

Laboratory Manual Introduction to Biotechnology (BT511P)



**VIRTUAL UNIVERSITY OF
PAKISTAN**

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Practical No. 1
Introduction to laboratory safety, containment and decontamination

Introduction:

Biotechnology is the use of an organism, or a component of an organism or other biological system, to make a product or process for a specific use. Biotechnological techniques are applicable not only to modern medical practice but also to the production of genetically modified organisms, forensics, and quality assessment of laboratory animals, pharmacogenomics, and other fields. Many applications of modern biotechnology depend on the ability to analyze, manipulate, and cut and paste pieces of DNA. Approaches that involve sequencing and manipulation of DNA are sometimes referred to as **DNA technology**.

Safety Considerations:

- ❖ Use personal protective equipment such as disposable gloves, lab coats, disposable masks, etc.
- ❖ Handle all sharps with care and dispose of sharps in the sharp's disposal containers.
- ❖ Handle hazardous chemicals and samples carefully. Blood and other body fluid must be considered potentially hazardous. Biological waste should be disposed of in the designated trash bags that could be incinerated later on.
- ❖ Decontaminate the work benches regularly and especially before and after work. For decontamination, wipe the surfaces with 10% bleach followed by water.

Containments: Containment is defined as the action of confining within a defined space a microbiological agent or other entity that is being cultured, stored, manipulated, transported, or destroyed in order to prevent or limit its contact with people and/or the environment.

Decontamination: Decontamination is a combination of processes that removes or destroys contamination so that infectious agents or other contaminants cannot reach a susceptible site in sufficient quantities to initiate infection, or other harmful response. Its types include, Physical cleaning, Ultrasonication, Disinfection, Antisepsis, Sterilizations.

Practical No. 2

Introduction and practical demonstration of use and handling of laboratory equipment/ glassware

Glassware

The glassware needs to be selected according to its accuracy and capacity. Narrow neck glassware is more accurate by rule. Some glassware can be used to measure fixed volumes while others can be used to measure a variety of volumes. Some glassware used in laboratory are enlisted;

- **Bulb and graduated pipettes.** These are used to transport specific amounts of fluids from one place to another.



Bulb and graduated pipettes

- **Burettes.** These are used to dispense exact quantities of liquid into another vessel.



Burettes

- **Beakers.** Simple containers used to hold samples and reagents.



Beaker

- **Volumetric flasks.** Similar to beakers, these are used to hold samples but usually come in a conical or spherical shape with a tapering neck.



Volumetric flasks

- **Funnels.** The tapered neck of a funnel allows easy pouring of a liquid into a narrow orifice.



Funnel

- **Graduated Cylinders.** Similar to beakers, these cylindrical vessels have volumetric markings to allow for monitoring volume.



Graduated cylinders

- **Vials.** Small bottles used to store samples or reagents.



Vials

- **Stirring Rods.** Used to mix solvents and samples together.



Stirring rods

Materials

1. **Agar Plate** — Petri dish containing solidified culture medium.



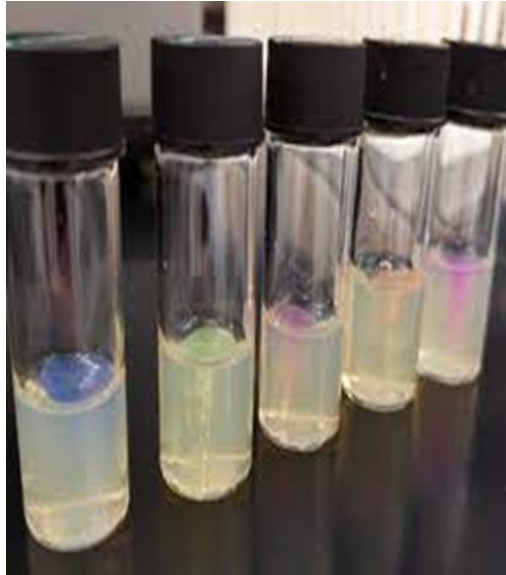
Agar plate

2. **Agar Slant** — Test tube containing solidified culture medium at a slope for preparing cultures and preservation of pure cultures in the lab.



Agar slant

3. **Agar Stab** — Test tube containing solid culture medium for growth of anaerobic organisms especially when sealed with oil or deep culture system.



Agar stab

4. **Broth** — Liquid medium containing nutrients in which bacteria are grown.



Broth

5. **Cotton Swabs** — Small stick with cotton wool for taking samples from 'any surface such as wounds, throat, skin, etc.



