جامعة الملك سعود
King Saud University
1957

Practical of General Microbiology

MIC 140

Instructor by

Ali Al-Harbi

Ahmed Al-Obaidi

(علم الاحياء الدقيقة) 140 حدق

نرحب بالطلاب المسجلين في مقرر ونتمنى لهم التوفيق والسداد وهناك بعض التعليمات التي يجب على الطالب الالتزام بها:

1- الالتزام بالحضور في وقت العملي والطالب المتأخر لن يسمح له بدخول المعمل.
2- يجب إحضار البالطو الخاص بالعملي.
3- سيتم تقسيم الطلاب إلى مجموعات داخل المعمل وكل مجموعة تكون مسئوله عن مكان العمل المخصص لها.
4- على كل طالب كتابة تقرير عن كل عملي بحيث يتم تسليمه في الأسبوع الذي يليه.
5- درجات الجزء العملي 30 درجة.

توزيع عملي مقرر 140 حدق على الاسابيع

No. week	Practice title
First week	Safety roles in the laboratory, Introduction of microbiology, laboratory equipment's and materials
Second week	Light microscope parts and examination.
Third week	Sterilization Techniques
Forth week	Preparation culture media of microbiology.
Fifth week	Isolation organisms from different sources.
sixth week	Purification of organisms.
Seventh week	Morphology colonies of bacteria & fungi.
Eighth week	**Midterm exam**
Ninth week	Smear preparation and simple stain.
Tenth week	Staining of bacteria by gram stain.
Eleventh week	Staining & identification of fungi.
Twelfth week	Examination of algae
Thirteenth	Revision
Fourteenth	**Final exam**

مدرس العملي:

أ/ علي عقيل الحربي **أ/ أحمد سعد العبيدي**

PRACTICAL No: 1

Lab safety

A- Safety rules in the laboratory in general:

1-Don't eat, smoke or drink inside the laboratory.
2-Don't put anything in your mouth such as pens, fingers, ect.
3-Don't take your personal belongings that that don't you need.
4-Wash your hand frequently after work by soap and water.
5-Wear lab coat, gloves, musk and goggles.
6- Change your coat twice per week.
7-Avoid un necessary talking and walking in the laboratory.
8-Clean the bench before and after the work by disinfectant

B- Equipment safety rules:

1-If the equipment not work properly stop using it and inform the senior.
2-Don't carry hot flasks of media by the neck, use a basket and wear heat resistant gloves.
3-Don't open the centrifuge until the rotator has stopped.
4-Don't open the autoclave until the contents have cool to about 45c.

C- Safety rules in the reagent and chemicals:

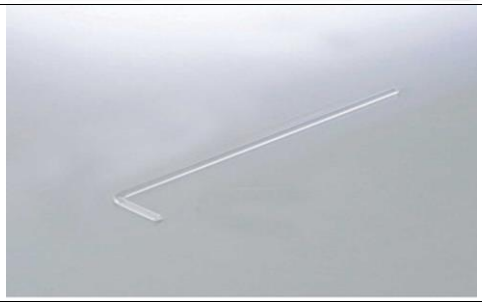
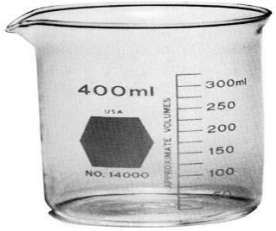
1-Read in the instruction on the bottle before transporting or using any chemicals or reagents.
2-Wear gloves and goggles when handling dangerous liquid or chemicals.
3-When diluting acid always add acid slowly to water.

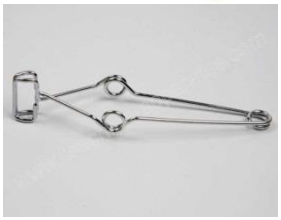
D- Safety rules at the end practical:

1-Put way all culture and equipment.
2-Wipe down the bench with disinfectant.
3-Turn off and unplug equipment that is not required.
4-Turn off and all Bunsen
5-Turn off any gas cylinder not in use.
6-Remove the gloves and wash your hands by soap and water.

Introduction of Microbiology

Laboratory Materials

Name	Equipment	Use
Forceps (metal/plastic)		Transfer of sterile paper/antibiotic discs; also plant material, e.g. short lengths of root with nodules
Spreader (glass/plastic)		Making spread/lawn plates
Bunsen burner		Sterilization of wire loops and (with alcohol) metal forceps and glass spreaders
- Flask **-Test tube** **- Beaker**	  	-Used for making solutions or for holding, containing, collecting, or sometimes volumetrically measuring chemicals, samples, solutions. -Is a cylindrical container used to store, mix and heat liquids in laboratories. -Small volumes of liquid media/agar slopes/sterile solutions for inoculation (held in test tube rack; dry non-absorbent cotton wool plug or plastic cap prevents contamination)

-Graduated Cylinder **- Pipette (glass/plastic)**	 	- used to measure the volume of a liquid. -Transfer of measured volumes/drops of culture/sterile solutions (dry,non-absorbent cotton wool plug in neck prevents contamination)
Petri dish (plastic/glass)		Plastic: pre-sterilized for streak/spread/lawn/pour plates Glass: only for materials for sterilization by hot air oven, e.g. paper discs
-Loop (wire/plastic) **-Needel (wire/plastic)**		Routine inoculation of agar slopes/deeps and small volumes of liquid media (up to ca 10 cm3); making streak plates
-Test Tube Rack **-Test Tube Clamp**	 	- Used to hold upright multiple test tubes at the same time. - Is used to hold test tubes

Funnel		Used for pouring liquids or powder through a small opening and for holding the filter paper in filtration. Used in transferring liquids in small containers
Autoclave		Sterilization of media, solutions and equipment before use and contaminated items afterwards; melting solidified agar media for use 121 °C for around 15–20 minutes.
Incubator		Incubation of cultures (but many cultures will grow at room temperature in the interval between lessons)
Personal protective equipment (hood)		protection of clothing, containment of dust on clothing Safety spectacles: not considered essential when dealing with suitable cultures and observing GMLP, but may be required by local regulations and for dealing with chemicals
Digital balance		Is a class of balance designed to measure small mass in the sub-milligram range

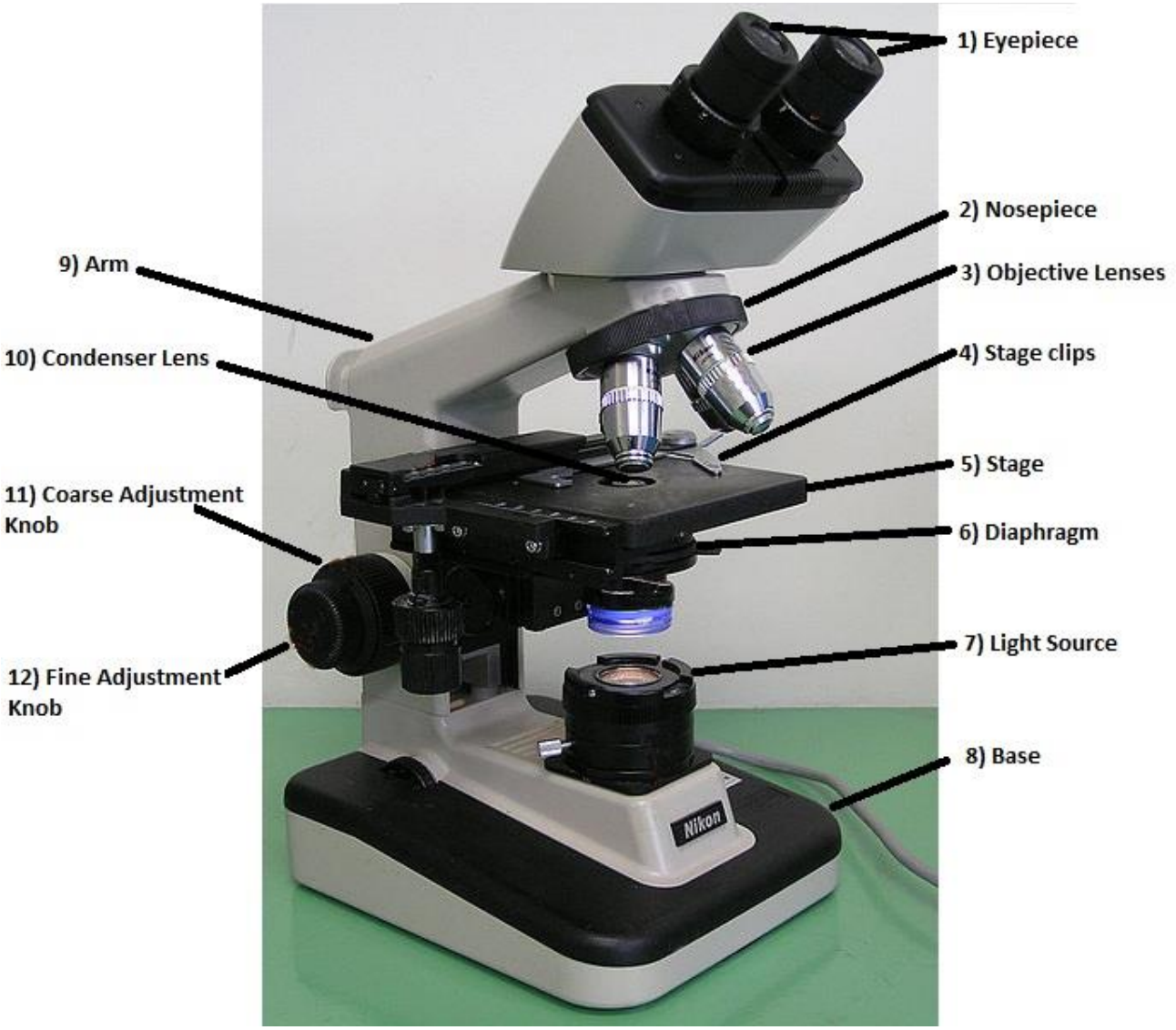
-Slides **-Cover Slips**		- Used to hold objects for examination under a microscope.
Centrifuge		Is a laboratory device that is used for the separation of fluids, gas or liquid, based on density.
Water bath		Suitable temperature for keeping melted agar media molten for use (50 °C); accurate temperature control
Hot air oven		Sterilization of glass Petri dishes and pipettes and paper discs (but not essential as autoclaves/pressure cookers serve virtually all needs)

PRACTICAL No: 2

Light microscope

Objectives:

The optical microscope, the most common type of microscope, contains several parts with specific functions. Observe the picture and find their functions.



