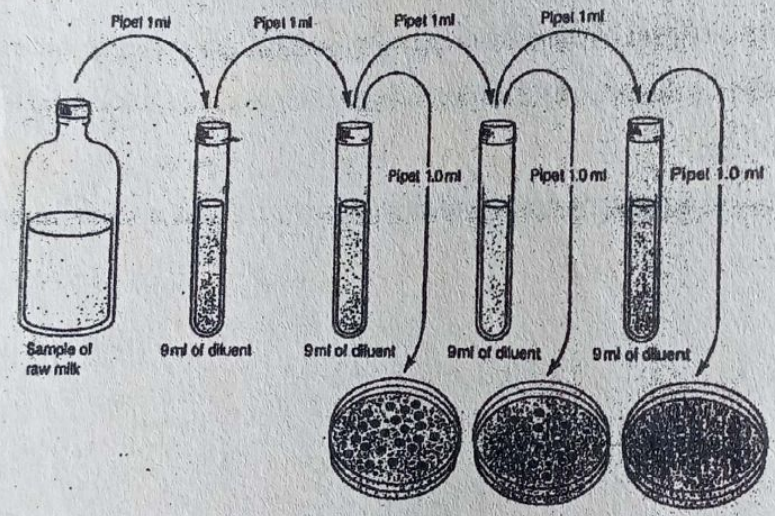
Make one or more ten fold dilutions by transferring one volume of water sample to 9 volumes of diluents.

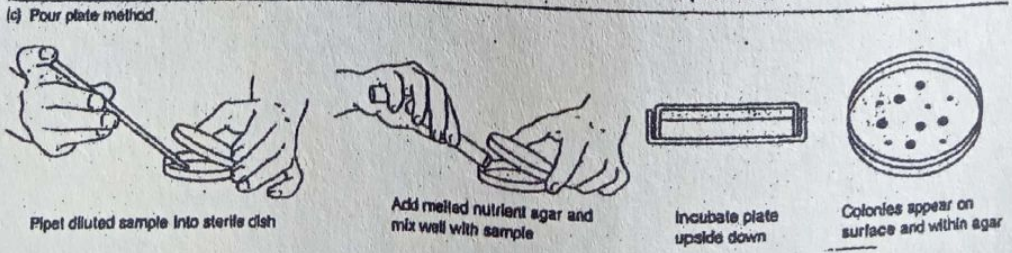
Prepared sufficient amount for each dilution for all the tests to be carried out on the sample.



Preparation of the sample

1. Use the raw sample; make one, two or more ten fold dilutions of it according to the bacteria content expected.
2. Measure1 ml volumes from the highest dilution into each of the two/three Petri dishes and, using a pipette.
3. Pour 15-20 ml of nutrient agar/Yeast-Extract Agar, (previously melted and cool to 45-50 °C) into each Petri dish.
4. Immediately mix the water (1ml) and the agar by rapid but gentle circular movements for 5-10 seconds keeping the Petri dish flat on the bench (Laminar flow) throughout.
5. Allow the agar to set, invert the Petri dishes and place them in the incubator.





Incubation and examination of the cultures

1. Incubate the two plates made with the highest dilution at 20-22 °C for two/three days or at 37°C for 24 ± 3 hours.
2. Count the colonies as soon as the plates are removed from the incubator. If this is not possible, keep the plates at 4°C for not longer than 24 hours.
3. Only the culture plates not exceeding 300 colonies per ml should be used to enumerate the micro-organisms.
4. Indicate the nutrient media used and the duration and the temperature of incubation in the analysis report.
5. Take the average of the counts in each pair of plates and multiply the result by the dilution factor. If the number of colonies exceeds 300 per ml', choose a dilution level in which the number of colonies lies between 30 and 300 and count the colonies in those plates.
6. If the plate made with the highest dilution contains more than 300 colonies per ml, either try to count them and report the results as an approximate or express the count as more than 300 n colonies forming units per ml where n represents the dilution factor.

AQS 103 10 Aquatic Microbiology and Water Quality
Practical NO. 2

Introduction
Isolation of pure culture of bacteria from the environment.

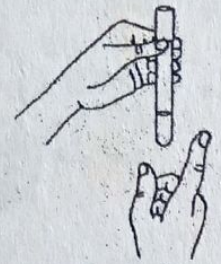
In nature microorganisms exist as mixed populations of many widely differing types. A study of a species can only be done by removing it from the mixed populations, and growing in an environment free from contamination by other living forms, i.e., pure ulture. All cell in the culture with common origin and are simply descendants of the same cell is referred to as pure culture. This involves isolation and purification of cultures.
Methods of isolation will depend on the source from which it is isolated. Purification of cultures is commonly done by methods such as streak plate, pour plate or spread plate. Once a pure culture has been made, it is desirable to maintain the culture in a viable condition for varying periods of time, from a few weeks to years. For short-term preservation and maintenance of cultures, one simply transfers the culture to an agar slant and stores it in a refrigerator. Periodic transfers are then made to fresh agar slants.

Streak plate method
The most widely used technique for the purification of a bacterial culture is the 'streak plate' method. A small amount of growth inoculum is obtained on a sterilised inoculating loop and the loop is then dragged lightly over the surface of the agar in the Petridish. This is done with the hope that the bacteria dropping off from the lop by this action, will finally drop off one at a time. After suitable incubation, single isolated colonies can be observed along the streaked line (Fig.1).

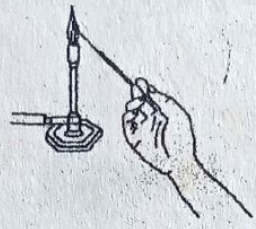
Pour plate method
The pour-plate technique is another method of obtaining pure cultures from a mixed culture of microbes. It differs from the streak plate in that the agar medium is inoculated while it is still liquid (but cool, at about 45°C) and therefore colonies develop throughout the medium and not only on the surface. Better distribution of colonies is obtained in a well-made pour plate and isolations are more easily made.
As there is no accurate way of predicting the numbers of viable cells in a given sample, one should always make several dilutions of the sample and pour several plates. Therefore, the first step in this procedure is to make serial dilutions of the sample. For general purposes serial dilutions up to 10^{-5} or 10^{-6} are made. Practical No. 1.
Thereafter two procedures may be followed.

1) Procedure 1: Pour-plate method- 1ml of each dilution is placed in me centre of an empty sterile petridish and molten agar medium at around 45° C is poured. (Ref. Practical No. 1 schedule for more information)

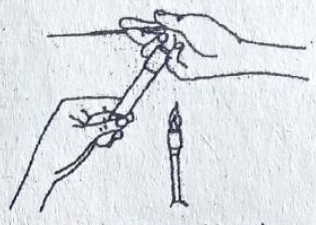
2) Procedure 11: loopful method- Loopful of dilutions are transferred to tubes of molten agar, the inoculum mixed and the mixture poured into separate sterile Petridishes. This method is also called loop dilution procedure.



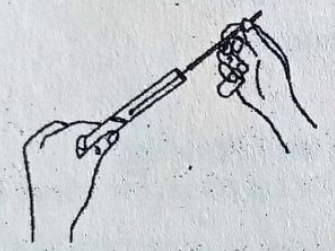
(a) Shake the tube to suspend the organisms as demonstrated by your instructor.



(b) Flame the entire loop and wire until it becomes red-hot. Begin heating at the middle of the wire and proceed slowly to the loop.



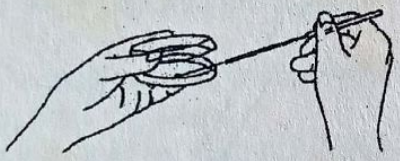
(c) Remove the plug or cap (closure). then flame the mouth of the tube. Don't contaminate the closure by placing it on the table.



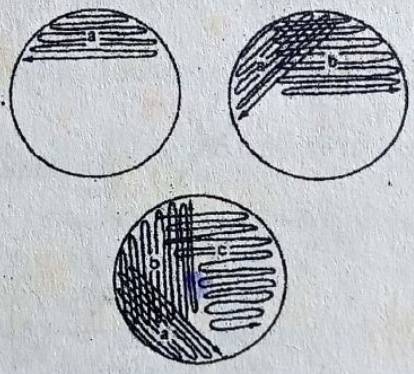
(d) After allowing the loop to cool, remove a loopful of organisms. Do not touch the sides of the tube.

(e) Flame the mouth of the tube again.

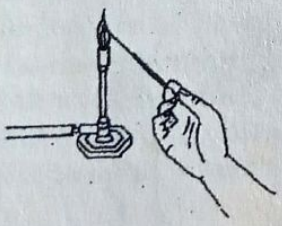
(f) Return the closure to the tube. Place the tube in a rack.



(g) Streak the plate as shown on the right or as illustrated by the instructor.



Streak plate procedure for pure culture isolation of bacteria. The direction of streaking is indicated by the arrows. Between each section, the loop is sterilized and reinoculated with a fraction of the bacteria by going back across part of the previous section. The plate is also rotated so the direction of the streak is always the same.

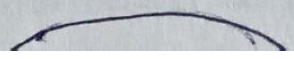


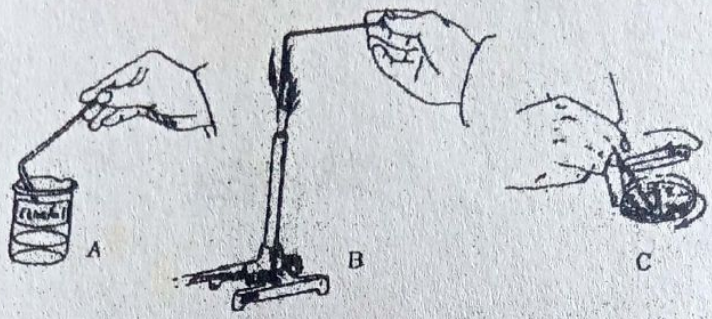
(h) Flame the loop before placing it down.

Fig. 1

Spread plate method

This is a simple method of isolation. Original sample or serial dilutions can be used. Two to three drops of the sample is pipetted on to the surface of an agar plate (previously poured and with dried surface). Using a spreader (dipped in 70 percent alcohol and flamed in a blue Bunsen flame), the inoculum is spread over the surface of the agar to form a layer. All the isolated colonies will be growing on the surface of agar (Fig. 2).

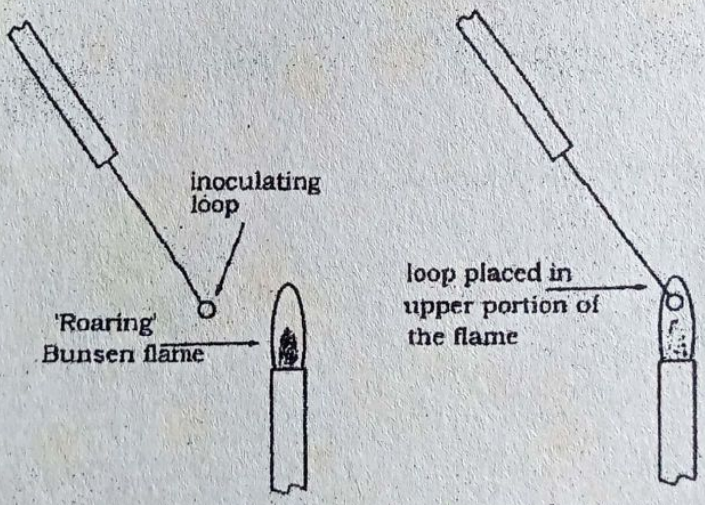




A- Dip spread in alcohol B- flam the spreader C- Spreading the plate

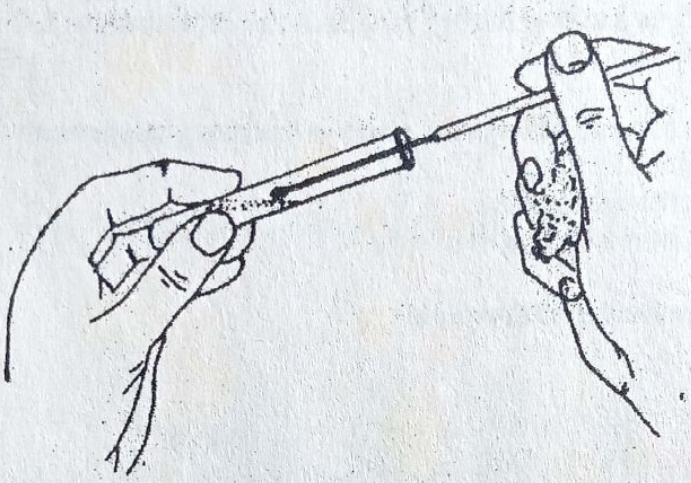
Exercise 1.
Isolation of pure cultures by streak plate techniques.