Cotton swabs

6. **Culture Medium** — Mixture of nutrients for the growth of microorganisms. Available in ready- to-use powdered form or is prepared from separate ingredients. Commonly used culture media are nutrient agar, nutrient broth, MacConkey agar, etc.



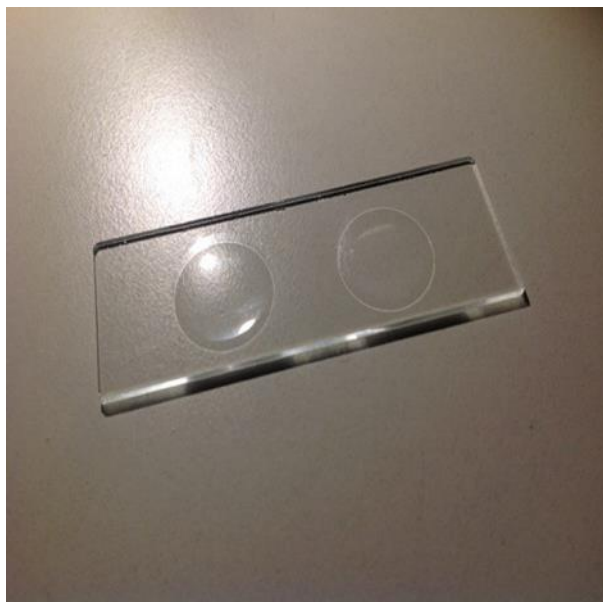
Culture medium

7. **Dropping Bottles** — Used for carrying staining solution in the lab. These deliver drop wise flow of the staining solutions.



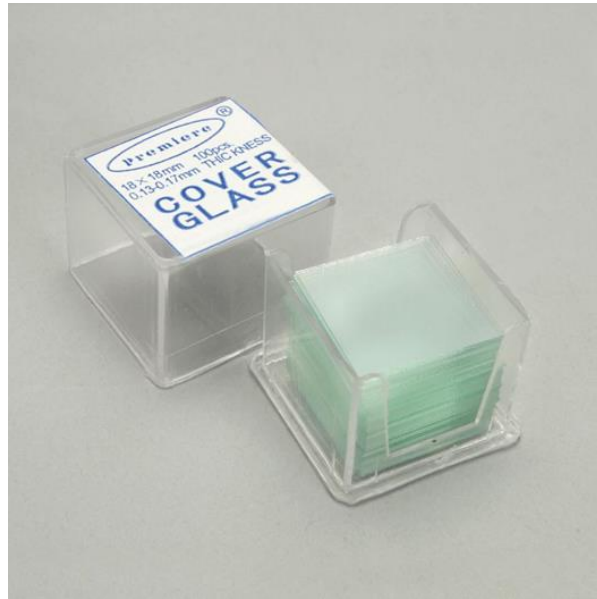
Dropping bottles

8. **Glass Cavity Slide** — Glass slide having concavity in its center. It is used for determining motility of microorganisms under microscope.



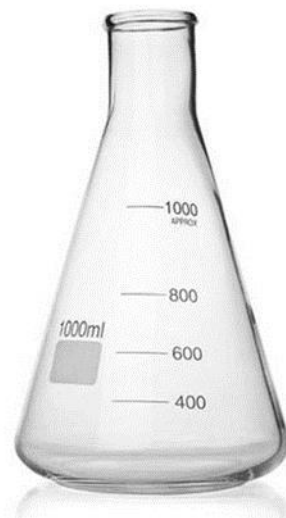
Glass cavity slide

9. **Glass Cover Slip** — Rectangular or circular thin piece of glass (usually 1 cm² or diameter) used for permanent mounting of smears or cell culture experiments.



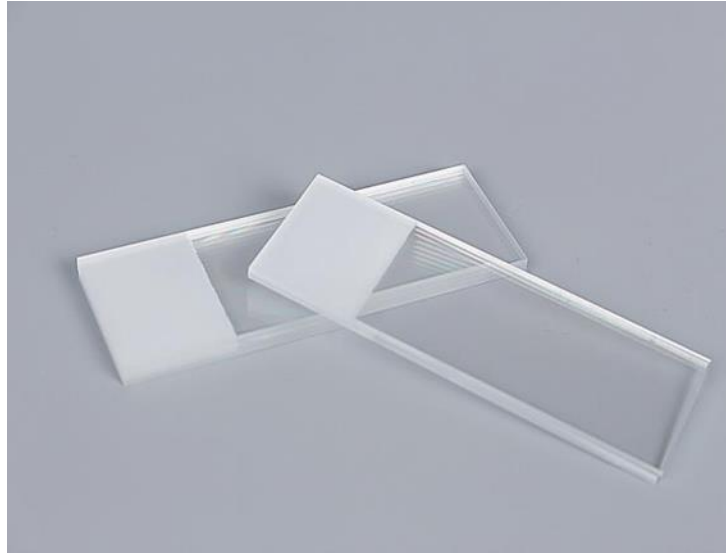
Glass cover slip

10. **Glass Flask**— Made of usually Pyrex glass, available in varying sizes and capacity as round or flat bottom. These are used for mixing purposes or making nutrient media. Flat-bottomed flasks are preferred.