1. **Eyepiece**: contains the ocular lens, which provides a magnification power of 10x to 15x, usually. This is where you look through.
2. **Nosepiece**: holds the objective lenses and can be rotated easily to change magnification.
3. **Objective lenses**: usually, there are three or four objective lenses on a microscope, consisting of 4x, 10x, 40x and 100x magnification powers. In order to obtain the total magnification of an image, you need to multiply the eyepiece lens power by the objective lens power. So, if you couple a 10x eyepiece lens with a 40x objective lens, the total magnification is of 10 x 40 = 400 times.
4. **Stage clips**: hold the slide in place.
5. **Stage**: it is a flat platform that supports the slide being analyzed.
6. **Diaphragm**: it controls the intensity and size of the cone light projected on the specimen. As a rule of thumb, the more transparent the specimen, less light is required.
7. **Light source**: it projects light upwards through the diaphragm, slide and lenses.
8. **Base**: supports the microscope.
9. **Condenser lens**: it helps to focus the light onto the sample analyzed. They are particularly helpful when coupled with the highest objective lens.
10. **Arm**: supports the microscope when carried.
11. **Coarse adjustment knob**: when the knob is turned, the stage moves up or down, in order to coarse adjust the focus.
12. **Fine adjustment knob**: used fine adjust the focus.

PRACTICAL No: 3

Sterilization and disinfectant Techniques

Objectives:

Sterilization

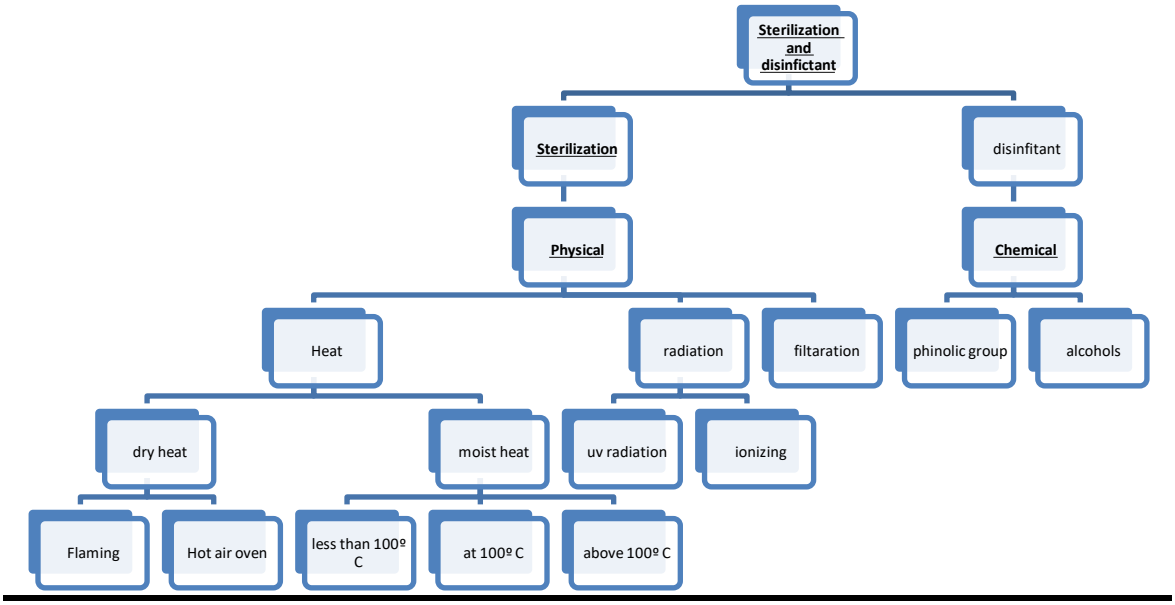
- **Freeing of an environment from all living microorganisms includes bacteria and their spores, fungi, parasites and viruses.**

Disinfection:

- **Is removing of pathogenic microorganism or reducing their number on the exposed area.**

Sterilization methods:

- **Physical methods.**
- **Chemical methods.**



Physical methods

- **Dry Heat (Flaming):**
  - **Exposure of the objects to heat will kills microbes by coagulation of protein, denaturation of enzymes and oxidation.**
  - **Exposure of wires and forceps to the Bunsen flame until it becomes red hot, then cool down and use.**
  - **Used for loop, forceps, and metal rods.**
  - **Used for sterilization of the mouth of bottle, flasks, containers and test tubes, smear slides etc,,,.**

Hot air oven:

- **Instruments consist of heater, oven.**
- **Used for sand, powder, metal, glass.**

- **Thermal death point and Thermal death time:**
  - **160C for 60 min.**
  - **180C for 30 min.**

Moist heat:

Less than 100C

- **Pasteurization of milk:**
  - **Holding method (65C for 30 min)**
  - **Flash method (72C for 20 sec)**
- **Preparation of vaccine:**
  - **By heating at 56C for 30-60 min.**

At 100 C

- **Steaming:**
  - **Single exposure of the microbe to steam at 100C for 90 min.**
- **Boiling:**
  - **Boiling water is the most common form of application of moist heat but is not capable of killing endospores or killing all viruses**
  - **At 100^{0} C for 30 min.**

Above 100C (Autoclaving)

- **Depends on steam and pressure.**
  - **Steam is a hot air able to penetrate through things.**

- **Pressure will rise the temperature from 100C to 121C.**

- **Moist heat is more effective than dry heat at a given temperature or length of exposure. also more penetrating than dry heat**
- **Make complete killing of bacteria, their spores, fungi and their spores, parasites and viruses including Envelop and non Envelop virus.**
- **Thermal death point and thermal death time:**
  - **121C for 15 - 20 min.**
  - **Flash autoclaving at 134C for 4-5min.**

- **Filtration:**
  - **Sterilization through removing of microbes from fluids by exposing to small size filter. Used for heat sensitive fluids like serum, antibiotic, suger, and urea.**
- **Radiation:**
  - **Exposure to irradiation causes denaturation of proteins and enzymes.**

Disinfection

Phenolic group of disinfectant:

- **E.g.: Phenol crystal, Dittol, Lysol, Cresol.**
- **Remain active, stable and persist for long period of time.**
- **Active against G+ve, G-ve, Mycobacterium & viruses.**

Alcohols:

- **E.g.: Anti-bacterial, sanitizer.**
- **They able to act and Evaporate, short period of time..**

PRACTICAL No: 4

Culture Media Preparation

Objectives:

- To be familiar with different types of culture media used in a microbiology laboratory.
- To be familiar with the pure technique for pouring sterilized media into Petri dishes or tubes.
- To prepare antigen, toxins, vaccines.
- To isolate pathogens from pathological specimens in pure culture to identify them and test their sensitivity to antibiotics for helping treatment the patients.

Introduction:

- **Common ingredients of culture media:**
  1- **Peptone:** provides nitrogen for growing microorganisms may be Carbohydrates, minerals and vitamins.
  2- **Meat extract:** provide microorganisms with amino acids vitamins and mineral salts.
  3- **Yeast extract:** a bacterial growth stimulant.
  4- **Mieral salts:** sulphates, phosphates, sodium chloride, potassium, iron and calcium.
  5- **Carbohydrates:** simple and complex sugars are add to media for following purposes:
  A- Provide pacteria with carbon and energy.

B- Assist in the differentiation of bacteria, example: lactose is added to MacConkey agar to differentiate enterobacteria. Fermentation of sugar with acid production is detected by change in color of indicator.

6- **Agar:** An inert polysaccharide extracted from some seaweed, assist solidify of culture media, and its solidify at 45˚C and dissolved at 90-100˚C.

7- **Water:** it is essential for growth all microorganisms.
Distilled water is better used in preparation of culture media and must be free from chemicals which inhibit bacterial growth.