Procedure
1. Pour a tube of melted (45-50 °C) nutrient agar into a Petridish.
2. Allow the agar to become firmly solidified. Do not attempt to streak a plate until the medium is firm.
3. Sterilise the inoculation loop by flaming the entire wire in the bunsen flame to redness and cool the inoculation loop thoroughly (Fig 3).



4. Remove bacterial culture with the tip of inoculating loop.
5. Place the sterile agar medium in a convenient place, so that the Petri-plate bottom rests on the bench and the top can be manipulated up and down with the thumb and third finger of the left hand.
6. Lift the Petri-plate cover and place the inoculum at the edge of the agar furthest from you (fig. 1) i.e,. area a.
7. Area A will contain the highest concentration of bacteria and is referred to as the pool. Sterilise the loop in the Bunsen flame and, when cool, streak over area B, so that some bacteria are removed from area A to area B (Fig 1).
8. Repeat the procedure for areas C. This will produce isolated colonies, after incubation, in area b or A or both. On each occasion loop must be re-sterilised prior to use.
9. Label the plate and incubate in an inverted position at 37 °C for 1-2 days.

10. After proper incubation, examine the plate and make a sketch of its appearance. If there are no single colonies along the streak, repeat the procedure.
11. Inoculate an agar slant (Fig. 4) for storing and maintaining.



Procedure for inoculating an agar slant
a) Take the sterile agar slant in your left hand; take the inoculating loop in your right hand and flame the needle to redness.
c) Cool the wire and remove inoculum from the pure culture plate (Previously prepared).
d) Remove the plug/cap from the slant tube by grasping it between the last two fingers and the palm of the right hand.
e) Flame the mouth of the slant tube; place the loop down onto the surface of the agar slant at the bottom of the tube.
f) Slide the loop on the surface of the slant from side to side in a zip-zag manner as you pull the loop out of the tube without digging into the agar surface.
g) Flame the tip of the slant tube and replace the cap or plug.
h) Flame the inoculating loop.
i) Label the tube with the microorganism used., type of agar, the date
i) Incubate at room temoerature for 48 hours.

Exercise 2.
Isolation of pure cultures by pour-plate (loop dilution) techniques

Materials
Bacteriological tubes with sterile nutrient agar; mixed culture plate; inoculating loop; 3 sterile Petridishes.

Procedure
1. Melt the 3 tubes of sterile nutrient agar (12-15 ml) and cool them to 45°C, wipe the tubes to remove any superficial moisture. Maintain them at 45-50°C during the dilution manipulations.
2. Aseptically transfer one loopful of a mixed microbial culture to the first tube of molten agar. Mix the inoculums well by rotating or swirling the tube between your palms ten times. label this, tube 1.

3. After mixing, transfer two loopfuls of the mixed inoculum-agar from the first tube to a second tube of molten agar and mix well in the same manner as in step 2. Label this, tube 2.
4. Transfer 3 loopfuls from the second tube to the third tube of molten agar and mix thoroughly as in step 2. Label this 3.
5. Remove the plug of each tube, flame the tip of the agar tube and aseptically pour the inoculated agar into three separate sterile Petridishes and label them as 1, 2 and 3 corresponding to the dilutions.

6. After the solidification of agar, incubate the plates at laboratory temperature for 24 - 48 hours in inverted position.
8. Observe and make a sketch of the plates.
9. Inoculate an agar slant for storing as given in fig. 4.

Common bacterial shapes and arrangements

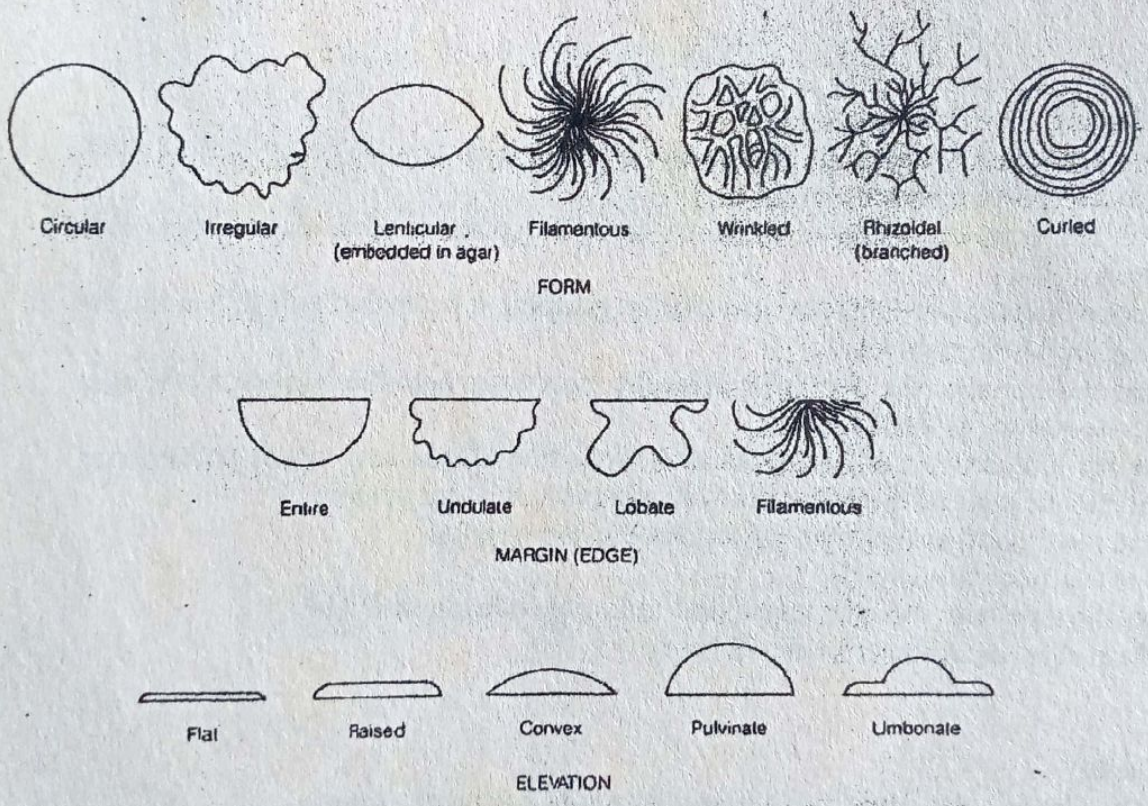
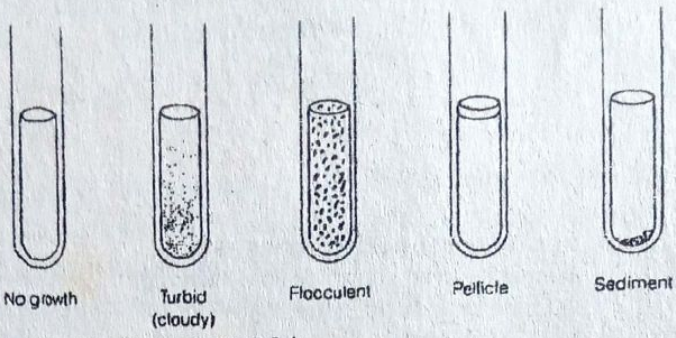
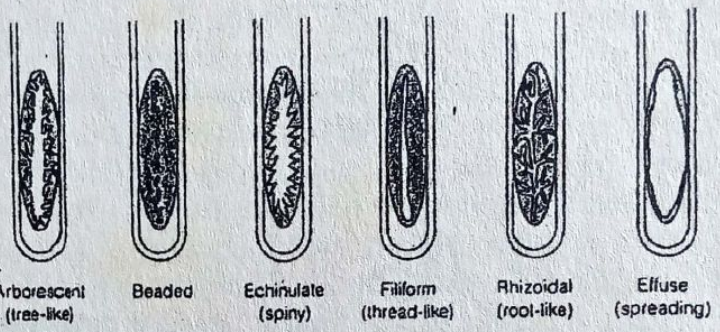


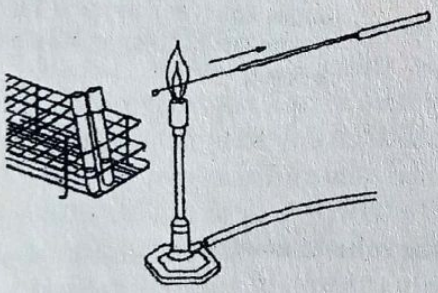
Fig. 5 Representative colony characteristics of bacteria



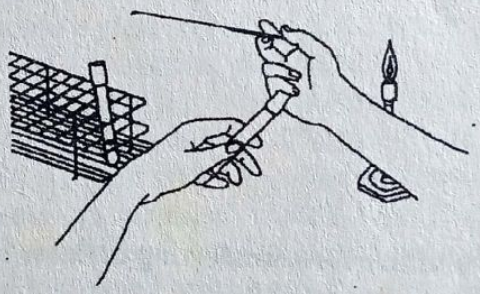
Growth Patterns of Bacteria Growing in Broth Cul-



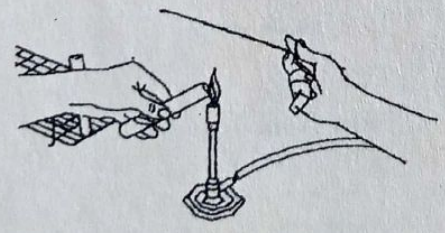
Growth Patterns of Microorganisms Growing on Agar



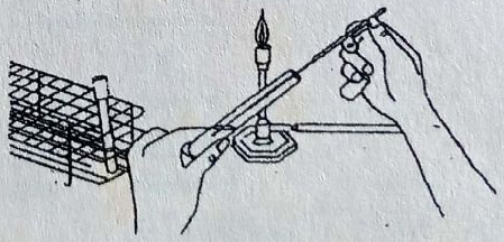
1 Flame the loop. Begin flaming at the center of the wire and proceed toward the loop



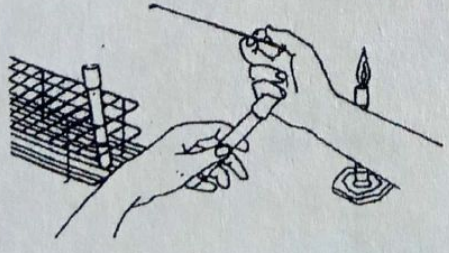
2 While holding the sterile loop, remove the closure from the tube as demonstrated by your instructor, or as illustrated



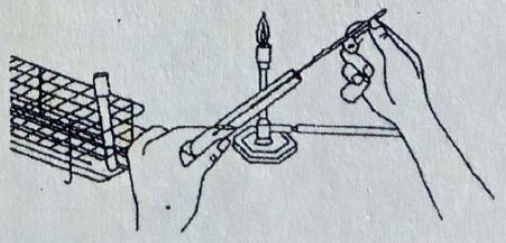
3 Heat the mouth of the tube by briefly holding it in the flame of a Bunsen burner.



4 Remove a loopful of culture, reheat the mouth of the tube, and replace the plug

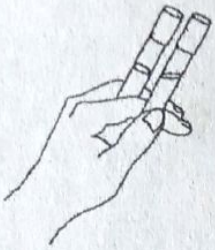


5 Pick up the sterile broth tube from test tube rack, remove closure, and flame the lip.

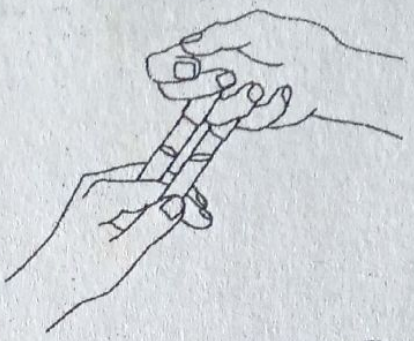


6. Introduce the loopful of bacterial culture to the sterile broth as shown. Shake the loop gently a few times to ensure a transfer of culture to the broth. Reheat the mouth of the tube, and replace the plug.

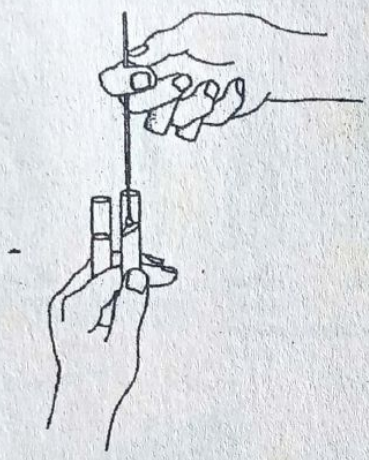
Procedure for Subculturing Organisms Grown in Tubes. The instructor may wish to show the student the correct method for holding two tubes simultaneously as illustrated in figure 7-5. Similar techniques may be used in subculturing from slants to broth, from broth to slants, and from slants to slants.