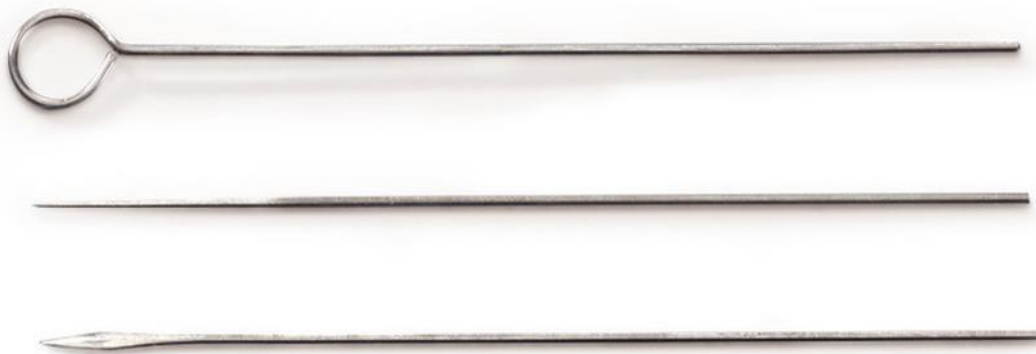
Glass flask

11. **Glass Slide** — Rectangular piece of glass sheet (3.0 x 1.5 cm) used for making bacterial or fungal smears, blood smears etc. for detailed microscopic study.



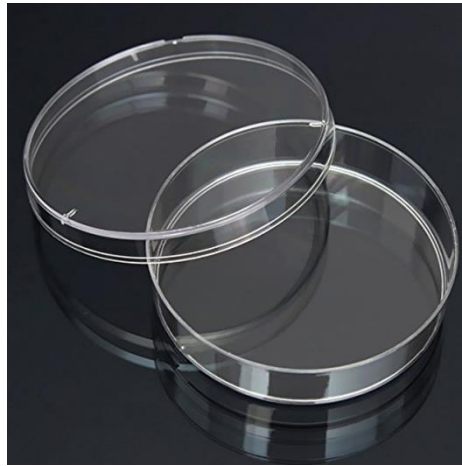
Glass slide

12. **Inoculating Needles and Loops** — These are used for transferring microorganisms. They must be flamed to red-hot before and after use. They should be cooled before being used to pick up organisms. These are also helpful in preparing slide smears.



Inoculating needles and loops

13. **Petri Dish** (named after its inventor) — Normally, made of Pyrex glass and consists of a bottom and a lid, it is used for growing microorganisms. It must be placed with lid down in the incubator. It is labelled on the underside. The glass Petri dish is reusable. Petri dish is also manufactured in plastic material as disposable. Different sizes such as small, medium, regular and king size are available for different purposes.



Petri dish

Equipments:

1. **Anaerobic Jar**— Closed chamber with controlled out-fits for air. It is used for anaerobic cultivation of microorganisms in the laboratory.



Anaerobic jar

2. **Autoclave** — Used for sterilization. Normally a pressure of 1.09-kg cm^{-2} (15 lb. per square inch) is used for 15 minutes or longer for moist, heat sterilization.



Autoclave

3. **Centrifuge machine** — Used to spin the suspension, blood, etc. for the separation of cells from the fluid part. Available in ordinary (5000 rpm) and high speed (15,000 rpm) versions. The ultracentrifuge (60,000rpm) is employed for sub-cellular particle separation.



Centrifuge machines

4. **Colony Counter** —Illuminated stage with magnifying lens attachment used for counting bacterial colonies on the surface of culture medium contained in a petri dish.



Colony counter

5. **Water Bath** — Used to maintain constant temperature of agar and other materials. Also useful for boiling sugar solutions and heat inactivation of serum.



Water bath

6. **Weighing Balance** — Required for weighing ingredients for making solutions and media etc.



Weighing balance

7. **UV Lamp** —Used for decontamination of laboratory environment.



UV lamp

8. **Vacuum Pump** — Used for creating negative or positive pressure in filtration systems.