- ## Classification of Culture media:

1-Culture media can be classified by consistency (physical state) as:

1) Liqid:	•also know as Broth •e.g. Peptone Water, Nutrient Broth (tubes or bottle)
2) Solid:	•consist (1-2%) agar. •e.g. Blood Agar (Petri dishes, bottle or tubes in tow forms slant and deep)
3) Semisolid:	• consist (0.5%) agar (tubes or bottle)

2-Culture media can also be classified based on the function and the chemical components:

Type of media	Remark
1- Simple (Basic) Media	These are simple media such as(**nutrient agar**, **nutrient broth** and **potato dextrose agar**)that will support the growth of microorganisms that don't have special nutritional requirement.
2- Enriched Media	An enriched media is a broth or solid medium containing a rich supply of special nutrients (e.g. **Blood**, **serum** or **egg**) that promotes the growth of **fastidious** organisms. It is made by adding extra nutrients to a nutrient agar (basic medium). They support the growth of pathogens that require additional nutrients as ***Hemophillus influenza***, pathogenic ***Neisseria*** several ***Strptococcus*** species. Commonly used examples are **Blood agar** and **Chocolate agar**, both of which are rich in nutrients and free from inhibitory substances.
3- Selective Media	These media contain substances (e.g. bile salt or other chemicals, dyes, antibiotics) which inhibit the growth of one organism to allow the growth of another to be more clearly demonstrated e.g. **XLD** (**Xylose Lysine Deoxycholate agar**) and **DCA** (**Desoxycholate Citrate Agar**) are used for the isolation of ***Salmonella*** and ***Shigella*** species.
4- Differential (Indicator) Media	These media contain a substance that is changed visibly as a result of the metabolism of particular organism e.g. **Kliger Iron Agar** (**KIA**) and **Triple Sugar Iron agar** (**TSI**), both differentiate between different types of Enterobacteriaceae on the basis of their ability to ferment carbohydrates.
5- Selective and Differential Media	An example of this is **MacConkey Agar** which is **selective** for enteric and other Gram-negative bacteria while inhibits gram-positive bacteria, and has an **indicator (neutral red)** to differentiate between **lactose** and **non-lactose** fermenting organisms. Another example, **MSA** (**Mannitol Salt Agar**) is selective and differential to grow ***Staphylococcus aureus***.
6- Enrichment media	Fluid media containing substance which stimulate the growth of certain bacteria such as ***salmonella*** and ***Shigella*** species. Example: **Tetrathionate broth** and **Selenite broth**.
7- Transport media	These are mostly semi-solid media> their use is important when specimens cannot be culture soon after collection, when transporting microbiological specimens (swabs) from health centers to microbiological laboratory. Example: **Amies transport medium** for swabs.
8- Anearobic media	These media are used to grow anaerobic organisms. Example: **Robertson,s cooled meat medium**.

Materials:

1-Powder media.
2- Balance.
3- Distilled water.
4- Spoon.
5- Bottle or flask.
6- Cylinder.
7-Filter paper.
8- Time Med labeling.
9-Marker pen.
10- Autoclave.
11-Petri-dishes or tubes.
12- Cotton or tissue.
13-Water path.
14- Safety cabinet.
15-Incubator.
16- Parafilm.
17-Refrigerator.

Procedure (Methods):

1- Read the label on the powdered media for instructions, on how to use, before preparation of the media.
2- Rinse all glassware in sterile distilled water before using.
3- Weighting powder media in a clean dry and dust free atmosphere. Use glassware (flask or bottle). The capacity of bottle or flask should be twice the volume media being prepare.
4- Label the flask or bottle (name of media).
5- Dissolving powder media with distilled water. Mix wills the bottle or flask until dissolve the powder. If media not dissolved, use heat and avoid boiling.
6- Autoclaving the media. Use autoclave by correct method. Use Time Med Labeling. Autoclaving at 121-126˚C under 15 Pa for 15-20 minutes.
7- Dispenses media. Cooled media until 48-50˚C. pour media in petri dishes about 15-20ml.
8- Labeling all petri dishes or tubes.
9- Perform sterile testing before using media. Incubation all petri dishes or tubes at 37˚C for 24h.
10-Store media at 2-8˚C.

Results:

Student group	Name of media	Solidify media	Type of media	Remark

Discussion:

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PRACTICAL No: 5

Isolation organisms from different sources

PRACTICAL No: 6

Purification of organisms

Objectives:

- To obtain a pure culture by isolating single colonies.
- To be familiar streak plate technique.
- To describe bacterial colony shapes.

Introduction:

There are two basic methods for obtaining a pure bacterial culture, the Streak Plate method, and the Pour Plate method. Both techniques are based on the assumption that a single bacterium on the agar surface will give rise to one colony. All bacterial cells within a colony are clones of the original bacterium that landed there when the plate was streaked or poured.

In the **Streak Plate** method, a sample of bacteria is streaked onto an agar plate using a loop and the loop is sterilized. Another set of streaks is made by crossing the first set of streaks a few times to pick up a few bacteria and then streaking back and forth to spread these out. The loop is flamed again, and the process repeated two more times. In the last set of streaks, the bacteria will be spread thinly enough that individual colonies will be well isolated from one another. The investigator can then pick individual colonies, which may represent pure cultures. Typically, additional streak plates are necessary before a culture can be termed "pure", and this must be confirmed with microscopic observations and physiological tests.

structure(cultural characteristics) of bacterial colonies on an agar plate. Features of the colonies may help to pinpoint the identity of the

bacterium. Different species of bacteria can produce very different colonies.

Materials:

1-Bacterial cultures. 2- media plates.
3-Wire or plastic loop. 4- Bunsen burner.
5-Marker pen. 6-Petri dishes.
7-Disinfectant. 8-Incubator.
9-Cotton or tissue. 10-Safety cabinet.
11-Colony count.

Procedure (Methods):

1- After sterilizing the loop on the flame, cool it, and then take a loopful from the culture.

2. Inoculate plate with a inoculating loop taken from the culture to make a primary inoculum.

3. Sterilize loop after step "2" and allow it to cool. Turn the plate anti-clock wise and by going back across the initial inoculum, streak across the surface of the agar.

4. Sterilize loop and allow it to cool. Repeat step”3” again, this time goes back across the first streak and spread across the surface of the agar.

5. Again sterilize, then repeat the streak one last time across the second streak and spread across the surface.

6. Finally, sterilize inoculating loop then streak the remainder of the plate in a zig-zag fashion.

7. Incubate the plate at 37 ˚C for 24 hours.

(See figure1)

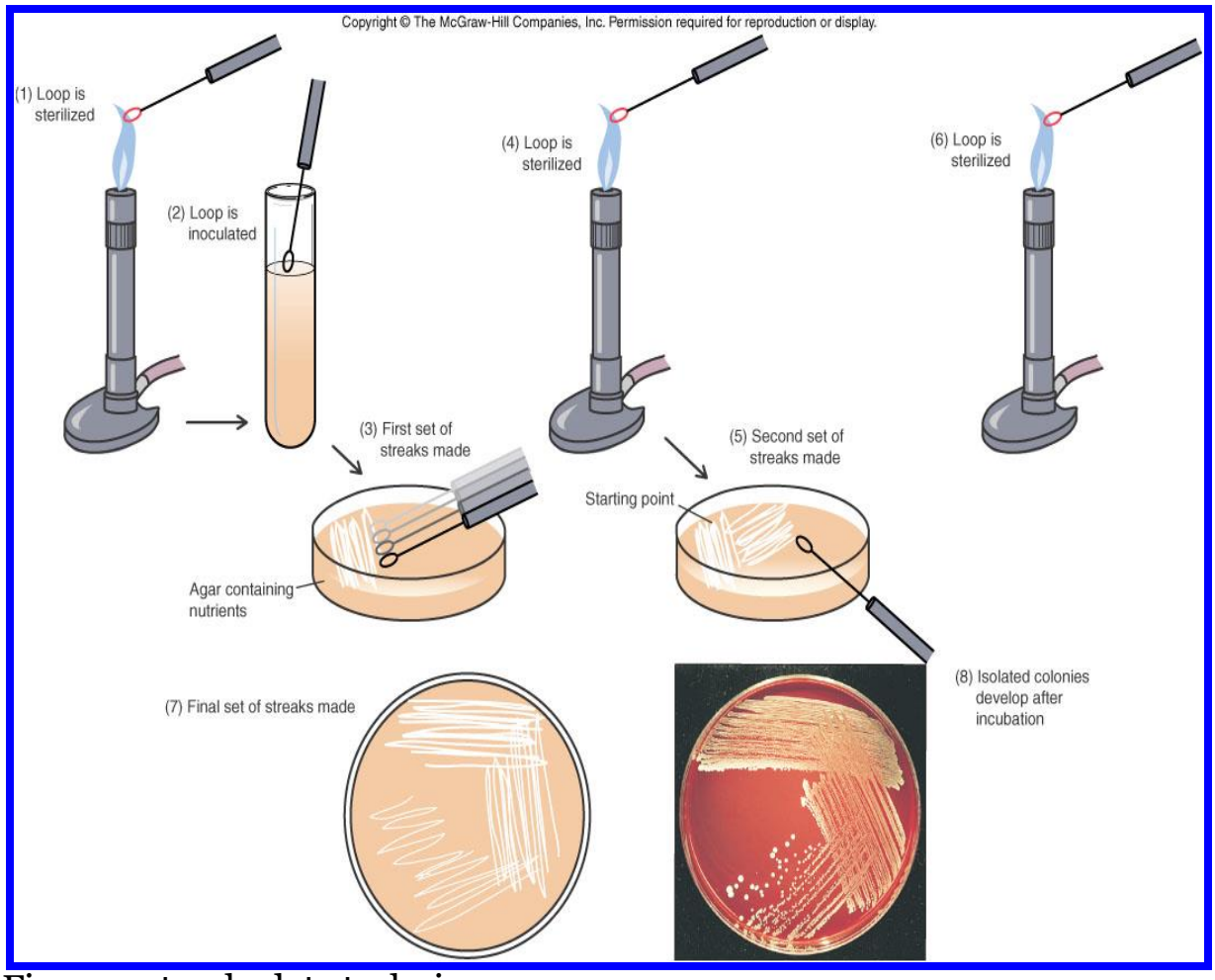

Figure1: streak plate technique

Result:

After incubation period, observe the plate for single isolated colonies on media agar.