1. In one hand, hold the tubes as illustrated, or as shown by your instructor. With the other hand, flame the inoculating loop.



2. Remove closures as illustrated. Flame the mouths of the tubes.



3. Transfer a loopful of culture from one tube to the other. Flame the mouths of the tubes again and replace the closures. Flame the inoculating loop before setting it down.

Procedure for transferring cultures from one tube to another

AQS 103 10 Aquatic Microbiology and Water Quality
Practical NO. 3

Positive and Negative staining

Introduction

A simple stain is a procedure in which only one stain is used to create a contrast between the specimen and its background. Generally, simple stains involve the use of basic dyes such as crystal violet, methylene blue, basic carbolfuchsin (red), safranin (red), or malachite green. Since simple staining procedures are rapid and easy to carry out, they are often used when information about cell shape, size, and arrangement is desired (fig. 8). A simple stain helps the microscopic determine the dimensions and shape of cells. Bacteria can generally be characterized as spheres (coccus, plural cocci), rods (bacillus, plural bacilli), spirals (spirillum, plural spirilla), helices (spirochete, plural spirochetes), or branched organisms. In addition, many organisms form very distinctive arrangements that can be used to identify them. For example, bacteria such as the streptococci (strepto- chain of) form chains of cells, the staphylococci (staphylo- bunch of grapes) develop in grape-like clumps, the neisseriae exist as pairs or diplococci (dipio- pair of), and some micrococci and sarcinae (sarcina a package) are typically found in packets of four or eight. Usually, a simple stain involves the staining of a dried preparation of cells on a glass slide (fig. 9). The dried preparation of cells is known as a smear. Smears can be prepared from cells in a liquid culture or from growth on an agar plate or slant. When using a liquid suspension, one to several loopfuls are smeared onto a glass slide and then allowed to air dry. The cells in the dried smear are attached or fixed to the slide by briefly heating the slide over a gas burner flame. This procedure is known as heat fixation. When using colonies or growth from a semisolid medium, a loopful of water is placed on the slide and a very small amount of material is mixed with the water to separate and suspend the cells. The suspension is then spread out, air dried, and heat fixed. In a good smear, individual organisms are visible microscopically and organisms are not piled on top of each other. In order to insure well-dispersed organisms, the drop used to make the smear should be only slightly turbid (cloudy).

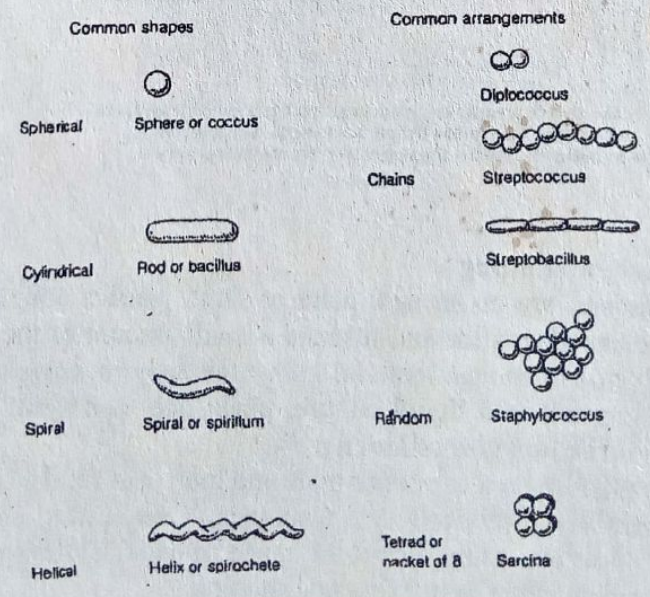


Fig. 8. Commomn bacterial shaps and arrangements.

Materials

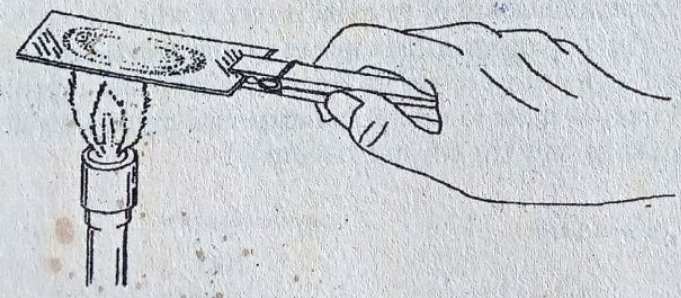
Solutions of clystal violet, methylene blue, basic carbolfuchsin, safranin, nigrosin, and India ink
Inoculating loop
Sterile toothpicks
Clean glass slides
Clothespins
Gas burner
Sink (with tap water)

Fig 9. Procedure for positive staining

1. Place several large loopfuls of a liquid culture on a clean slide. If the culture is on an agar plate or slant, place several loopfuls of water on the slide and then mix in a very small amount of the solid growth (e.g. part of a colony).

2. Spread the liquid culture or mixture over the slide so as to create a thin film.

3. Air dry the smear. Use a warming plate if possible to speed up the drying, but do not use a gas burner.



4. Heat fix the dried smear by placing the bottom of the slide over the gas burner flame for three seconds. An alternate method is to pass the slide through the flame three times.

Exercise 1

Procedure for positive staining

1. If the organisms are on an agar plate or slant, place a couple of loopfuls of water on clean glass slide and suspend a small amount of the organism in the water. Only mix in enough material so that the drop becomes slightly turbid. If the organisms are in a liquid culture, place one to several loopfuls of the suspension on a clean glass slide (fig. 9).
2. Air dry the mixture on a warm hot-plate and then heat fix the organisms over a burner flame. Heat fix for only 3 to 5 seconds. Overheating a smear or heating a still-wet smear can badly distort the shape of cells. Clothespins can be used to hold your slide when heat fixing and staining.

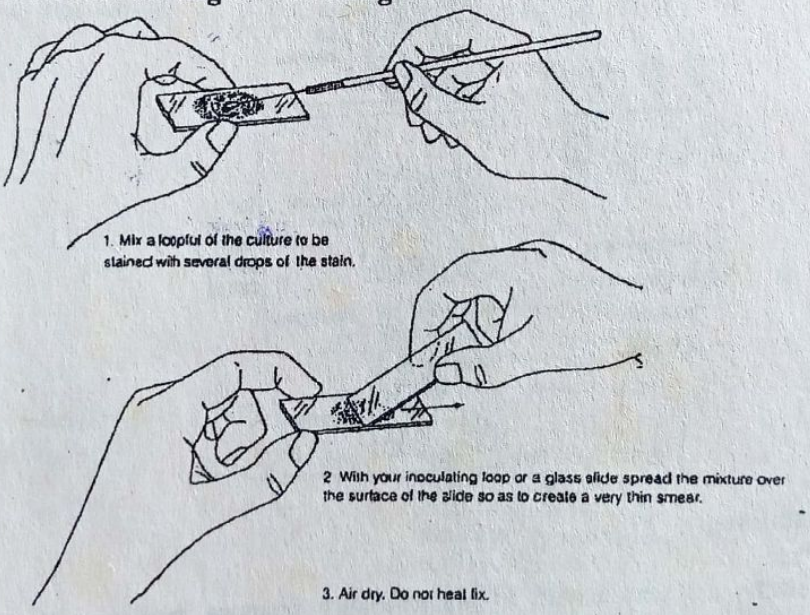
3. Cover the cooled, heat-fixed smear with one of the basic dyes. Crystal violet is a good choice. After staining for 30 seconds wash off the crystal violet gently with tap water or dip the slide into standing water. Drain the excess water from the slide.

4. Carefully blot the slide dry with bibulous paper or a paper towel. When you hold the slide up to the light, you should be able to see your stained smear.

5. Locate the smear with the low power lens, then place a small drop of oil on the stained smear and view the organisms with the oil immersion lens (100 X objective). If you have carried out the simple staining procedure correctly, the microorganisms should be coloured (dark blue-violet if you used crystal violet) and the background should be clear and bright.

6. Draw, label, and describe what you see. Examine several oil immersion fields in different areas of the smear to obtain a representative view of the specimen as a whole.

Fig. 10 Procedure for negative staining



Procedure for negative staining

Exercise 1

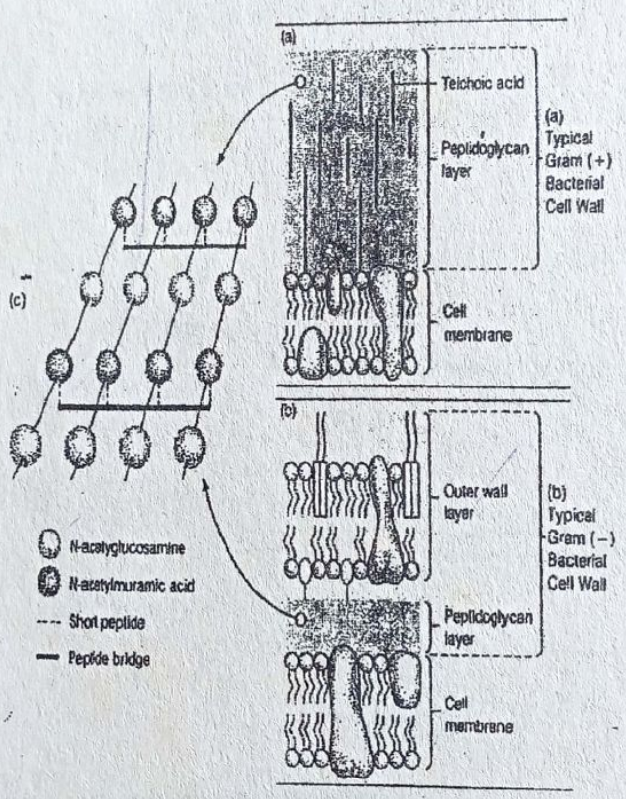
1. Place one to several loopfuls of an acidic stain at one end of a clean glass slide. The use of nigrosin is suggested. If the microorganisms are suspended in a liquid, mix a loopful of the suspension with the drop of acidic stain. If the microorganisms are on a plate or slant, use aseptic technique to obtain a very small sample of the material with an inoculating loop and mix it in the stain. Do not mix in too much of the specimen. If you do, the cells will be piled on top of each other and it will be difficult to see individual organisms.

2. Spread the mixture over the slide by drawing a second glass slide into the drop and then pulling the drop back along the slide (Fig. 10). Try to make as thin a smear as possible.

3. Air dry the mixture on a warm hot plate but DO NOT HEAT FIX.

4. Locate the smear with the low power lens (10 x), then place a small drop of oil on the stained smear and view the organisms with the oil immersion lens (100 X). The condenser diaphragm should be open so that as much light as possible is captured by the objective lens.
If you have carried out the staining procedure correctly, the micro organisms should be clear or lightly stained while the background should be heavily stained.

5. Draw, label, and describe what you see.



Deferential staining
Gram staining
Differential stains are very useful in microbiology because they can be used to distinguish between groups of bacteria. An important differential stain is the Gram stain. The Gram stain is one of the most important steps in the characterization and identification of bacteria.
The Gram stain separates bacteria into one of two large groups: the. Grain-positive bacteria that retain the color of the first stain used (crystal violet), and the gram-negative bacteria that assume the color of a second stain (safranin). Bacteria stain differentially because of differences in the structure and chemical composition of their cell walls. The Gram-positive bacteria have a thick cell wall that consists primarily of peptidoglycan (fig. 11)

The walls of Gram-negative bacteria have much less peptidoglycan than those of Gram-positive bacteria. The peptidoglycan layer, usually about 2 nm thick, is surrounded by a complex lipid bilayer called the outer membrane. Some microbiologists regard this outer membrane as part of the cell wall while other scientists consider the outer membrane to be a separate and distinct envelope. No teichoic acids are associated with the cell wall of Gram-negative bacteria.

The Gram stain procedure illustrated in figure 12 consists of nine steps.