Vacuum pump

9. **pH Meter** — Calibrated instrument with buffers, used for determining the acidity or alkalinity (pH) of solutions, broths or culture media.



pH meter

10. **Pipettes** —Used for transferring liquid materials. Pasteur pipettes are used for pouring small amounts while graduated pipettes are employed for transferring specific amount of material.



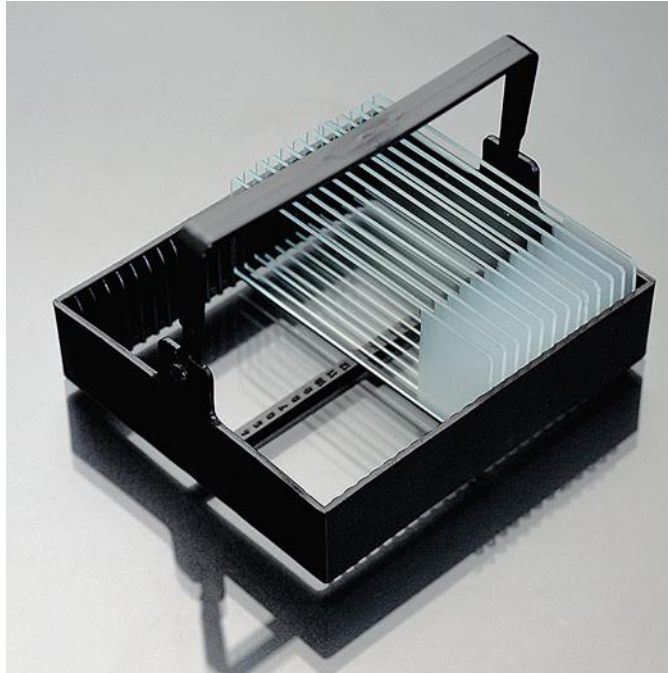
Pipettes

10. **Refrigerator** — Essential for keeping samples and cultures etc. at 4°C.



Refrigerator

12. **Staining Rack** — Used to support slides during staining.



Staining rack

13. **Lyophilization apparatus** — Meant for the preservation of cultures for extended period of time.



Lyophilization apparatus

14. **Magnetic stirrer** — Instrument provided with hot plate arrangement, used for proper homogenous mixing of solutions, media, etc. under warm conditions.



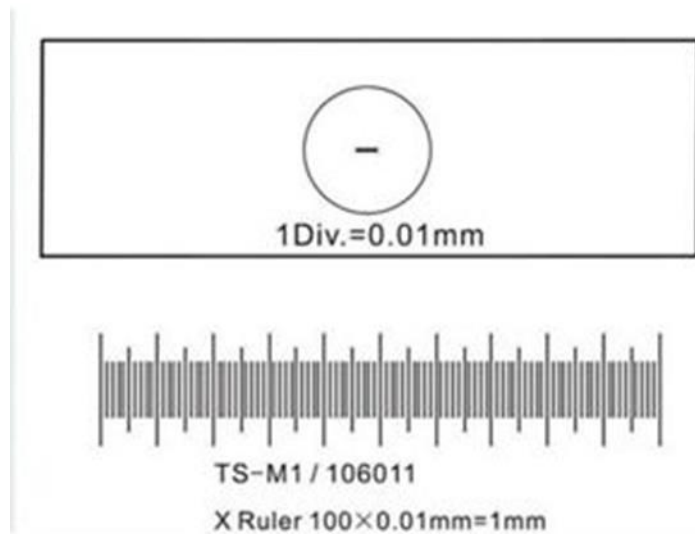
Magnetic stirrer

15. **Micro dispenser** — Micropipettes delivering minute amounts of solutions in microliters (μL) for making multiple dilutions, These are available in single, 4, 8 and 12 channels.



Micro dispenser

16. **Micrometers** — Stage and ocular micrometers, used for micrometrical study.



Micrometer

17. **Microscope** — Simple, compound or binocular used for detailed study of microbial (bacterial or fungal) or blood smears, etc.



Microscope

18. **Laminar Air Flow Cabinet**—Semi-closed cabinet with filtered airflow arrangement used for

open culture manipulation.



Laminar air flow cabinet

19. Hot Air Oven — For sterilizing glassware, metal-ware and oils at usually a temperature of 171 to 200°C. Also used for drying objects.



Hot air oven

20. Culture Hood — A closed cabinet with UV light arrangement in order to maintain sterilized

environment for culture manipulation.



Culture hood

21. Incubator — For growing microbial cultures at specific and constant temperature. Ordinary incubators work at 25-40°C, while the low temperature incubators are set at 0°C to 10°C. Carbon dioxide incubators work at 25-40°C.