Student group	Type of Samples	Single Colonies				
		Colony shape	Colony size	Colony color	Colony surface	Colony property

Discussion:

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Inoculation and incubation of bacteria (Aseptic Technique)

Objectives:

- **Introduce bacteria into a growth medium using "aseptic technique" to prevent contamination.**
- **Compare bacterial growth with types of media.**
- learn aseptic techniques for working with bacterial cultures.
- learn methods for transferring and working with bacterial cultures aseptically.
- **learn to transfer broth culture to broth, slant culture to broth, broth culture to slant, and slant culture to plate culture.**

Introduction:

Microorganisms include bacteria, fungi, and molds, and they are everywhere in the air, in water, and on every kind of surface imaginable. Their widespread presence means that microbiologists have to take certain precautions when working with bacteria to avoid contamination. When studying bacteria, it is important that the bacterial culture be pure, meaning that it contains only cells of that bacterial species. Otherwise, the results of any tests or procedures are meaningless. From a clinical standpoint, it is imperative to avoid cross-contamination of samples, lest the wrong diagnosis be made.

Materials:

1- media plates and tubes (solid/broth).
2- Wire or plastic loop.
3- Bunsen burner.
4-Differinatial specimens.
5-Marker pen.
6-Petri dishes or tubes.
7-Disinfectant.
8-Incubator.
9- Cotton or tissue.
10- Gloves.
11- Safety cabinet.

Procedure (Methods):

Part 1: Transfer of bacteria from broth to broth:

1- **label plates and tubes, and light Bunsen burner.**
2- **After sterilizing the loop on the flame, cool it, and then take a loopful from the broth culture.**
3- With the loop in your dominant hand, pick up the broth culture in the other hand and shake it gently to make sure it's mixed.
4- With the little finger of the hand holding the loop, grasp the cap of the tube and pull it off.
5- Immediately flame the mouth of the tube, but do not hold it in the flame too long time.
6- **Insert the loop into the culture, then remove the loop.**
7- flame the mouth of the culture, and replace the cap. Set the tube down in the rack.
8- Pick up the new tube of sterile broth, remove the cap as before, and flame the mouth of the tube.
9- Without flaming the loop, insert the loop containing some of the original culture into the new tube.

10-Remove the loop and flame the mouth of the tube before replacing the cap, finally flame the loop to sterilize it.

10- Incubate the plate at 37 ˚C for 24 hours.

Part 2. Transfer of bacteria from agar(slant) to broth:

1- With a loop sterilized and cooled as described in Part 1 in one hand, pick up agar slant culture of bacteria in the other. Remove the cap as before, and flame the mouth of the tube.
2- Insert the loop and pick up a small amount of bacterial growth. Remove the loop, flame the mouth of the tube, and replace the cap. Put the culture back in the rack.
3- Pick up a fresh tube of nutrient broth, remove the cap, and flame the mouth of the tube.
4- Insert the loop containing the bacteria from the slant culture and stir gently to dislodge the bacteria into the broth.
5- Remove the loop and flame the mouth of the tube before replacing the cap, finally flame the loop to sterilize it.
6- Incubate the plate at 37 ˚C for 24 hours.

Part 3. Transfer of bacteria from broth to agar:

1- With a loop sterilized and cooled as described above, pick up broth culture of bacteria. Remove the cap as before, and flame the mouth of the tube.
2- Insert the loop to get a drop of culture. Remove the loop, flame the mouth of the tube, and replace the cap. Put the culture back in your rack.

3- Carefully lift the lid of agar plate with the hand that is not holding the loop. Do NOT set the lid down anywhere at anytime, and try to avoid breathing on the plate while the lid is off.

4- Carefully streak the loop back and forth over the surface of the plate.

5- Put the lid back down and flame loop.

6- Incubate the plate at 37 ˚C for 24 hours.

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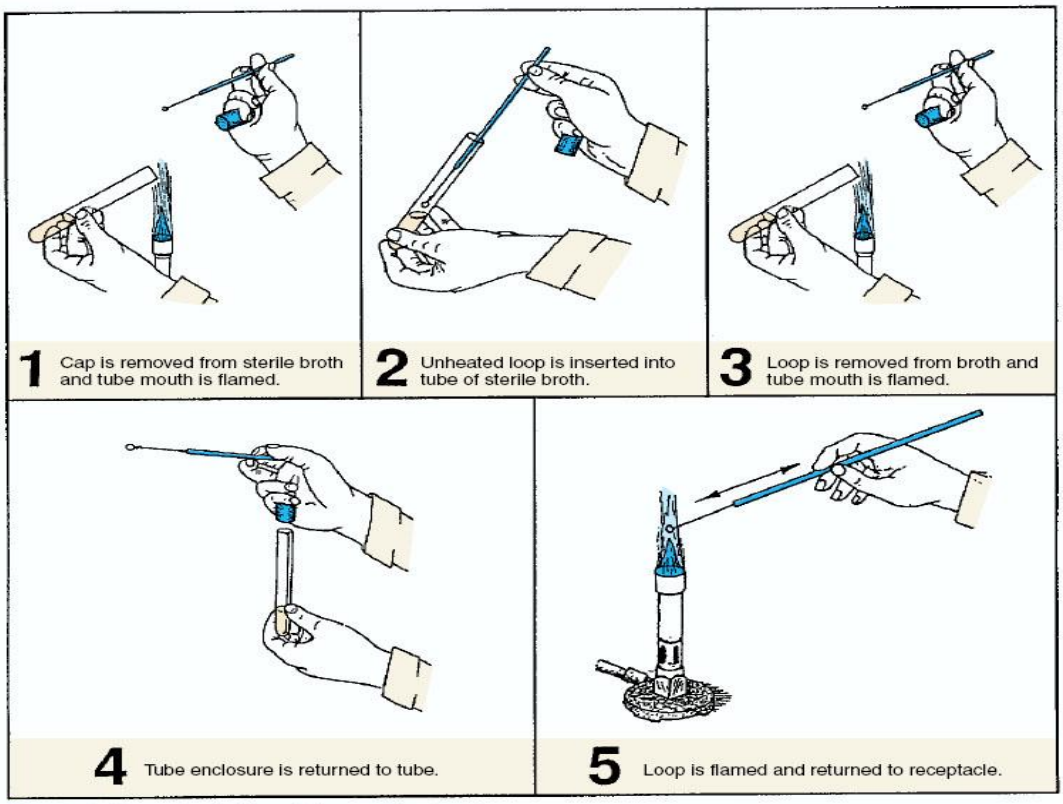

Figure 2 Procedure for inoculating a nutrient broth

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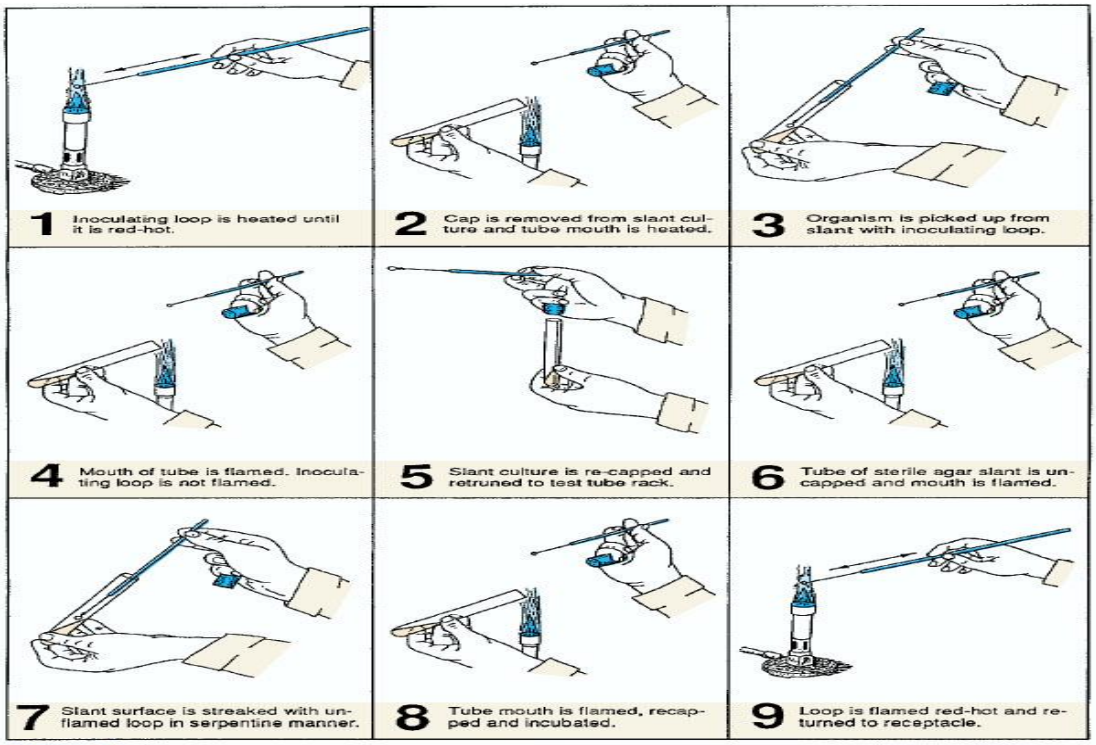