STEPS		COMMENTS
1. Crystal violet	60 sec.	Primary dye. (Primary stain)
2. Water rinse	brief	
3. Gram's iodine	60 sec.	Mordant. Gram's iodine functions as a mordant, that is, it forms a chemical complex with the crystal violet that helps the dye attach to the charged groups in the wall, membrane, and cytoplasm.
4. Water rinse	thorough	
5. 95% ethanol	10 sec.	Decolorizing agent. The ethanol functions as a decolorizing agent that draws the iodine-crystal violet complexes from the gram-positive and gram-negative cells. Since the gram-negative cell has a much thinner wall than the gram-positive cell, the iodine-crystal violet complexes are more rapidly removed from the gram-negative cells.
6. Water rinse	thorough	
7. Safranin	60 sec.	Secondary dye. The safranin functions as a counterstain to color the clear gram-negative bacteria red. It is believed that the gram-positive bacteria pick up very little safranin because most of the charged groups are still occupied by the crystal violet.
8. Water rinse	brief	
9. Drain and blot dry		

In order to obtain a reliable Gram stain, it is necessary to use a young culture of organisms, no older than 24 to 48 hours. If older cultures are used, there is the possibility of ambiguous results. As cultures age, progressively more cells sustain damage to their cell walls. This damage apparently allows dyes to be more easily

leached from the cells by decolourising agents. "Thus, Gram-positive cells w
damaged cell walls tend to lose their ability to retain the crystal violet-Gram's iod
complex and consequently stain as if they were Gram-negative. Damaged cells i
pure culture of Gram-positive bacteria are sometimes the reason a mixture of bl
violet and red cells is seen in Gram stained smears of pure microorganisms.

Materials

Young cultures (24 to 48 hours old) of the Gram-positive bacteria and Gram-negat
bacteria *Escherichia coli*.
Solutions of crystal violet, Gram's iodine, 95% ethanol, and safranin
Clean glass slides
Inoculating loops
Gas burner
Sink with tap water

Exercise 3

Procedure

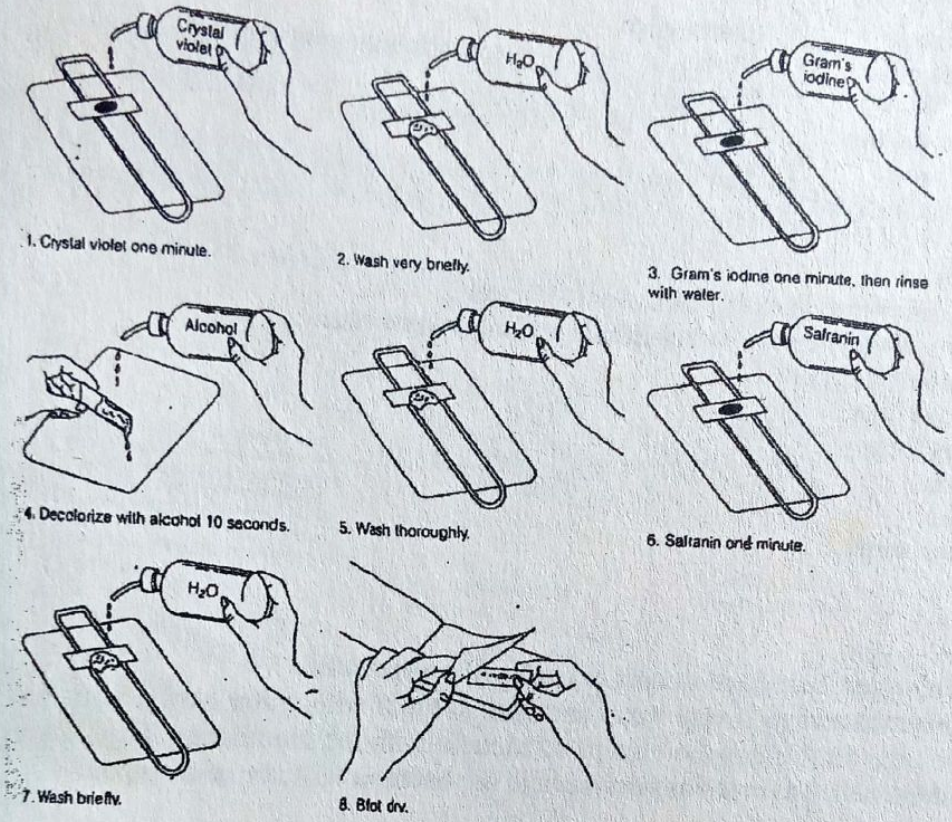
1. Prepare a thin smear of the bacteria provided. When making a mixed smear m
together on a clean slide. When using organisms from a plate or slant, mix in o
enough bacteria to make the drop slightly turbid.

2. Air dry the smears on a hot plate and then heat fix the bacteria over a burner flam
Heat fix for only 3 to 5 seconds.
3. Cover the heat fixed smear with crystal violet and stain for 60 seconds. Rinse
slide gently and briefly with water. Allow the excess fluid to drain from the slide.

4. Cover the smear with Grain's iodine. After 60 second thoroughly rinse the slide.

5. Cover the smear with the decolorizing agent, 95% ethanol, for 10 seconds. After
seconds, immediately wash all the ethanol from the slide with water to stop
decolourising action of the ethanol. If the alcohol remains on the smear for more th
10 seconds, it will remove the crystal violet-Gram's iodine complexes from Gra
positive as well as Gram negative bacteria and the staining procedure will not gi
accurate results.
If the alcohol does not remain on the smear for at least 10 seconds or is diluted whe
added because of rinse water remaining on the smear, the crystal violet will not
leached out of either type of cell. Again the staining procedure will not give accura
results. If the decolorizing has been done property, the Gram-positive cells will s
be dark blue violet while the Gram-negative cells will be clear and difficult to see.

6. Cover the smear with safranin. After 60 seconds wash the slide thoroughly w
water.
7. Carefully blot the slide dry with a paper towel.
8. Locate the smear with the low power lens (10 x). Then place a small drop of oil
the smear and rotate the oil immersion lens (100 x objective) into place.
9. Draw, label, and describe what you see.



Acid-fast stain

The acid-fast stain is a useful procedure for distinguishing bacteria in the genera *Mycobacterium* and *Nocardia* from all other types of bacteria. These genera contain important human pathogens. *Mycobacterium leprae* is the cause of leprosy, while *Mycobacterium tuberculosis* is responsible for tuberculosis. *Nocardia asteroides* causes pulmonary nocardosis, a disease of the lungs that resembles tuberculosis. Bacteria in these genera are said to be acid-fast bacteria because they retain a primary stain (ZiehI-Neelsen carbolfuchsin) that is removed from other bacteria by a brief treatment with an acidified decolorizing agent (acidalcohol). Acid-fast bacteria stain a bright red. Bacteria that lose the primary stain when treated with an acidified decolourising agent are said to be non-acid-fast bacteria. Non-acid-fast bacteria are detected by use of a counterstain (methylene blue) that colors them a bright blue.

Mycobacterium and *Nocardia* are unusual because they have a high concentration of waxes in their cell wall. In some species the waxes may account for as much as 60% of the wall's weight. The waxes make the bacteria difficult to stain since charged dye ions do not readily penetrate the waxy layers of the wall. The primary stain is usually applied with the aid of heat to allow the dye to penetrate the waxy layer of the cell wall. Once stained, the acid-fast bacteria are difficult to decolourise. The acid-fast stain procedure

(Fig. 13) consists of the following steps.

STEPS		COMMENTS
1. Carbolfuchsin	5 min, (steamed)	Primary stain must be steamed for 5 min
2. Water rinse	thoroughly	
3. Acid-alcohol	20 sec.	Decolorizing agent
4. Water rinse	thoroughly	
5. Methylene blue	1 min.	Counter stain
6. Water rinse	briefly	
7. Drain and blot dry		

Materials
Young cultures (less than 48 hours old) of bacteria.
Solutions of Ziehl-Neelsen carbolfuchsin and methyiene blue.
Solution of acid alcohol
Clean glass slides
Inoculating loops
Clothespins
Gas burner
Sink and tap water

Exercise 4
Procedure
1. Prepare air-dried, heat-fixed smears of each culture provided.
Since the Mycobacterium is very waxy and does not mix with water easily, you must spend some time breaking up the clumps of bacteria with the inoculating loop. If you spread the bacteria out as much as possible and the bacteria will not be clumped.

2. Place the slide on a rack over a sink.

3. Cover the fixed smear with carbolfuchsin and heat the stain. The carbolfuchsin must steam for at least 5 minutes.
In order to get the carbolfuchsin to steam, heat it from above with the flame from the gas burner. NOT ALLOW THE DYE TO BOIL. Also, remove the flame occasionally so that the slide does not become so hot that it breaks.

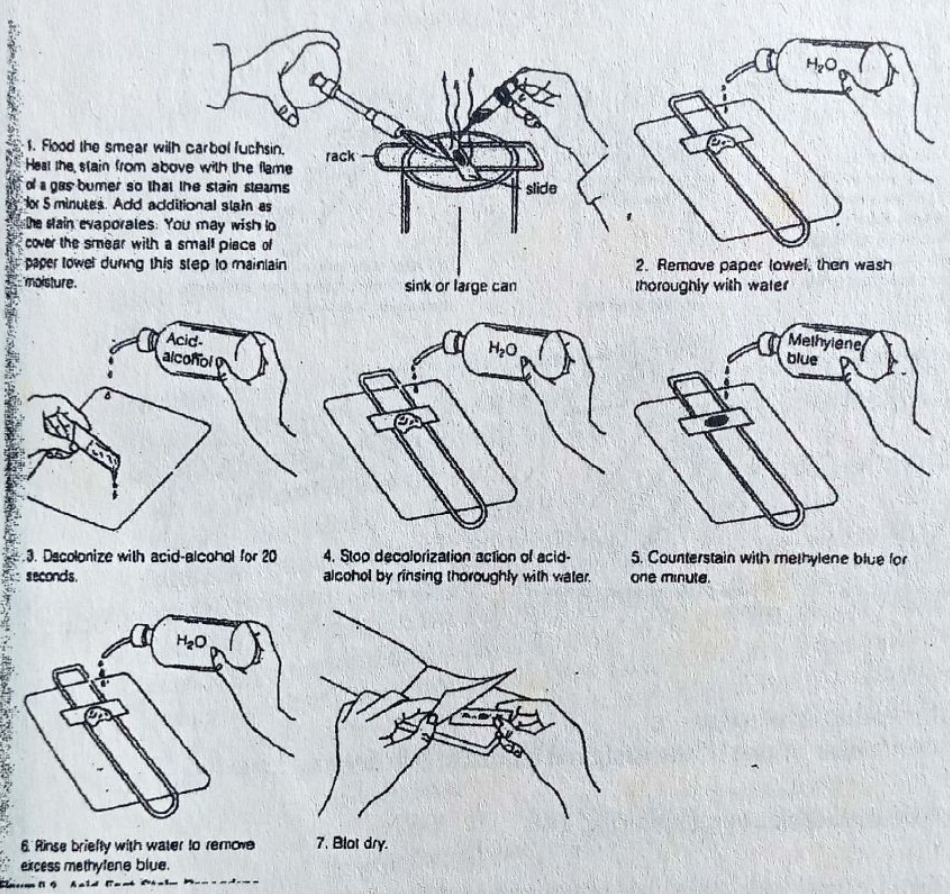
4. If towel paper was used, remove it and discard in appropriate containers (not in the sink). Rinse the stained smear thoroughly with tap water and then drain the excess water from the slide.
5. Flood the slide with acid-alcohol. After decolourising far 20 seconds, immediately and thoroughly wash with tap water to stop the decolourising action of the acid alcohol.
The acid-alcohol removes the carbolfuchsin from cells that lack waxes in the cell wall but is unable to remove the stain from the vegetative cells with high concentrations of waxes. At this point in the procedure, the waxy cells are red while wax-free cells are clear and very difficult to see.

6. Counterstain the smear with methylene blue for 60 seconds.
Methylene blue is a basic dye and binds to the negative charges on the wax-free (non-acid-fast organisms) vegetative cells. The counterstain does not readily penetrate or bind to the waxy cells. Thus, the waxy cells are red while the wax-free cells are blue.

7. Rinse the smear briefly with tap water. Methylene blue is a weak dye and it is easy to over rinse. Carefully blot dry with a paper towel.

8. Locate the smear with the low power lens (10 x), then place a small drop of oil on the smear and rotate the oil immersion lens (100 X objective) into place.