Incubator

Validation of Critical Reagents and Procedures

All technical procedures and critical reagents should be tested and validated before performing the actual case work or research work experiments.

Calibration of Instruments

All instruments should be calibrated according to required schedule and before performing the validation studies, case work and research experiments.

Practical No: 3 Preparation of Solutions

Solutions: A homogenous mixture of two or more substances in relative amounts that can be varied continuously upto what is called the limit of solubility. The term solution is commonly applied to the liquid state of matter, but solutions of gases and solids are also possible.

The following general instructions are applicable in the preparation of all reagents. Use graduated cylinders or pipettes closest to the volume being measured for preparing liquid reagents. Store all reagents in sterile containers unless otherwise noted. Label all reagents with name of reagent, date prepared, initials of scientist that prepared reagent, lot number and expiry date. Record each preparation in the lab's reagent logbook.

1M Tris-HCl (Tris Hydroxymethyl amino methane) pH 8

Tris base 121.1g

H₂O to 800ml

Adjust to desired pH with concentrated HCl. Mix and add H₂O to make final volume upto 1 Liter.

Store at room temperature.

0.5 M EDTA (Ethylenediamine Tetra Acetic Acid) pH 8.0

Na₂EDTA.2H₂O 186.1g

H₂O to 700ml

Adjust pH to 8.0 with 10M NaOH (almost 50 ml). Mix and add H₂O to make final volume upto 1 Liter.

Store at room temperature.

10M NaOH

NaOH 400 g

H₂O to make final volume upto 1 Liter.

Store at room temperature.

10% NaOH

NaOH 40 g

H₂O to 1 L

Mix well and store at 4°C in dark.

1 M solution of KCl

Dissolve 74.55 g of KCl in 900 ml of H₂O. Make up the volume to 1 L with H₂O and autoclave for 20 min.

10% Glucose

Weigh 10 g glucose (solute) and add enough water (solvent) to make a 100 mL solution.

0.1N NaOH Solution

Dissolve 40 grams of NaOH in 1L of water. For 100 ml of water = $(4/1000) \times 100 = 0.4$ g of NaOH. Thus, the amount of NaOH required to prepare 100ml of 0.1N NaOH solution is 0.4 g of NaOH.

Preparation of 10% hydrochloric acid solution (100 ml) from 37% HCl

1. Take 50 ml of distilled water in a volumetric flask.
2. Calculate the volume of HCl required as follows;

$$C_1V_1 = C_2V_2$$

$$37 \text{ g/mol} \times V_1 = 10 \times 100 \text{ ml}$$

$$V_1 = 1000/37 = 27.02 \text{ ml}$$

4. Add 27.02 ml of HCl to the volumetric flask slowly and raise the volume to 100 ml with distilled water to prepare 100 ml of 10% HCl solution.
4. Record the pH of the solution using pH meter.

Preparation of 5% NaCl solution

1. Weigh 5 grams of sodium chloride.
2. Dissolve the NaCl in 25 ml of distilled water in a beaker with the help of stirrer.

3. Transfer the contents into a volumetric flask. Add some more water (10-20 ml) to the beaker and rinse the walls of beaker and add that water from beaker to the same flask.
4. Raise the volume to 100 ml in the volumetric flask to prepare 5% NaCl solution.
5. Check the pH of the solution with pH meter.

Practical No: 4

Preparation of Buffers

Buffer: A buffer is a solution that can resist pH change upon the addition of acidic or basic components. It is able to neutralize small amounts of added acid or base, thus maintaining the pH of the solution relatively stable. This is important for processes and/or reactions which require specific and stable pH ranges. Buffer solutions have a working pH range and capacity which dictate how much acid/base can be neutralized before pH changes and the amount by which it will change.

Types of Buffer Solutions:

Buffer solutions are broadly classified into two categories. i.e., acidic and alkaline buffers.

Acidic Buffers

As the name suggests, these solutions are used to maintain acidic environments. Acid buffer has acidic pH and is prepared by mixing a weak acid and its salt with a strong base. An aqueous solution of an equal concentration of acetic acid and sodium acetate has a pH of 4.74.

- The pH of these solutions is below seven.
- These solutions consist of a weak acid and a salt of a weak acid.
- An example of an acidic buffer solution is a mixture of sodium acetate and acetic acid (pH = 4.75).

Alkaline Buffers

These buffer solutions are used to maintain basic conditions. Basic buffer has a basic pH and is prepared by mixing a weak base and its salt with strong acid. The aqueous solution of an equal concentration of ammonium hydroxide and ammonium chloride has a pH of 9.25.