Figure 4 Procedure for inoculating a nutrient agar slant from a slant culture

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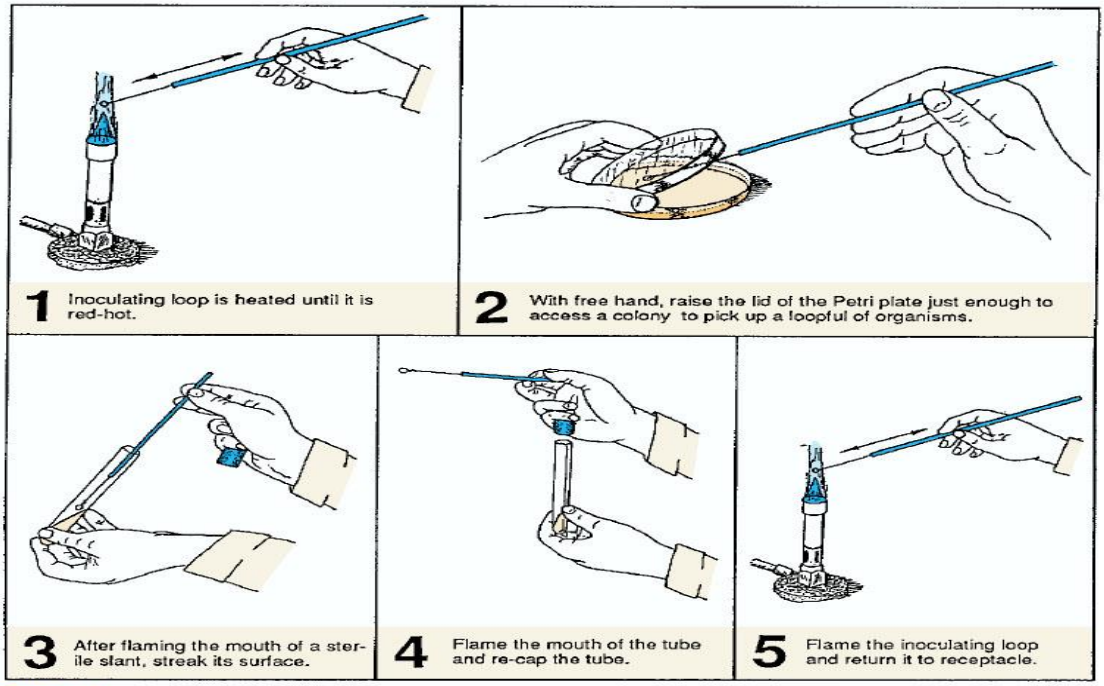

Figure 3 Procedure for inoculating a nutrient agar slant from an agar plate

Result:

After incubation period, observe the plates and tubes and determine bacterial growth.

A) Plates:

B) Tubes:

1- Broth:

2- Slant and deep:

Discussion:

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PRACTICAL No: 7

Morphology colonies of bacteria & fungi

PRACTICAL No: 8

Staining of bacteria

Smear and simple stain preparation

Objectives:

- Prepare bacterial smears for the microscopic visualization of bacteria.
- Perform a simple staining procedure.
- Compare the shapes and arrangements of bacterial cells.

Introduction:

Smear:

Much of your success in staining microbes will come from preparing a good smear. In doing so, you must keep in mind three key goals. First, you want to adhere the cells to the slide so that they are not washed off in subsequent procedures. Second, in doing so, you do not want to overheat the smear so that the cells shrink and you are left with distortion and artifacts. Third, you need to prepare a thin smear, because the thickness will determine whether or not you can visualize individual cells, their arrangement, or details regarding gram reaction or internal structure.

Simple stain:

The use of a single stain or dye to color a bacterium is called a simple stain. These types of dyes, called basic dyes. Some common basic dyes used in staining are methylene blue, crystal violet, and basic fuchsin.

Materials:

1- Bacterial growth (fresh)
2- Wire or plastic loop.
3- Bunsen burner.
4-Microscope slide.
5-Marker pen.
6-Distilled water.
7-Disinfectant.
8-Pasture pipettes.
9- Filter paper or tissue.
10- Gloves.
11- Safety cabinet.
12- simple stain
13- Light microscope.
14- oil emersion.

Procedure (Methods):

A-Smear preparation:

1. Clean and dry microscope slides thoroughly.
2. With the pen (permanent ink)/pencil, mark the name of the bacterial culture in the far left corner on each slides.
3. Flame the inoculating loop.
4. For the broth culture, shake the culture tube and, with an inoculating loop, aseptically transfer 1 to 2 loopfuls of bacteria to the center of the slide. Spread this out to about a 1/2-inch area.
5. preparation a smear from a slant or plate, place a loopful of water in the center of the slide.
6. With the inoculating loop, aseptically pick up a very small amount of culture and mix into the drop of water. Spread this out as above.
7. Allow the slide to air dry, or place it on a slide warmer.
8. Pass the slide through a Bunsen burner flame three times to heat-fix and kill the bacteria.

B- simple stain preparation:

1. Prepare a heat fixed smear of the culture to examination.
2. Cover the smear with methylene blue .
3. Allow the dye to remain on the smear for approximately 1 minute. (Note staining time is not critical. Somewhere between 30 seconds and 2 minutes should give you an acceptable stain. The longer you leave the dye on, in general, the darker the stain).
4. Wash the excess stain off the slide Pick up the slide by one end and hold it at an angle over the staining tray. Using the distilled water wash bottle, gently wash off the excess methylene blue from the slide by directing a gentle stream of water over the surface of the slide.
5. Wash off any stain that got on the bottom of the slide as well.
6. Blot off excess stain using bibulous paper. DO NOT rub the slide, rather place the slide between two sheets of bibulous paper and press down gently. Paper will absorb excess dye.
7. Examine the slide under the brightfield microscope.
8. Record the shape, arrangement, and approximate size of the organisms.(see figure1).

Results and observations:

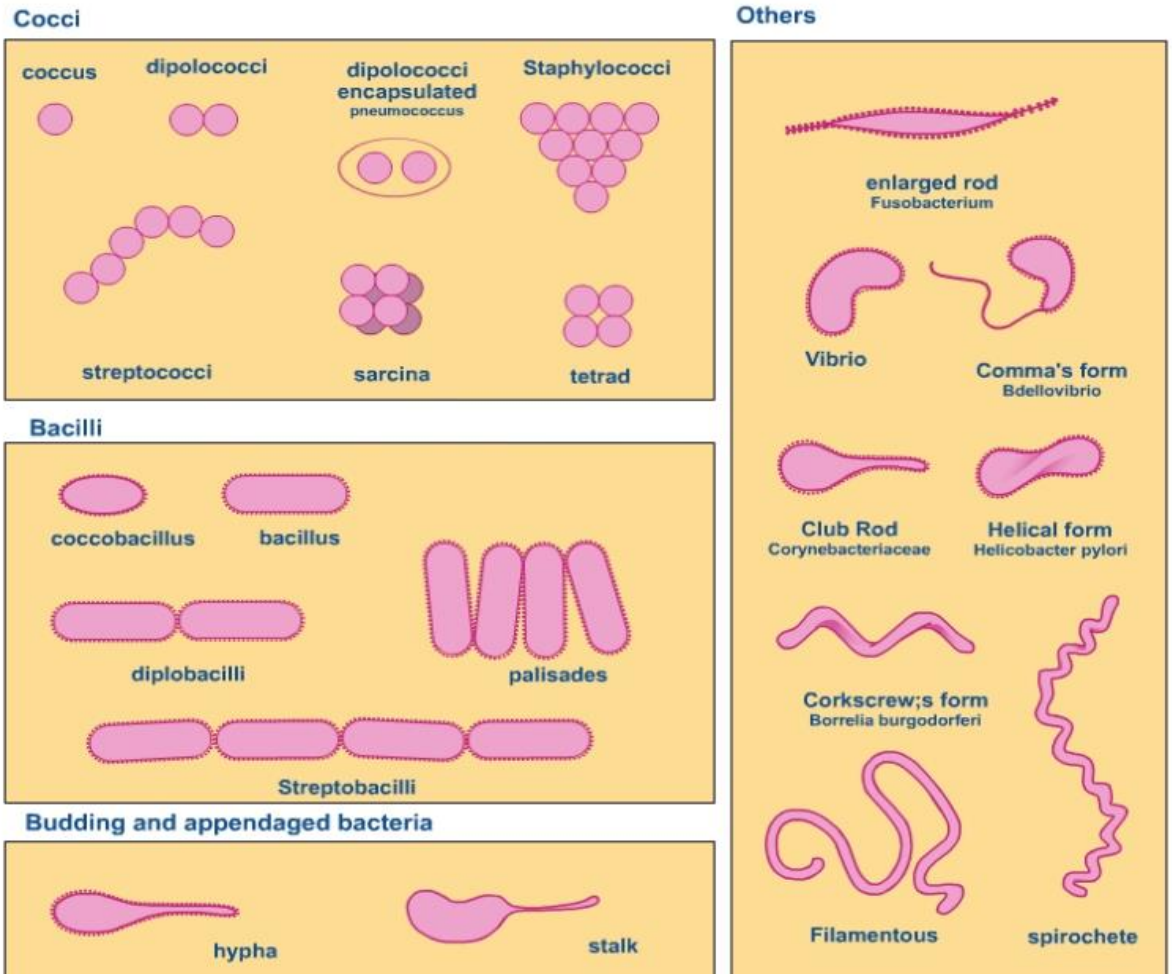

Fig1: Different bacterial morphology

Student group	No. of slide	Shape of bacteria	Arrangement

Discussion:

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PRACTICAL No: 9

Gram Stain Technique

Objectives:

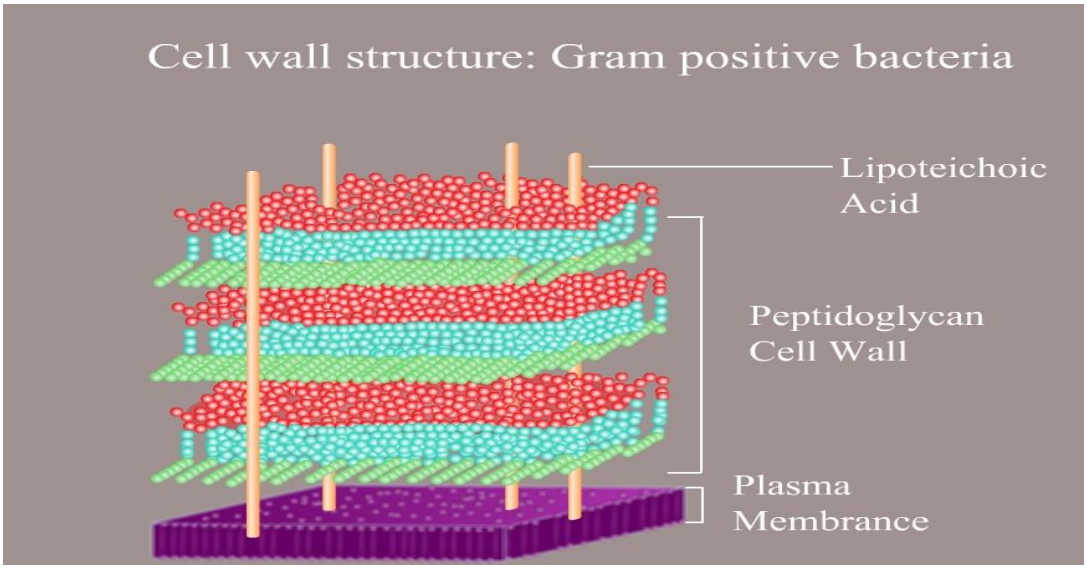
- To differentiate between the two major categories of bacteria: Gram positive and Gram negative.
- To understand how the Gram stain reaction affects Gram positive and Gram negative bacteria based on the biochemical and structural differences of their cell walls.

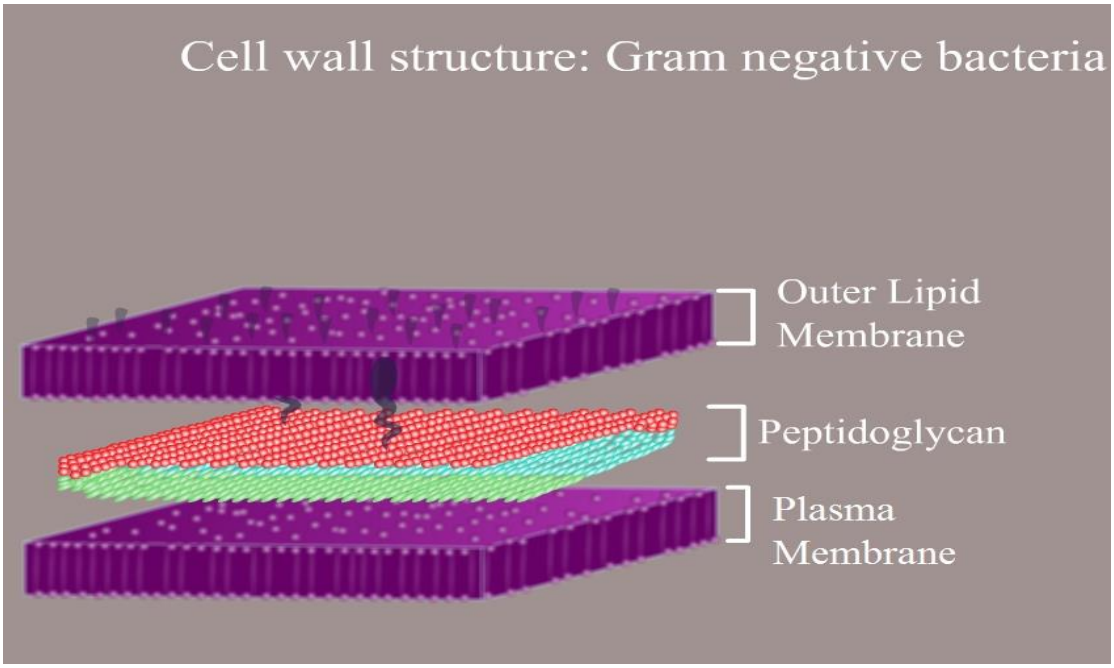
Principle:

Staining is an auxiliary technique used in microscopic techniques used to enhance the clarity of the microscopic image. Stains and dyes are widely used in the scientific field to highlight the structure of the biological specimens, cells, tissues etc.

The most widely used staining procedure in microbiology is the Gram stain, discovered by the Danish scientist and physician **Hans Christian Joachim Gram** in 1884. Gram staining is a differential staining technique that differentiates bacteria into two groups: gram-positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by the alcohol, losing the color of the primary stain, purple. Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization

step, a counter stain is used to impart a pink color to the decolorized gram-negative organisms.





Materials Required:

1-Clean glass slides. 2- Inoculating loop. 3- Bunsen burner.

4- Bibulous paper. 5- Microscope. 6- Immersion.

7- Lens paper and lens cleaner. 8- Distilled water.

9-18 to 24 hour cultures of organisms

Reagents:

1. Primary Stain - Crystal Violet. 2. Mordant - Grams Iodine.
1. Decolourizer - Ethyl Alcohol. 4. Secondary Stain – Safranin.

Procedure:

Part 1: Preparation of the glass microscopic slide:

Grease or oil free slides are essential for the preparation of microbial smears. Grease or oil from the fingers on the slides is removed by washing the slides with soap and water. Wipe the slides with spirit or alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use.

Part 2: Labeling of the slides:

Drawing a circle on the underside of the slide using a glassware-marking pen may be helpful to clearly designate the area in which you will prepare the smear. You may also label the slide with the initials of the name of the organism on the edge of the slide. Care should be taken that the label should not be in contact with the staining reagents.

Part 3: Preparation of the smear:

- **Bacterial suspensions in broth:** With a sterile cooled loop, place a loopful of the broth culture on the slide. Spread by means of circular motion of the inoculating loop to about one centimeter in diameter. Excessive spreading may result in disruption of cellular arrangement. A satisfactory smear will allow examination of the typical cellular arrangement and isolated cells.
- **Bacterial plate cultures:** With a sterile cooled loop, place a drop of sterile water or saline solution on the slide. Sterilize and cool the loop again and pick up a very small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion.
- **Swab Samples:** Roll the swab over the cleaned surface of a glass slide.

Please note: It is very important to prevent preparing thick, dense smears which contain an excess of the bacterial sample. A very thick smear diminishes the amount of light that can pass through, thus making it difficult to visualize the morphology of single cells. Smears typically require only a small amount of bacterial culture. An effective smear appears as a thin whitish layer or film after heat-fixing.

Part 4: Heat Fixing:

Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains.

- Allow the smear to air dry.
- After the smear has air-dried, hold the slide at one end and pass the entire slide through the flame of a Bunsen burner two to three times with the smear-side up.

Now the smear is ready to be stained.

Please Note: Take care to prevent overheating the slide because proteins in the specimen can coagulate causing cellular morphology to appear distorted.

Part 5: Gram Stain Procedure:

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.
6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.

8. Gently flood with safranin to counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion. (see figure1)

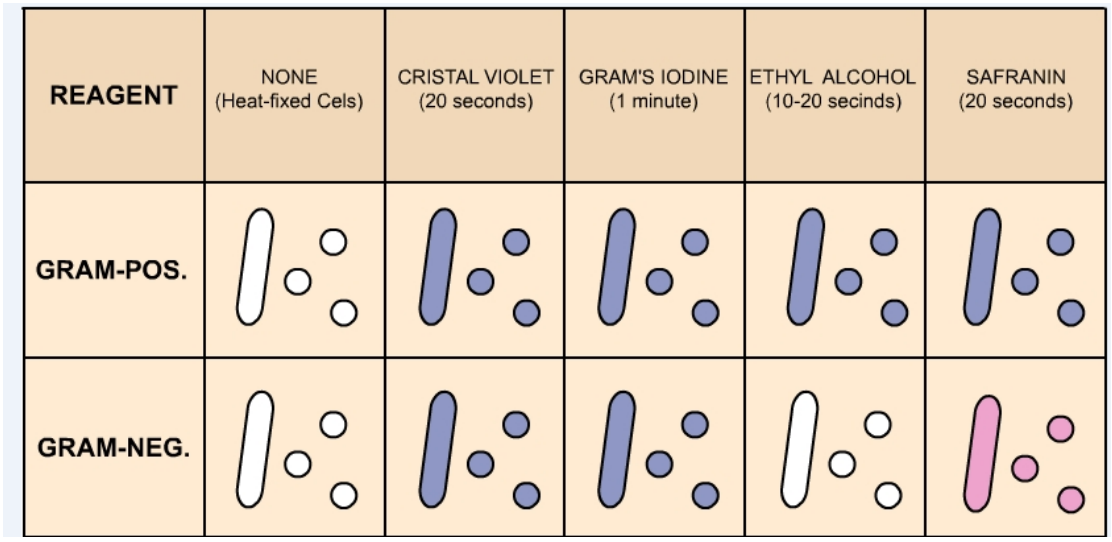


Fig1: Colour changes that occur at each step in the staining process

Typical Gram-negative bacteria:

1. ***Bordetella pertusis***, the causative agent of whooping cough
2. ***Salmonella typhi***, the causative agent of typhoid
3. ***Vibrio cholera***, the causative agent of cholera
4. ***Escherichia coli***
5. ***Proteus* sp**

Typical Gram-positive bacteria:

1- *Staphylococci* such as ***Staphylococcus epidermidis*** and ***Staphylococcus aureus*** which is a common cause of boils.