9. Draw, label, and describe what you see.



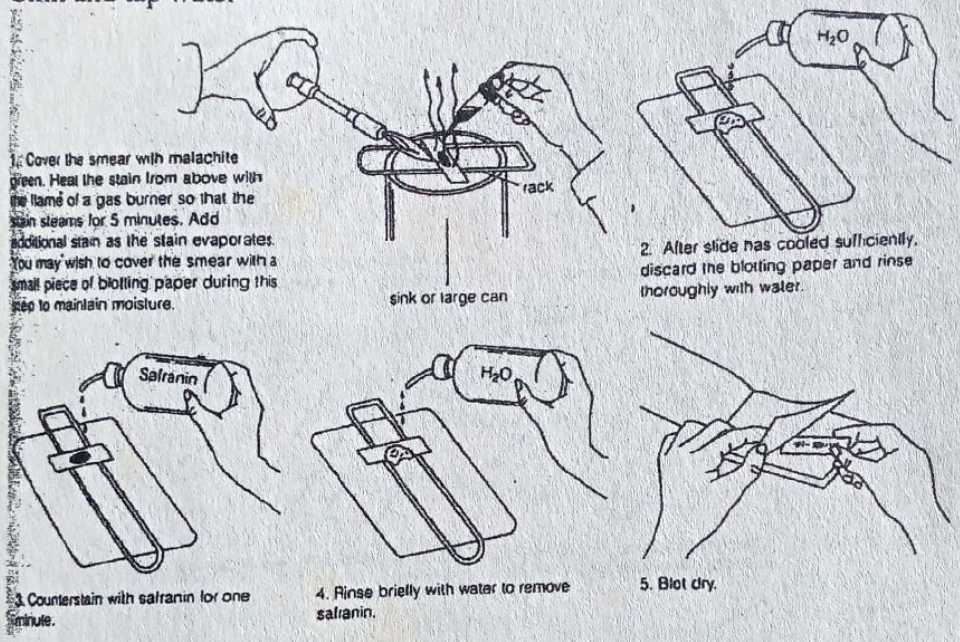
Exercise 5
Endospore stain

An endospore is a heat- and chemical-resistant form produced by members of certain bacteria genera in response to adverse environmental conditions. The only bacteria known to produce endospores belong to the following genera: *Bacillus, Clostridium, Desulfotomaculum, Sporolactobacillus, Thermoactinornyces,* and Sporosarcina. Endospoprs develop within the cell and only one endospore form per cell. Consequently, this type of sporolation is not involved in reproduction.
Under favorable conditions, the endospore forming bacteria proliferate like other common bacteria. When the environment becomes unfavorable for vegetative growth, the endospore former begins and sporulate or form an endospore. An unfavorable environment may be one in which the carbon, energy, phosphate source is running low; toxic waste beginning to accumulate; the temperature is becoming unfavorable;

or the environment is becoming hypertonic (as a result of desiccation). Under laboratory

Materials

Old cultures (older than 48 hours) of bacteria (*Bacillus subtilis, Clostridium sporogenes, Clostridium butyricum*)
Solutions of malachite green (potential carcinogen) and safranin
Clean glass slides
Clothespins
Inoculating loops
Gas burner
Sink and tap water



Procedure for endospore stain

1. Prepare a thin smear of one of the assigned cultures. Air dry and heat fix.

2. Place the slide on a rack over a sink (fig. 14).

3. Cover the dried smear with malachite green and heat the stain so that it steams. The malachite green must steam for at least 5 minutes.
One method of steaming is to heat the malachite green from above with the gas burner flame for 5 minutes. If the malachite green begins to evaporate, add fresh stain and reheat so that the stain shows rising vapours. After vapours are detected, remove the flame so that the slide does not become so hot that it breaks. Another method of steaming is to heat the malachite green for 5 minutes from below with the steam from a boiling water bath.

4. Cool the slide for about I minute before continuing.

5. If towel paper was used, remove it and discard it in an appropriate container. Wash the smear thoroughly with tap water.

The tap water readily removes the malachite green from most bacterial vegetative cells but is unable to decolorize the endospores. At this point in the procedure, the endospores are green while the vegetative cells are clear and very difficult to see.

6. Counterstain the smear with safranin for 60 seconds. Do not heat the safranin.
The counterstain is a basic stain and binds to the negative charges on the vegetative cell. The counter stain does not penetrate the endospore. Thus, the vegetative cells should stain red while the endospores, if present, should stain green.

7. Rinse the smear briefly with water. Carefully blot dry with a paper towel. Safranin is a weak dye and is easily removed from vegetative cells by over rinsing.

8. Locate the smear with the low power lens (10 X), then place a drop of oil on the smear and switch to the oil immersion lens (100 x objective) to examine the cells. The condenser diaphragm should be open so that as much light as possible is captured by the oil immersion lens.

Exercise 6
Flagella stain

Bacteria flagella are long thin protein appendages that some bacteria use to propel themselves (fig.15). Even though bacterial flagella may be 2 um to 5 um long, They cannot normally be viewed with the light microscope because they are generally less than 0.025 um in diameter.
Flagella are found in arrangements characteristic of the species, with a single flagellum (monotrichous), or flagella at one end (polar), or flagella at both ends (amphitrichous), or flagella that originate over the entire surface (peritrichous). Flagella at the poles of the cell may be single or multiple. The arrangement of flagella is sometimes useful in the identification of certain species of bacteria. Because of this, stains have been developed that allow flagella to be viewed with the light microscope. A chemical called a **mordant**, which precipitates on the flagella, is used to thicken them. The mordant is then stained and the thickened flagellar structures can be seen with the light microscope.
Motile species within the family Enterobacteriaceae (such as Proteus) have peritrichous flagella, while bacteria of the genus Pseudomonas typically show polae flagellates.

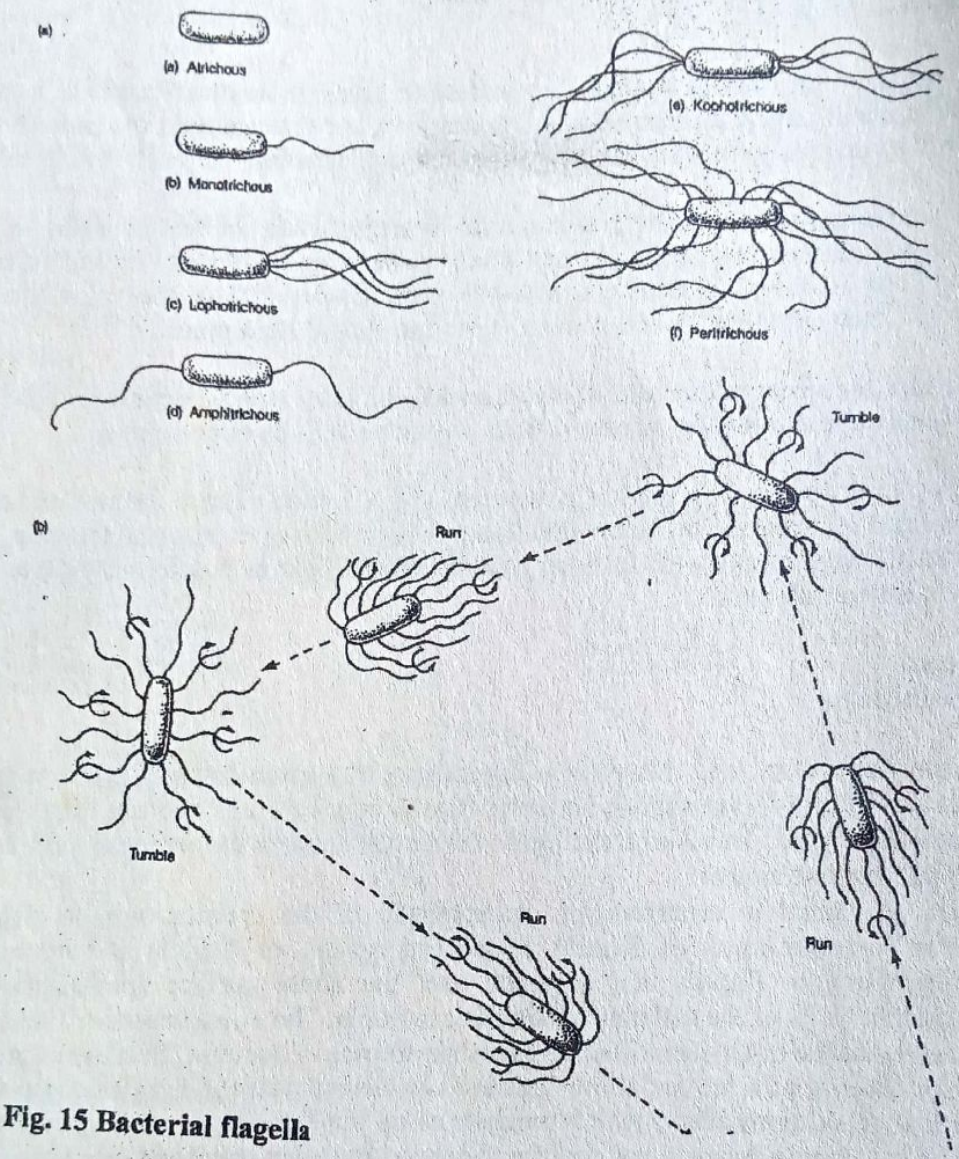


Fig. 15 Bacterial flagella

Procedure

1. Gently obtain a small mass of bacteria from a slant or plate and place the mass, without agitation, in 0.5ml of saline in a small test tube. Allow the mass to sit undisturbed for 15 minutes sothat bacteria diffuse from the mass (fig. 16).

2. While you are waiting for the bacteria to make the saline turbid, prepare the Gray's flagellar stain. Mix 9 ml of the mordant (5 ml of potassium alum + 2 ml of tannic acid + 2 ml mercuric chloride) with 0.8 ml of the stain (0.8 ml basic fuchsin). Then filter the mixture.

3. Rinse clean slides in 95% ethanol. Then flame the slides. Place the slides at a steep angle against a test tube rack.

4. Place a large drop of the slightly turbid bacterial suspension near the top of a slide and allow it to run down the slide. Allow the slide to air dry but DO NOT HEAT FIX.

Make three slides for each organism.

5. Place the slides on a staining rack over the sink.

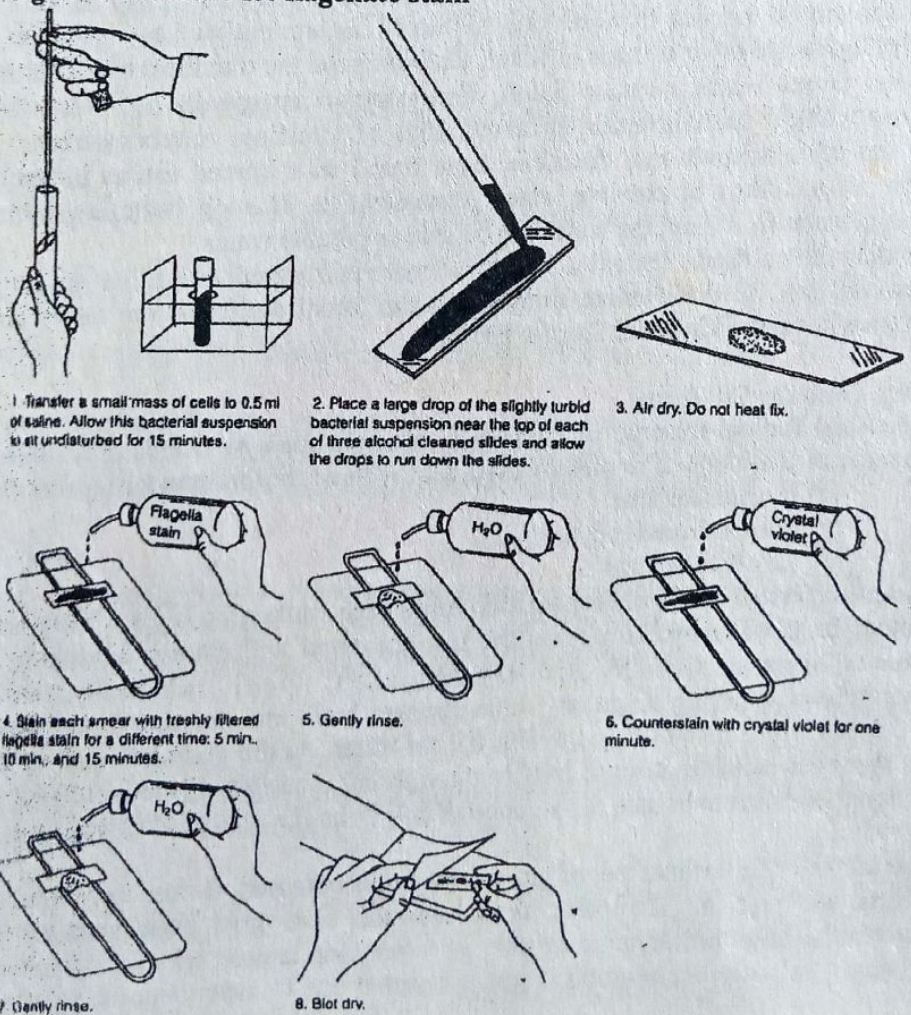
6. Flood the smears with freshly prepared flagella stain. Stain one smear for 5 minutes, one for 10 minutes, and one for 15 minutes. Rinse GENTLY AND BRIEFLY with water (or dip into water).