- The pH of these solutions is above seven.
- They contain a weak base and a salt of the weak base.
- An example of an alkaline buffer solution is a mixture of ammonium hydroxide and ammonium chloride (pH = 9.25).

Preparation of Buffers:

If the dissociation constant of the acid (pK_a) and base (pK_b) are known, a buffer solution can be prepared by controlling the salt-acid or the salt-base ratio. As discussed earlier, these solutions are prepared by mixing the weak bases with their corresponding conjugate acids or by mixing weak acids with their corresponding conjugate bases.

An example of this method of preparing buffer solutions can be given by the preparation of a phosphate buffer by mixing HPO_4^{2-} and $H_2PO_4^-$. The pH maintained by this solution is 7.4.

Phosphate Buffer (pH 5.8 to 7.4)

1. Prepare 800 ml of distilled water in a suitable container.
2. Add 20.214 g of $Na_2HPO_4 \cdot 7H_2O$ to the solution.
3. Add 3.394 g of $NaH_2PO_4 \cdot H_2O$ to the solution.
4. Adjust solution to final desired pH using HCl or NaOH.

Citrate Buffer (0.1 M, pH 6.0)

1. Prepare 800 ml of distilled water in a suitable container.
2. Add 24.269 g of Sodium Citrate dihydrate to the solution.
3. Add 3.358 g of Citric Acid to the solution.
4. Adjust solution to desired pH using 0.1N HCl (typically $pH \approx 6.0$).

Acetate Buffer (pH 3.6 to 5.6)

Acetate buffers are inexpensive and simple to prepare and can be stored at room temperature.

1. Prepare 800 ml of distilled water in a suitable container.
2. Add 7.721 g of Sodium Acetate to the solution.
3. Add 0.353 g of Acetic Acid to the solution.
4. Adjust solution to final desired pH using HCl or NaOH.

Practical No: 5
Use of Simple and compound microscope and micrometry
Micrometry:

Micrometry is the science in which we have some measurement of the dimensions of an object being observed under the microscope. The method employs some special types of measuring devices which are so oriented that these can be attached to or put into the microscope and observed. The object, to be measured, is calibrated against these scales. Once we are observing an object under a microscope using the 5X objective and the 10X eyepiece, we say that the image being perceived is $5 \times 10 = 50$ times of the object. Although, magnified view obtained has perfect coordination of dimensions. However, precise measurement of exact size of object can only be achieved through application of some small scales. i.e., micrometers.

Microscopy:

Microscopy is the technical field of using microscopes to view objects and areas of objects that cannot be seen with the naked eye (objects that are not within the resolution range of the normal eye).

Microscope:

Microscope is an instrument used to obtain an enlarged image of a small object. Microscopes are classified as:

- a. **Light microscope** — Simple and compound microscopes where the ordinary light source (visible light spectrum) is utilized to make the final image of the object. These are the most commonly used microscopes in the microbiological laboratories.

Different parts of microscope and their functioning is described as follows;

1. **The lenses:** The ordinary light microscope is a compound microscope. It has two sets of lenses in contrast to the simple magnifying glass that is a simple microscope. The set of lenses nearer the eye is the ocular or eyepiece. The usual magnification of the ocular lenses is $20 \times$, $25 \times$, $6 \times$, $10 \times$ and $12 \times$. The set of lenses nearer the object is the objective. The objectives are carried on a revolving nosepiece. The nose-piece usually carries three to four objectives.

Table: Types of objective lenses used in microscope with magnification powers and focal lengths

Objectives lens	Usual magnification of objective lens	Focal length
Low power	10 x	18
Medium power	20 x	9 mm
High dry	40 x	4 mm
Oil immersion	100 x	1.2-1.6 mm

2. **The mirror:** It transmits light from a light source which may be an electric lamp or natural daylight. When a condenser is present, the flat side of the mirror is used. If a condenser is absent then concave side of the mirror is employed. In the modern microscopes, mirror is absent and the lamp transmits light.
3. **The sub stage condenser (also called the Abbe condenser after its discoverer):** It focuses light on the object.
4. **The iris diaphragm:** The amount of light entering the microscope is controlled with the iris diaphragm. It should be adjusted to suit the nature of the specimen being observed, depending on whether more or less light is required. It is fitted beneath the condenser.
5. **The mechanical stage:** This is the platform where the slide is placed. It is provided with two mechanical manipulator knobs (one for forward backward and the other for side movements). These are usually located on or immediately below the stage. It helps in locating exact position of the specimen or object under microscopic field.
6. **The adjustment Knobs:** These are three: the coarse adjustment knob, the fine adjustment knob and the sub-stage adjustment knob (used to raise the condenser). All of these are working to get fine image of the object.
7. **Magnifying power of the microscope:** It is obtained by multiplying the magnification powers of ocular and objective lenses of the microscope.
8. **Resolving power of the microscope:** It is the ability of microscope to distinctly separate closely related objects. It is calculated as follows;

$$RP = \lambda / 2NA$$

Whereas;