2- *Streptococci* such as the many species of oral streptococci, ***Streptococcus pyogenes*** which causes many a sore throat and scarlet fever and ***Streptococcus pneumoniae*** which causes lobar pneumonia.

3- *Clostridia* such as ***Clostridium tetani***, the causative agent of tetanus.

Results and observations:

Gram positive bacteria→→→→ Violet or blue color.

Gram Negative bacteria→→→→ Red or pink color.

Student group	Sample number	Color cells	Shape cells	Result read	Example

Discussion:

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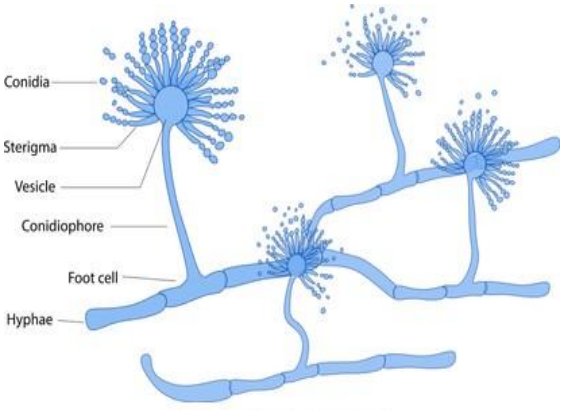
PRACTICAL No: 10

Staining of Fungi

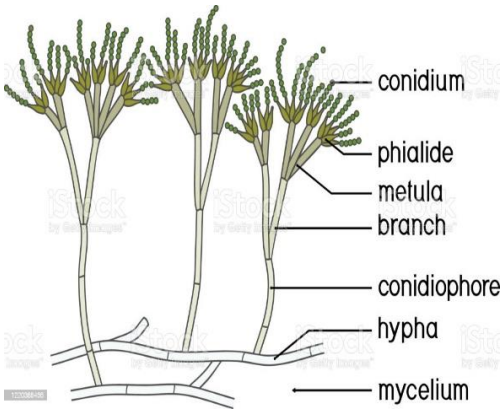
Objectives:

Fungi are eukaryotic organisms that includes yeasts and molds. Their cell walls contain chitin.

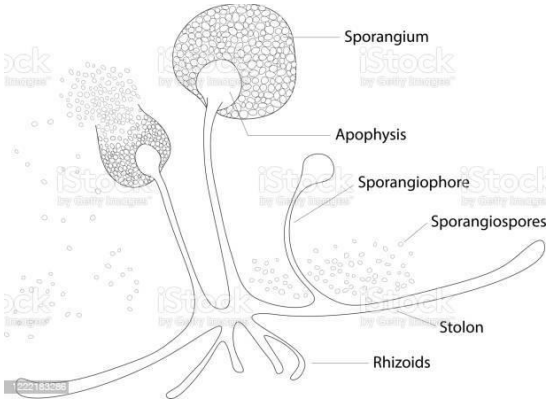
Usually, **Lactophenol Cotton Blue (LPCB)** Staining method is used to detect fungi under the microscope, as **Lactic acid** is used for preserve the fungal structures, **Phenol** is used as a disinfectant by killing any living organisms and **Cotton blue** is give color to the chitin.



Aspergillus sp.



Penicillium sp.



Rhizopus sp.

Materials Required:

1-Clean glass slides. 2- Inoculating needle. 3- Bunsen burner.

4-Microscope. 6- Lens paper and lens cleaner.

7-Cultures of organisms A*spergillus, Penicillium, Rhizopus, Fusarium* and *saccharomyces* (yeast)

Reagents:

1- **Lactic acid** 2- **Phenol** 3- **Cotton blue**

Procedure:

1-label the needed clean slides.

2-Add a drop of the **Lactophenol Cotton Blue (LPCB) on slide.**

3-Transfer a part of mycelium by sterile needle to slide and mix with the stain drop.

4- Cover the stain with a clean sterile coverslip without making air bubbles>

5- Examine the stain microscopically at 40X.

Results and observations:

Discussion:

PRACTICAL No: 10

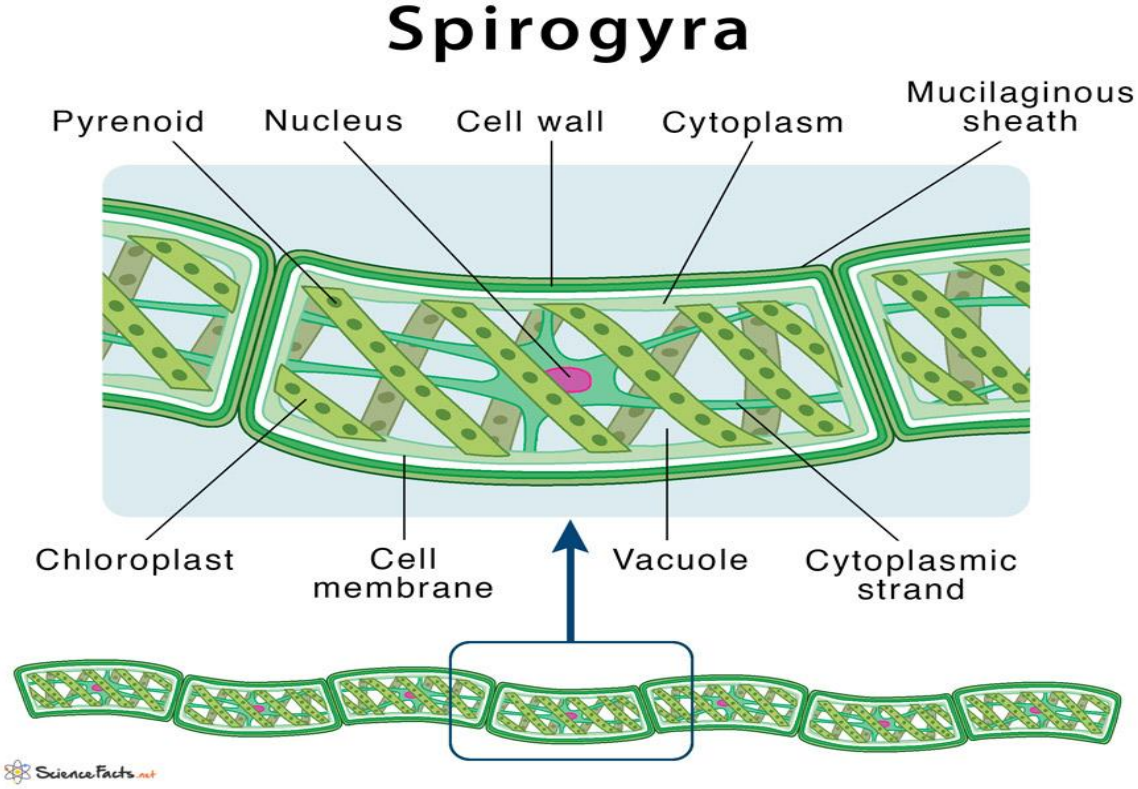
Algae

Culture media: cho 10

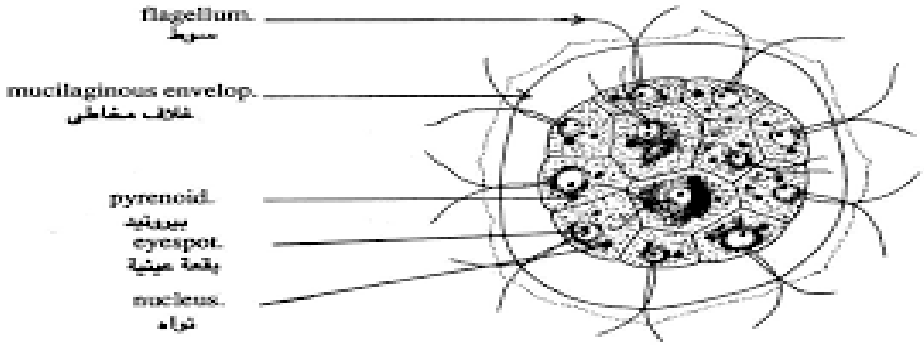
Some of Classification algae:

1) Green algae:

Example:



Pandorina sp



Pandorina sp.
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2) Red algae:

Example:

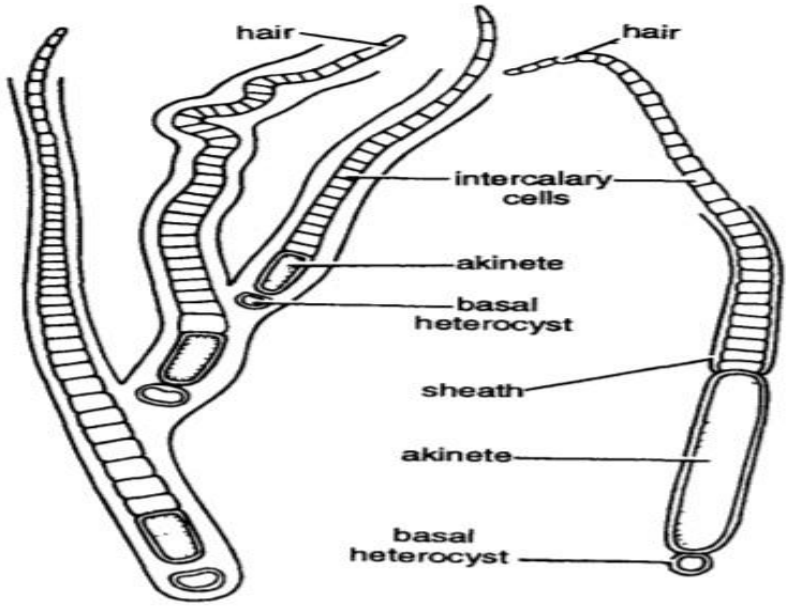
Polysiphonia sp

3) Brown algae:

Example: **Diatoms**

4) Cyanobacteria:

Example: ***Gloeotrichia* sp**



Gloeotrichia. A filament to show heterocyst and akinete.

PRACTICAL No: 12

serial dilutions of bacteria

Objectives:

- There are various ways of counting or monitoring microbial growth in a culture.
- Serial dilution involves taking a sample and diluting it through a series of standard volumes of sterile diluent, e.g. distilled water or 0.9 % saline. Then a small measured volume of each dilution is used to make a series of pour or spread plates.
- By diluting the sample in this controlled way it is possible to obtain an incubated plate with an easily countable number of colonies (30–100) and calculate the number of microbes present in the sample.

Materials Required:

1- sample of natural material
2- 6 sterile test tubes containing 9 ml
3- labelled
4- 12 sterile, Pasteur pipettes
5- Marker pen
6- Petri-dishes
7- Cotton or tissue
8- Incubator
9- media

Procedure (Methods):

1. Take a sterile pipette.

2. Draw up 1 ml of a well mixed sample

4. Add this sample to the first tube. The volume of this tube is now 10 ml . This provides an initial dilution of 10–1.

5. Mix the dilution thoroughly, by emptying and filling the pipette several times.

6. Discard this pipette into the pot of disinfectant, but keep the pipette for making the next dilution.

7. Take a new pipette, fit it to the pipette and draw up a 1 ml sample of the 10–1 dilution and place it in the second tube.

8. Mix well as before. This gives a 10–2 dilution.

9. Discard the pipette in disinfectant.

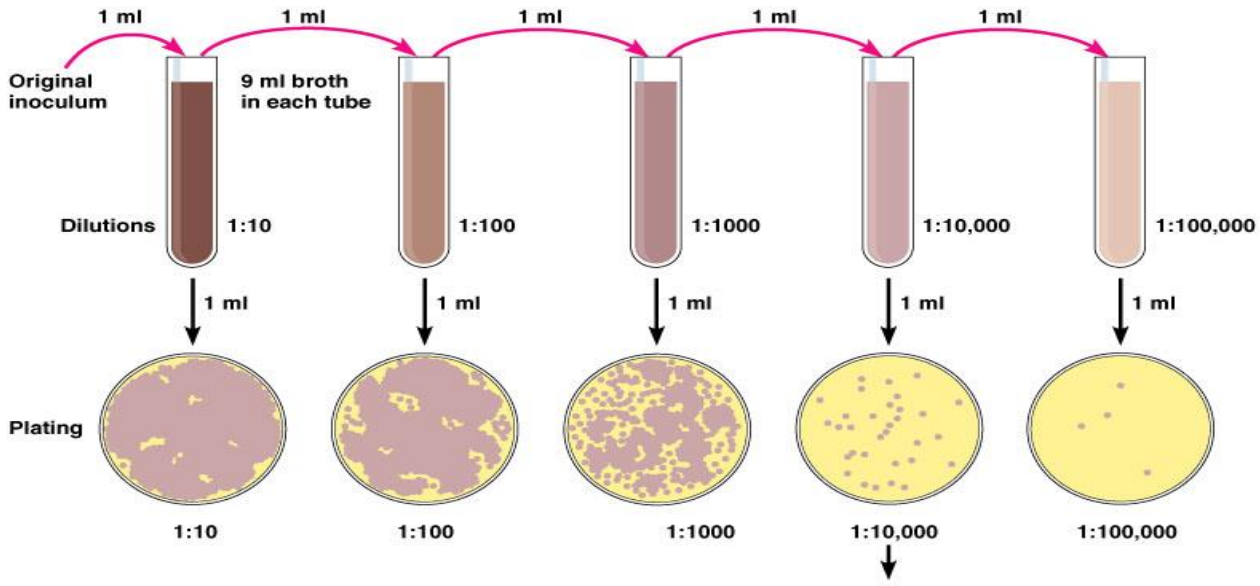
10. Repeat this for the remaining tubes, removing 1ml from the previous dilution and adding it to the next 9 ml of diluent.

If 6 tubes are used, the final dilution for the bacteria will be 10–6 (1 in 1,000,000).

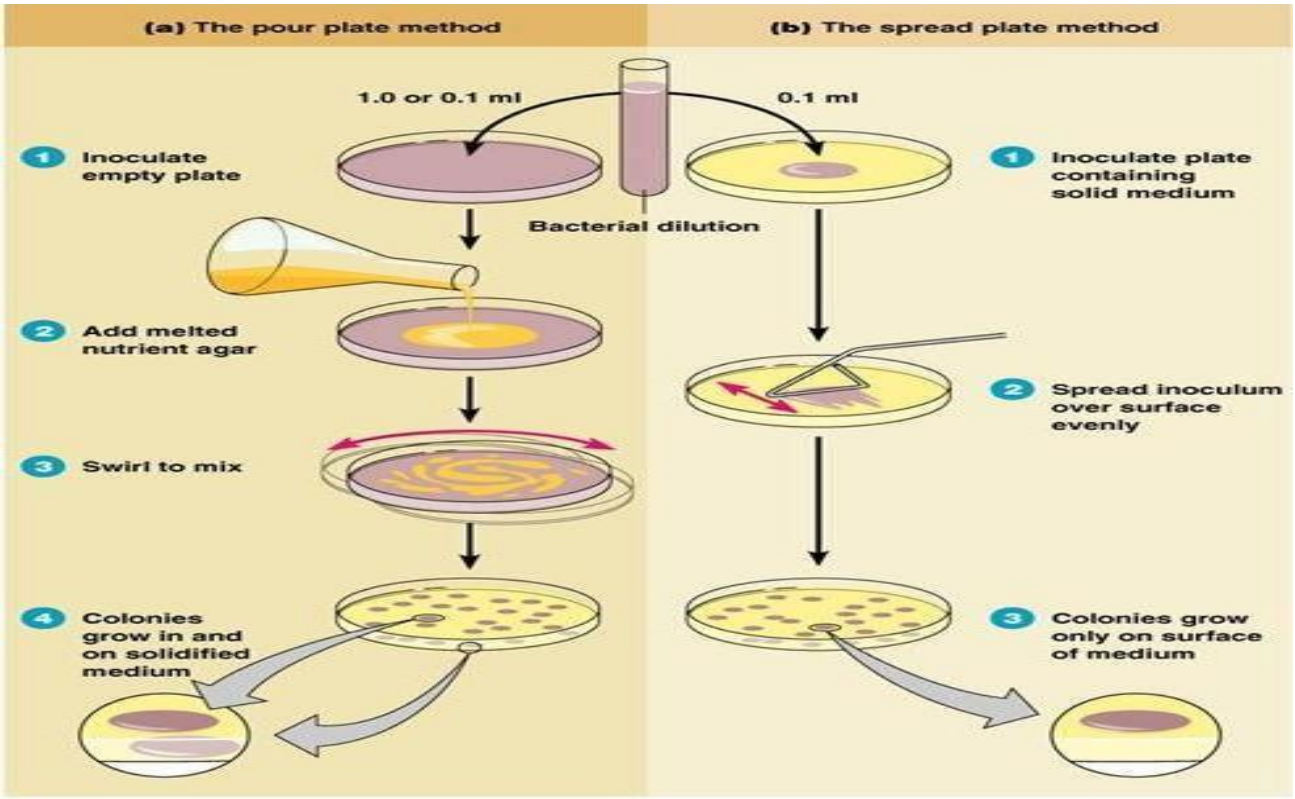
Plating and counting procedure :

Use a known volume of each dilution to make either pour plates or spread plates .By starting with the highest dilution, the same pipette may be used throughout. For statistical purposes, replicate plates should be prepared. After incubation the plates will show a range of numbers of colonies. Choose the plate that has an easily countable number (about 30–100) and carefully count every colony. Using a marker pen helps to avoid counting the same colony twice. Then calculate the number of micro-organisms in the sample:

Number of microbes/ml = number of colonies × dilution of sample



Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000/ml in sample.)



Results and observations:

Student group	Samples number	Dilution	Colonies number	Calculation CFU/ML

Discussion:

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