7. Counterstain with crystal violet for 1 minute. Rinse GENTLy AND BRIEFLY with water and carefully blot dry with a paper towel or bibulous paper.

8. Locate the smears with the low power lens (10X), then switch to the oil immersion lens (100 x objective).

9. Describe, draw, and label your findings.

Fig. 16 Procedure for flagellate stain



1. Transfer a small mass of cells to 0.5 ml of saline. Allow this bacterial suspension to sit undisturbed for 15 minutes.

2. Place a large drop of the slightly turbid bacterial suspension near the top of each of three alcohol cleaned slides and allow the drops to run down the slides.

3. Air dry. Do not heat fix.

4. Stain each smear with freshly filtered flagella stain for a different time: 5 min., 10 min., and 15 minutes.

5. Gently rinse.

6. Counterstain with crystal violet for one minute.

7. Gently rinse.

8. Blot dry.

AQS 103 10 Aquatic Microbiology and Water Quality
Practical No. 4

Microbiological analysis of water purity

Introduction

The natural water bodies such as lakes, streams, rivers contain sufficient amount of nutrients that support the growth of microorganisms. There are different ways by which microorganisms enter in water supply, for example broken sewer lines, congested centres, inappropriate treatment, etc. In addition, lack of awareness among people also add in contamination of water. Unhygenic environment at public places of water collection is also one of the reasons. People suffering from communicable diseases also discharge pathogenic microbe in water through their excreta, for example amoebic dysentery, typhoid fever, bacillary dysentery poliomyelitis, etc. Bacteria in water multiply at 35°C and at 20°C

The faecal Coliforms: On the basis of microbiological examination of water, its potability (suitability for drinking) may be ascertained. Intestinal bacteria present in water generally do not survive in aquatic environment due to physiological stress, but if entered human system in the mean while, they cause serious problems. The characteristic group of intestinal bacteria are the coliforms.

Coliforms are defined as facultatively anaerobic. Gram-negative, nonsporing, rod shaped bacteria that ferment lactose with gas formation within 48 hours at 37°C. The colifom groups are present in water due to faecal contamination i.e. discharge of faeces by human and other animals in water. Coliforms are the members of the family Enterobacteriaceae which includes *E.coli, Enterobacter aerogenes*, and *Klebsiella pneumoniae*. These bacteria make up about 10% of intestinal microorganisms of humans and other animals and, therefore, have found wide spread use as indicator organisms, as an index of possible water contamination. If such bacteria are not detectable in water in 100 ml, the water can be said as potable water.

Unfortunately the coliforms include a variety of bacteria irrespective of their primary source as intestine. To differentiate from others the faecal coliforms are tested for their presence in water (Klein and Casida, 1967).

1. Sanitary Tests for Coliforms

The original test for the presence of colifom in water is done by standard multiple tube fermentation technique. This method involves the three routine standard tests:

(a) the presumptive
(b) the confirmed test, and
(c) the complete test

(i) Presumptive test: A series of fermentation tubes each containing lactose broth or lauryl tryptose broth of known concentration, are inoculated with known amount of water. These tubes are incubated for 24 to 48 hours at 35°C (Fig. 17). Generally, five fermentation tubes containing single or double strength broth are inoculated with 10 ml water, 5 tubes with I ml water and 5 with 0.1 ml water. At the end of 24 hour of incubation, the tubes indicates that the coliforms are absent. These lubes are incubated for an additional 24 hours to be sure for absence of coliforms (i.e. gas production).

(ii) Confirmed test: If a positive test of gas production is obtained, it does not mean that coliforms are present. The other organisms too also give false positive presumptive test because they are also capable of fermenting lactose with formation of acid and gas. The positive presumptive test is resulted due to synergism i.e. joint

action of two microorganisms on a carbohydrate with production of gas which is not formed if both are grown separately. In addition if yeasts, species of Clostridium and some other microorganisms are present, gas is also produced. Therefore, a confirmed test is performed for the presence of coliforms. All fermentation tubes showing gas within 24 hours at 37°C are used for confirmed test. It is of two types as described below:

(iii) Completed Test: In the last the completed test is performed to ascertain about the presence of coliforms in water. The purpose of the completed test is to determine whether

(a). the colonies growing on EMB or endo agar are again capable of fermenting lactose and forming acid and gas, and

(b) the organisms transferred to agar slants show the morphological appearance of coliform group. Each colonies form positive confirmed test is transferred to lactose fermentation tube and to nutrient agar slants. The tubes are incubated at 37°C for 48 hours.

Production of gas in fermentation tubes and, demonstration of Gram-negative, non spore forming rods on the agar slants constitute a positive completed test for coliforms.

The absence of gas and the rod production confirms for negative test of coliforms.

Exercise 1

Total colony count Colonies 100 ml^{-1} – Membrane filter method

Application

The method is applicable to potable water, surface water and effluents.

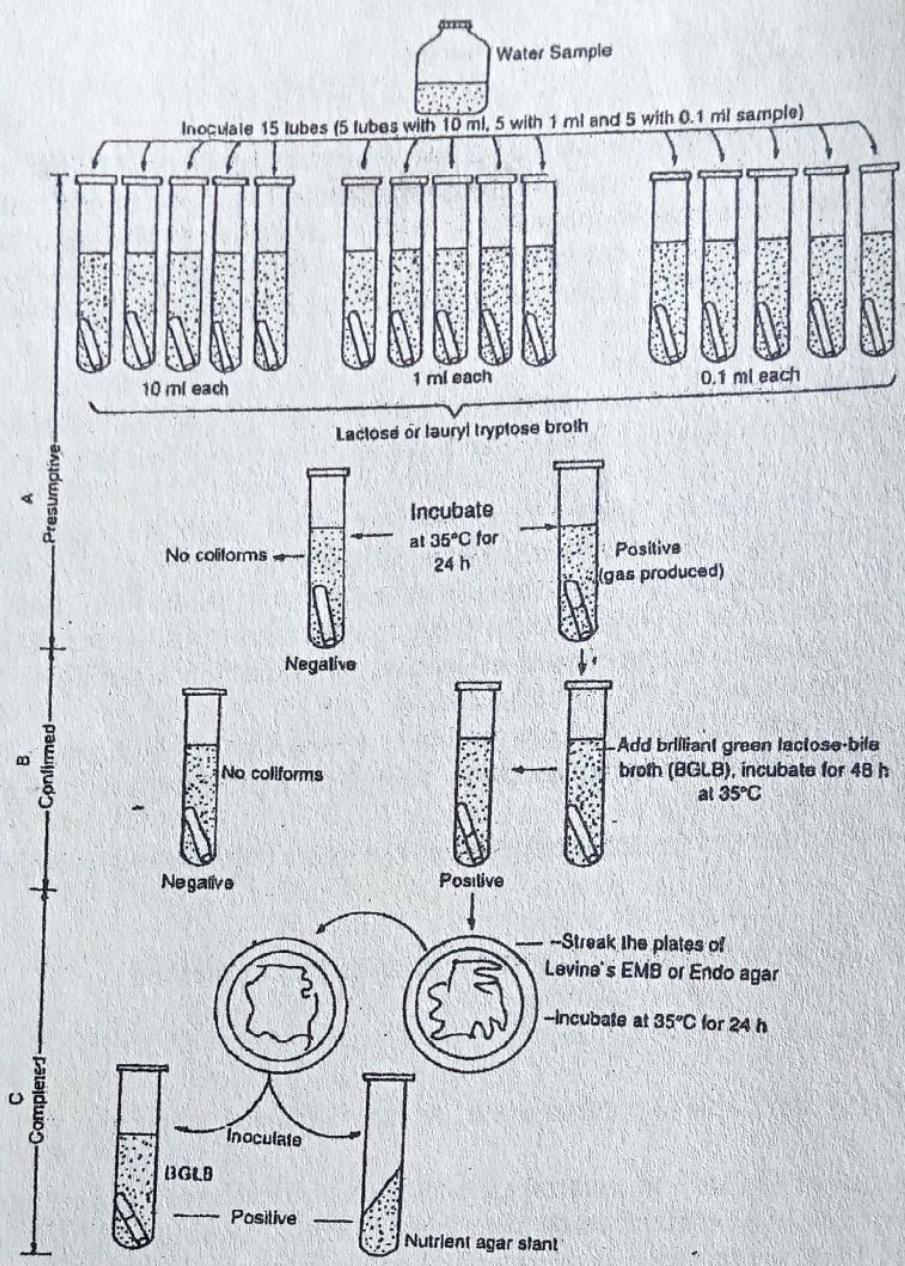
In the context of the method, micro-organisms which produce acid from lactose and form yellow colonies on membranes after incubation for 4 hours at 30 °C followed by 14 hours at 37 °C are regarded as presumptive coliform organisms.

The method

Isolate the micro-organisms on a membrane placed on an absorbent pad saturated with a broth containing lactose and phenol red as an indicator of acidity and subsequently confirm the ability to produce gas and indole where necessary.

Apparatus

Membrane-filteration unit (Milipore)
Membrane filters (47 mm diameter-, 0.45 um pore size)
Absorbance pad (47 mm diameter; I mm thickness)
Incubator
Petri dishes
Pipettes (bacteriological)
Water bath
Autoclave



Reagents and media

Membrane Lauryl Sulphate Broth

Peptone	40 g
Yeast Extract	6 g
Lactose	30 g
Phenol red (0.4% w/v aqueous solution)	50 ml
Sodium lauryl sulphate - specially pure	1 g
Distilled water	1l

Add the ingredients to the water and mix gently to avoid froth. The final pH of the medium should be 7.4 to 7.5 and it may be necessary to adjust the pH to about 7.6 before sterilization to achieve this. Distribute in screw-capped bottles and autoclave at 115 °C for 10 minutes.

Note: The media used with membrane fillters differ in composition from those of the Multiple Tube Method and Pour Plate Culture Method because membranes selectively absorb some substances but not others.

Procedure

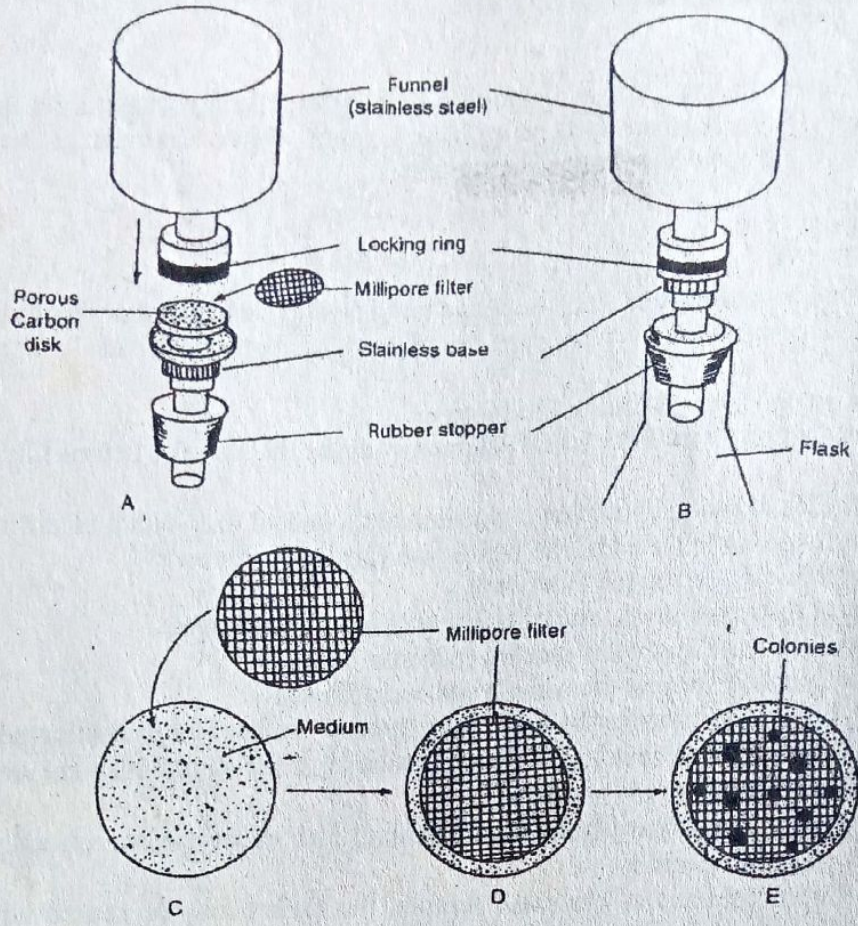
Preparation of the samples- The volumes should be choosen such that the number of colonies to be counted on the membrane lies, if possible, between 10 ml and 100 ml.
For treated waters, filter 100 ml of the sample.
For polluted waters, either filter small volumes or dilute the samples before filtration.