A = wavelength of visible light spectrum

NA = numerical aperture of the lens

Microscope Parts

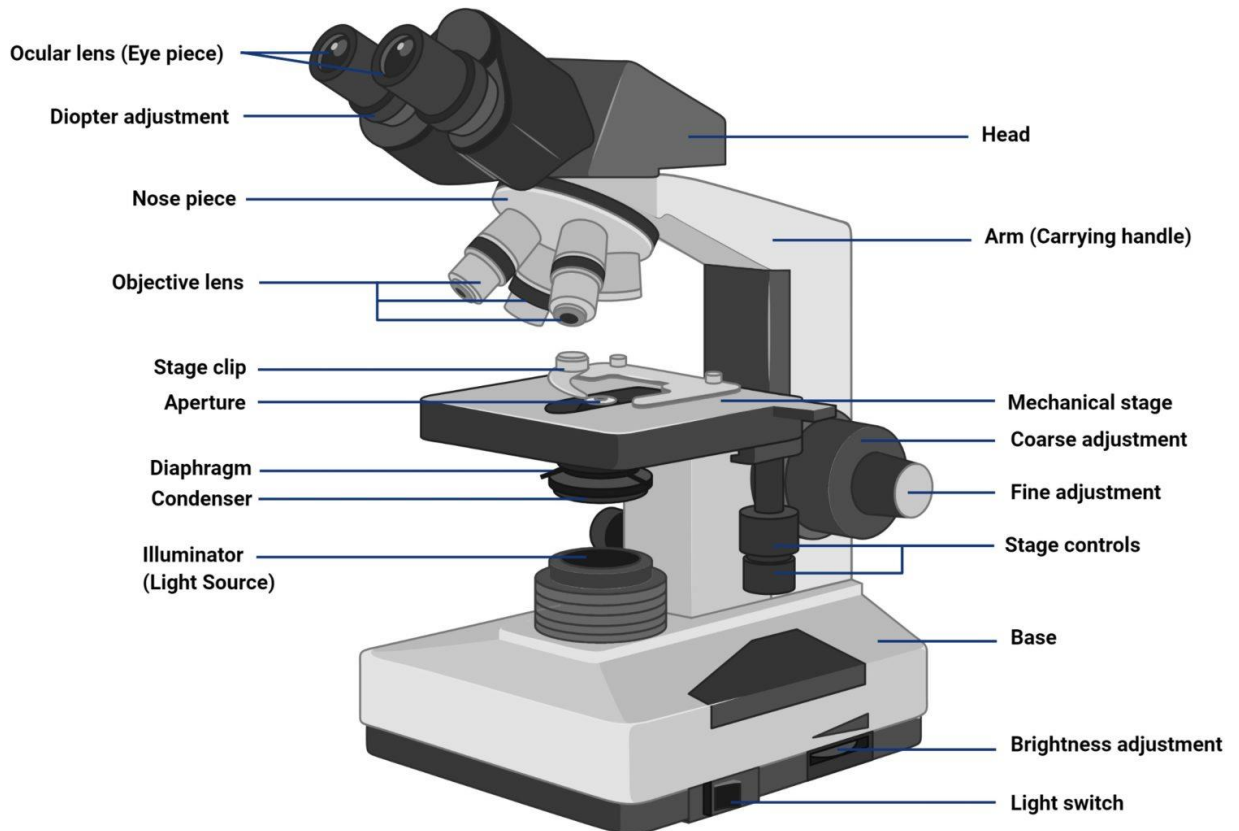
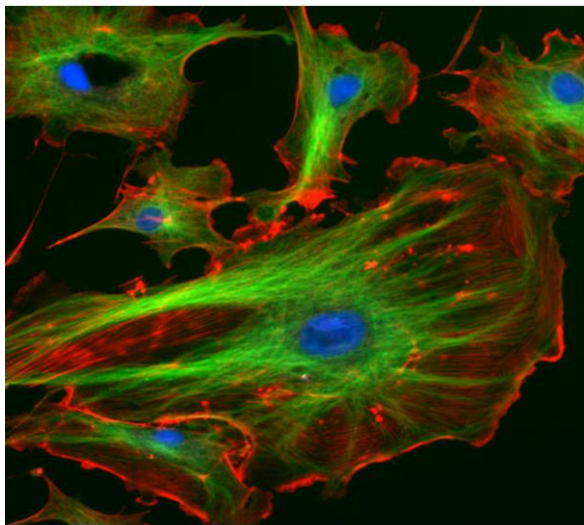
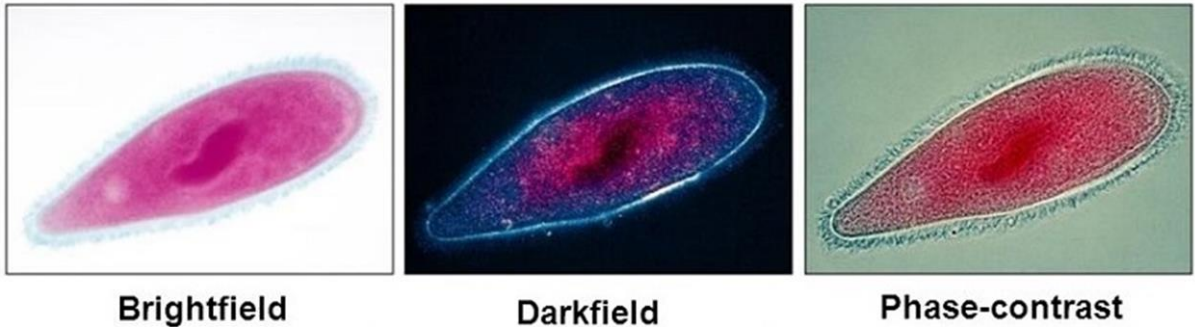