Filteration
Place the sterile filteration apparatus in position and connect to a source of vaccum.
Remove the funnel and place a sterile membrane (gird side upwards).
Replace the sterile funnel on the filter base.
Pour or pipette the required vouime of water sample into the funnel.
Open the stop cock and apply the vaccum (500 mm Hg).
Close the stop cock as soon as the sample has been filtered.
Remove the funnel and transfer the membrane carefully either to a pad saturated with the medium or to a well dried agar plate (ensure that no air bubbles are trapped between the membrane and the medium).
Pour any excess of medium from the saturated pad either before or after the membrane is placed in position.
Note;For different volumes of the same sample, the funnel may be reused without boiling provided that the smallest volumes are filtered first.
For different samples, remove a funnel from the boiling water bath, allow to cool and repeat the filteration procedure.
After filtration of each sample, disinfect the funnel by immersion in boiling distilled water for at least one minute.

Incubation and examination
Place the Petri dishes with the membrane inside a container with a tightly fitting lid to prevent drying out.
Incubate the membrane at the temperature for the duration of time specified for the organism sought.
After incubation count the characteristic colonies on the membrane within a few minutes under good light (if necessary use a hand lens).
Express the results as the number of indicator organisms per 100 ml of water sample.

Advantages
A quick method by which results can be obtained as direct counts. There is a considerable saving on labour, in the amount of media, and the glassware required. The conditions of incubation can be varied easily to encourage the growth of slow growing organisms.

Limitations:
Gas production, for example, by coliform organisms is not detected. Membranes are not suitable for use with waters of high turbidity or those containing small numbers of indicator organisms sought.



Incubation and Examination of Membranes for Thermo tolerant Coliforms and *E.coli*

Incubate the membranes for 4 hours at 30 °C and then for 14 hours at 44 ± 0.25 "C°C. Count the yellow colonies within a few mintues of removal from the water bath or incubator.

Note: Provided that the membranes are not overcrowded, colonies of E.coli usually have characteristic apperance of bright yellow in colour and are more than I mm in diameter. Total incubation period of 18 hours is recommended for the enumeration of coliform organisms and *E.coli*. It is important to note that the counts of yellow colonies at 37 °C and 44 °C are only presumptive results and for potable water it is essential to carry out confirmative tests for coliform organisms and *E.coli* in order to asses the sanitary significance of the results.

Confirmation and Determination of Coliform Organisms

Subculture all yellow colonies or a representative number of them, in tubes of Lactose Pepton Water containing an inverted (Durham) tube in order to detect gas formation. Incubate these tubes at 37 °C and examine them for the presence of acid and gas after 24 hours, and if the results are negative re-examine after a further period of 24 hours. It is advantages to subculture after 6 hours of incubation from the Lactose Peptone Water and plate on Nutrient-Agar and Mac Conkey-Agar to check the purity and colonial appearance.

Confirmation of *E.coli*

Subculture all yellow colonies or a representative number of them in tubes of Lactose Peptone Water and in tubes of Tryptone Water.

Incubate them at 44 °C for 24 hours and examine the tubes of Lactose Peptone water for the presence of acid and gas. Add 0.2-0.3 ml of Kovac's reagent to the tubes of Triptone Water. Development of red colour indicates the production of indole.

Note: Yellow colonies on membranes incubated at 44 °C are regarded as *E. coil* if acid and gas are produced in Lactose Peptone Water and if the Indole Test is positive.

Exercise 2

TOTAL COLIFORM Counts 100 ml^{-1} - Multiple Tube Fermentation

Application

The method is applicable to potable water, surface water and effluents.

The method

Water samples or their dilutions are inoculated in a suitable broth (liquid nutrient medium). At the end of the incubation period, the tubes are examined for gas production by the coliform organisms. This test is known as a Presumptive Test since gas can also be produced by bacteria in addition to coliform organisms. The positive tubes of the presumptive test are subjected to a Confirmatory Test followed by a Completed Test.

Apparatus

Fermentation tubes
Inoculation loops
Water bath with a thermostat
Durham vials
Microscope
All the other basic materials required for total coli form counts Reagents and culture media
Potasium dihydrogen phosphate KH_2PO_4
Sodium hydroxide
Magnesium chloride
Mac Conkey's Broth (single strength medium: pH = 7.4-7.5)

Brilliant-green Lactose Bile Broth (pH = 7.2)

Peptone	10.0 g
Lactose	10.0 g
Ox bile (dehydrated)	20.0 g
Brilliant-green	13.3 mg
Distilled water	1l

Eosine Methylene Blue (EMB) Agar (pH = 7.1-7.2)

Peptone	10.0 g
Lactose	10.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	2.0 g
Agar	15.0 g
Eosin Y	0.4 g

Methylene blue	0.065 g
Distilled water	1l

Endo Agar (pH = 7.4)

Peptone	10.0 g
Lactose	10.0 g
Dipotassium hydrogen phosphate (K_2HPO_4)	3.5 g
Agar	15.0 g
Sodium sulphate	2.5 g
Basic fucshin	0.5 g
Distilled water	1l

Procedure

Presumptive Test

1. Select the dilution according to the expected bacterial count using
2. Select the number of tubes for each sample
3. Put Durham vials inverted in each test tube and put the media required. Sterilize the tubes at 121 °C for 15 minutes.
4. Shake all the water samples vigorously immediately before removing sample aliquots to inoculate the series of test tubes.
5. Add samples using sterilized pipettes to the test tubes selected for the test and mix thoroughly. Use separate pipettes for different samples as well as for dilutions. While withdrawing sample portions, the tip of the pipette should never be submerged more than 1 inch below the surface of the sample. This procedure minimizes the accumulative drainage from exterior of pipette into the media.
6. Place within 30 minutes, all these tubes in an incubator at 35-37 °C.
7. After 48 hours, examine each tube carefully. Those showing gas in the Durham vials are recorded as positive (+). Gas in any quantity even a tiny bubble is recorded as (+). The tubes showing Positive Test are subjected to Confirmatory Test, as gas production is not the only criterion for a positive test.
8. Discard all the Durham tubes first at the completion of 24 hours. Subject the tubes showing positive test immediately to a confirmatory test. Incubate negative tubes to a further period of 24 hours and observe any positive results.

Dilution of water samples for the Presumptive Test

Type of water (ml)	Sample volume (ml)	Culture volume (ml)	No. of tubes
Potable water	10	10 (double strength)	05
Surface water	10	10 (single strength)	05
(Unpolluted)	01	10 (single strength)	05
	0.1	10 (single strength)	05
	0.01	10 (single strength)	05
Polluted with	0.1	10 (single strength)	05
Domestic sewage	0.01	10 (single strength)	05
	0.001	10 (single strength)	05
	0.001	10 (single strength)	05

Confirmatory Test

Exercise 3

For this test. Brilliant-green Lactose Bile Broth (BGLB) is used.

1. Prepare fermentation tubes with 10 ml BGLB medium and put Durham vials inverted in each tube. The number of tubes to be prepared is equal to all positive tests in the presumptive tests.
2. Shake gently, the fermentation tubes of presumptive test with positive results and transfer one loop-full of medium to BGLB broth.
3. Incubate the tubes at 35-37 °C for 48+2 hours and record the tubes with gas formation as positive. Remember that wrong concentration of BGLB or the exposure of the media to excessive heat or light may give false positive tests.

Completed Test

Exercise 4

Since some of the positive results from the Confirmatory Test may be false, it is desirable to repeat the completed test occasionally. For this, inoculum from each positive tube of the confirmatory test is streaked on a plate of Eosine Methylene Blue (EMB) or Endo Agar.

1. Prepare Endo-Agar (5.7) or EMB (5.6) Agar Petri dishes. The number of Petri dishes to be prepared is the same as that of tubes showing gas production in BGLB medium.

2. Label the dishes with the corresponding numbers of the tubes of confirmatory test. Streak inoculum of the BGLB tubes on the Petri dishes in such a way that the colonies after separation have a distance of 0.5 cm.

3.Incubate these Petri dishes at 37 + 2 °C for 24 hours.

Now examine the dishes for bacterial growth and colony appearance. Well isolated colonies with a dark centre (nucleated) are the typical coliform colonies.

They may have a metallic surface sheen.

The colonies that are pink or opaque are not nucleated. They are typical colonies and may belong to the coliform group.

Clear, watery colonies are not of the coliform group and are reported as negative in the completed test.

Now inoculate a coliform colony isolated (avoid picking a mixture of colonies) from each place into the tubes of Mac Conkey's broth and record the gas production (a repetition of the presumptive test but with the colonies) within 48 hours at 37 °C.

Also examine the colonies by Gram Staining. For this, transfer the colonies to a nutrient agar slant. Subject the colonies obtained from agar slants to the Gram Staining.

If organisms appear rod (bacilli) shaped, red stained and occurring single or in pairs or in short chains, the test is confirmed. Since the coliform organisms are Gram-negative, if the Gram-positive organisms or spore forming organisms appear,this portion (completed) of the test is noted as negative.

FAECAL COLIFORM (*E. coil*) counts 100 ml^{-1} - Multiple Tube Fermentation

Application - The method is applicable to potable water, surface water and effluents.

The method

Conduct the presumptive test in the same way as the total coliform count but the confirmatory test should be carried out as outlined below.

E. coil medium
Lauryl Tryptose (Lactose) Broth (double strength)

Tryptose	40 g
Lactose	10 g
Sodium chloride	10 g
Di-potassilim hydrogen phosphate	5.5 g
Potassium dihydrogen phosphate	5.5 g
Sodium lauryl sulphate - specially pure	0.2 g
Distilled water	1l

Add the tryptose, sodium chloride, lactose and phosphates to the water and warm to dissolve. Add the sodium lauryl sulphate and mix gently to avoid froth. Adjust to pH 6.8. Prepare single strength medium by dilution of the double strength medium with an equal volume of distilled water. Distill single strength medium in 5 ml volumes and double strength medium in 10 ml and 50 ml volumes. Each tube or bottle should contain an inverted fermentation tube. Autoclave at 115 °C for 10 minutes.

Procedure- Presumptive Test

Perform presumptive test as of the total coliform count.

Confirmatory test

Carry out the test exactly as of the confirmatory test for total coliform count but use *E. coli* medium instead of BGLB an incubate the tubes at 44.5 "C in a water bath within 30 minute after inoculation.
Remove the tubes after 24 hours.
Shake the tubes gently and observe the gas production. The test is positive if any gas is produced. Such tubes may be subjected to the completed test.

Calculation of Most Probable Numbers (MPN)

The calculation of MPN of coliform organisms is done by combination of positive and negative results in the Multiple Tube Test. The values can be calculated for any of the combinations given in Table. (If three combinations, e.g., 10 ml, 1.0 ml and 0.1 ml have been used). The important thing to remember is that the positive and negative combinations of any one test can be used e.g., if a test has been carried out only upto the presumptive test stage, than the positive and negative combinations of this test can be used to calculate the MPN. If all three tests had been carried out, the MPN can be calculated on the basis of either presumptive, confirmatory or completed test.

MPN per 100 ml for various combinations of positive results when 5 tubes each of 10, 1 and 0.1 ml sample fractions are used

Combination	MPN per 100 ml	Combination	MPN per 100 ml
0-0-0	2	4-3-0	27
0-0-1	2	4-3-1	33
0-1-0	2	4-4-0	34
0-2-0	4	5-0-0	23
1-0-0	2	5-0-1	31
1-0-1	4	5-0-2	43
1-1-0	4	5-1-0	33
1-1-1	6	5-1-1	46
1-2-0	6	2-1-2	63
2-0-0	5	5-2-0	49
2-0-1	7	5-2-1	70
2-1-0	7	5-2-2	94
2-1-1	5	5-3-0	79
2-2-0	9	5-3-1	110
2-3-0	12	5-3-2	140
3-0-0	8	5-3-3	180
3-0-1	11	5-4-0	130
3-1-0	11	5-4-1	170
3-1-1	14	5-4-2	220
3-2-0	14	5-4-3	280
3-2-1	17	5-4-4	350
4-0-0	13	5-5-0	240
4-0-1	17	5-5-1	350
4-1-0	17	5-5-2	540
4-1-1	21	5-5-3	920
4-1-2	26	5-5-4	1600
4-2-0	22	5-5-5	2400
4-2-1	26		

AQS 103 10 Aquatic Microbiology and Water Quality
Practical NO. 5

Introduction

The staining procedures are used to characterizing and identifying bacteria. Within ew minutes it is possible to determine the group of organisms to which an unknown solate. Gram staining and observing cell shape and arrangement divide bacteria into at least four groups. An acid-fast stain of rods can determine whether or not you have an organism like *Mycobacterium*, while an endospore stain can indicate whether or not your unknown might be an endospore former such as *Bacillus*.

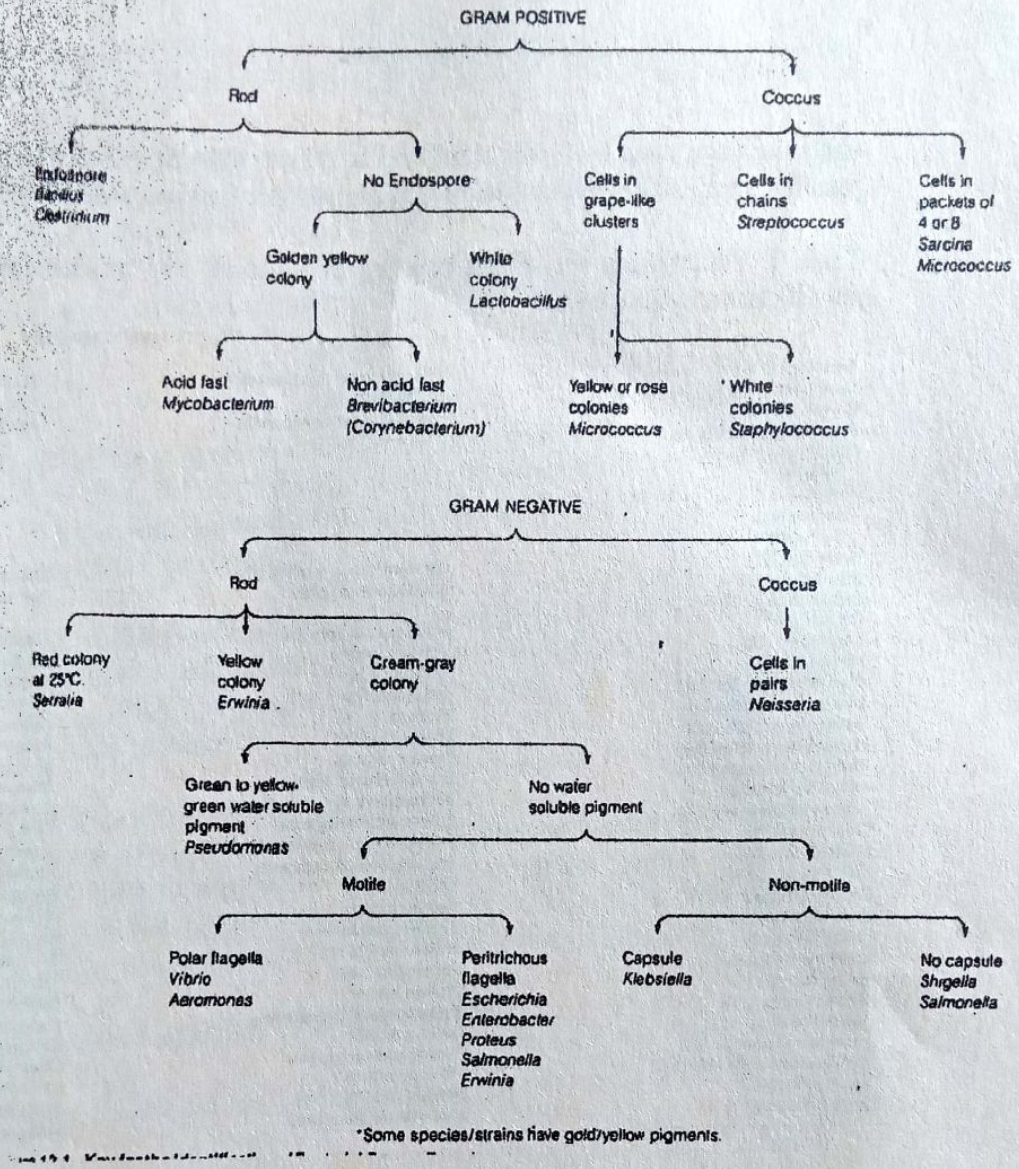
Grain-positive nonsporulating, non-acid-fast rods that have various shapes (pleomorphic), that line up parallel to each other like a picket fence (palisade arrangement), or that contain metachromatic granules are very likely coryneforms such as *Corynebacterium*.

Gram-positive cocci that form long chains are called *Streptococci* and are probably related to *Streptococcus*, while Gram-positive cocci that form cuboidal packets of 4, 8, 16, or 32 cells are known as sarcinae and may be related to *Sarcina* or *Micrococcus*. Gram positive cocci tending to occur in irregular "grapelike" clusters probably are related to *Staphylococcus* or *Micrococcus*. Cocci which are found in pairs are known as diplococc.
Gram-negative diplococci may be related to *Neisseria*, while Gram-positive diplococci may be related to *Streptococcus* or to *Staphylococcus*.

Exercise 1

Procedure

1. Carry out a Grain stain, acid-fast stain, capsule stain, endospore stain, and flagella stain on your unknown.
2. Determine the shape of your unknown and any distinctive arrangements of the cells.
3. Streak the unknown onto on nutrient plate and incubate it 24 to 48 hours at 35°C. characterize the colonial morphology and colour of the unknown.
4. Use the key to determine which organism you have.



Key for the identification of bacterial genera based on morphological and staining characteristics

Exercise 2
Identification of an unknown bacterium

The identification of unknown bacterial isolates is a common task of microbiologists. In the clinical laboratory, it is essential that microorganisms isolated from a patient be identified promptly and accurately so that the proper treatment can be initiated without delay. It is also important that microorganisms isolated from foods and beverages implicated in food poisoning be identified so that the appropriate control measures can be initiated and the outbreak stopped.

Bacteria may be identified on the basis of morphological, cultural, and biochemical characteristics. Morphological criteria such as the shape, size, and arrangement of cells are usually not sufficient to identify a bacterium unequivocally. Other

characteristics, such as Gram stain and acid-fast staining properties, colonial gro patterns, fermentation reactions, and assimilation of amino acids must be used.

Table 1. Summarizes the characteristics of cultural and biochemical tests used identify unknown bacteria.

TEST*	CONTROL MICROORGANISMS POSITIVE	NEGATIVE
Growth at 20°C (16)	*Pseudomonas fluorescens*	*Bacillus stearothermophil*
Growth at 35°C (16)	*Escherichia coli*	*Pseudomonas fluorescen*
Growth at 55°C (16)	*Bacillus stearothermophilus*	*Escherichia coli*
Colony characteristics (6)		
form		
elevation		
margin		
consistency		
pigmentation		
Gram stain (9)	*Staphylococcus epidermidis*	*Escherichia coli*
Acid-fast stain (9)	*Mycobacterium phlei*	*Escherichia coli*
Cell morphology (3)		
Capsule (10)	*Klebsiella pneumoniae*	*Staphylococcus aureus*
Endospores (10)	*Bacillus subtilis*	*Escherichia coli*
Motility (3)	*Proteus vulgaris*	*Staphylococcus aureus*
Glucose fermentation (24)	*Escherichia coli*	*Micrococcus luteus*
Lactose fermentation (24)	*Escherichia coli*	*Micrococcus luteus*
Maltose fermentation (24)	*Escherichia coli*	*Micrococcus luteus*
Sucrose fermentation (24)	*Proteus vulgaris*	*Micrococcus luteus*
Mannitol fermentation (24)	*Staphylococcus aureus*	*Micrococcus luteus*
Methyl red test (24)	*Escherichia coli*	*Enterobacter aerogenes*
Voges-Proskauer test (24)	*Enterobacter aerogenes*	*Escherichia coli*
Catalase test (25)	*Staphylococcus epidermidis*	*Streptococcus faecalis*
Oxidase test (25)	*Pseudomonas fluorescens*	*Escherichia coli*
Starch hydrolysis (23)	*Bacillus subtilis*	*Escherichia coli*
Casein hydrolysis (23)	*Bacillus subtilis*	*Escherichia coli*
DNA hydrolysis (23)	*Serratia marcescens*	*Escherichia coli*
Fat hydrolysis (23)	*Proteus mirabilis*	*Staphylococcus epidermid*
Indole production (26)	*Escherichia coli*	*Enterobacter aerogenes*
H_2S production (26)	*Proteus vulgaris*	*Escherichia coli*
Nitrate reduction (25)	*Pseudomonas fluorescens*	*Staphylococcus epidermid*
Gelatin hydrolysis (27)	*Bacillus subtilis*	*Escherichia coli*
Lysine decarboxylation (26)	*Enterobacter aerogenes*	*Citrobacter freundii*
Phenylalanine deamination (26)	*Proteus vulgaris*	*Escherichia coli*
Urea hydrolysis (27)	*Proteus vulgaris*	*Escherichia coli*
Citrate utilization (27)	*Enterobacter aerogenes*	*Escherichia coli*

An unknown may exist as a pure culture or as a mixed culture. If the unknown is r pure, it must first be purified before proceeding with its identification. Purificati usually is accomplished by streaking the mixture onto agar media. The purity of a cu ture (even if it was pure at one time) should be verified by performing the Gram sta and determining if all the bacterial cells seen in the stained smear have the sa general shape and arrangement.

Once the purity of the isolate has been established, the culture can be subjected to variety of tests to obtain a morphological and biochemical profile that will aid identification. Each test should be controlled to make sure that the culture medium sterile and suitable for the test performed, the reagents are able to detect the desi products, and that your techniques are adequate.

Once a biochemical and morphological profile is obtained for each of the isolat they are identified using a dichotomous key (Above table).

By choosing the characteristics that fit the unknown, eventually you are led to possible genus. It is always a good idea to verify your results by reading t description of the identified isolate in a reference book. Bergey's Manual Systematic Bacteriology is the major reference (Available). Use the latest editi available in your library or classroom and compare the description of your isol with the results that you obtained. Try to explain any discrepancies between yo results and those published in Bergey's Manual.

Procedure

First Period

1. Label the bottom of two TSA plates with your name, date, and unknown identification number. Label one plate for incubation at room temperature and the other at 35°C.

2. Streak both plates with your unknown sample in order to obtain well-isolated colonies of the unknown culture.

3. Incubate one of the plates at room temperature and the other at 35°C.

Second Period

1. Examine the plates for the presence of well-isolated colonies.
Since your unknown may be contaminated with a mixture of bacterial species, carefully examine the colonies on each plate to determine the extent of contamination and the characteristics of the unknown.

2. Mark well-isolated colonies of each of the types appearing on the plate by circling their location with a grease pencil on the bottom of the Petri plate.
Make sure that the colonies you select are on the streak pattern. Colonies off the streak lines may be contaminants.

3. Characterize selected colonies as follows:
   a. determine the colony's shape, margin, elevation, consistency, and color.
   b. determine the temperatures at which the organism grows by inoculating trypticase soy broths. Incubate the broths at 4°C, 20°C, 35°C, 45°C, and 55°C.
   c. determine the Gram reaction for each of the isolated organisms.

4. Subculture the purified organism onto slants of TSA.

5. Record your observations on the unknown report form.

Third Period

1. Verily the purity of your cultures by doing the following:
   a. examine the plates for the presence of one predominant colony. Make sure that you examine only those colonies appearing along the path of streaking.
   b. perform a Gram stain on several representative colonies in each of the plates. If the culture is pure, all the colonies will have cells of the same morphology and Gram staining characteristics.

2. a. If the cultures are pure, prepare fresh subcultures of each isolate on TSA slants. These two subcultures represent a stock that will serve as a source of bacteria for the tests to be performed in the fourth and any subsequent) period of this exercise.
   b. If your cultures are not pure, repeat the procedures outlined the second period of this exercise.

Fourth Period

1. Using the plates or slants prepared in the previous period, perform each of the tests indicated on the unknown report form.

Fifth Period

1. Using the dichotomous key in the above table, identify your unknown bacteria.
2. Compare the results you obtained with those published in Bergey's Manual of Systematic Bacteriology.

Using the unknown, report form, write a paragraph or two explaining your results, how you arrived at the identity of your unknowns, and any discrepancies that may be evident between your results and the descriptions in Bergey's Manual.