Figure: Parts of a microscope, Image Copyright © Sagar Aryal, www.microbenotes.com

There are seven categories of light microscopes intended for various kinds of studies. These are;

- a. **Bright field microscope** — Commonly, used for viewing stained bacteria and fungi for their morphological and micrometrical studies.
- b. **Fluorescent microscope** — Used for the identification of bacteria, fungi and specific antigens, coated with fluorescent dye. It provides much greater details.
- c. **Dark field microscope** — Very small objects including bacterial capsules may be viewed through negative staining under dark field microscope.
- d. **Phase contrast microscope** — Viable bacteria may be viewed directly without any staining through phase contrast microscope.

- e. **Nomarski differential interference contrast microscope** — A three dimensional view of the object may be observed irrespective of the staining method adopted under this microscope.
- f. **Inverted microscope** — Cell cultures and the fungi growing at the base of the Petri dish may be viewed directly under the inverted microscope.
- g. **Ultraviolet light microscope** — Very high resolving power of light microscope may be obtained and detailed structures of the cells may be viewed.

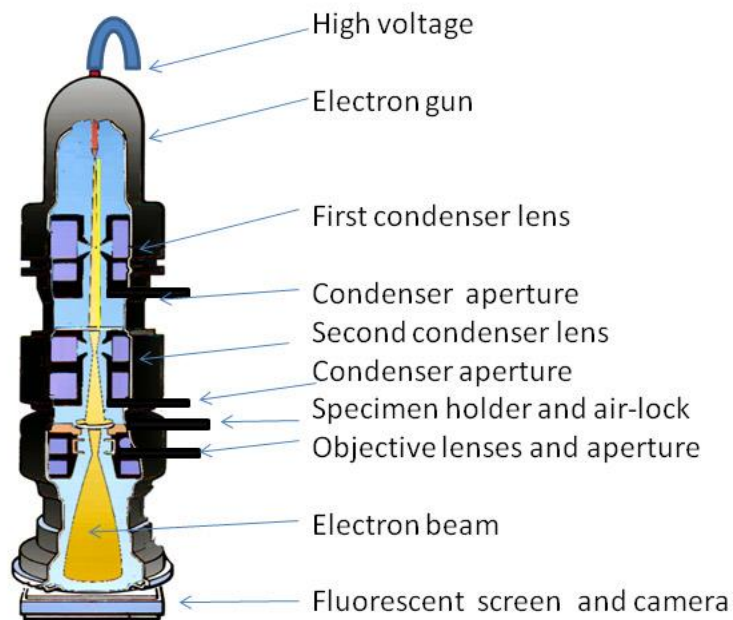


Fluorescence Microscopy



Differential interference contrast Microscopy

- h. **Electron microscope** — Transmission and the scanning electron microscopes are employed to resolve very minute particles like viruses and peptides by using electron beam under vacuum. These are primarily used for detailed examination of sub-cellular particles in research laboratories.



Electron microscope

Practical No: 6

Enumeration of bacteria/ yeast cells through hemocytometer

Bacterial enumeration involves **the counting of bacterial cells**. Viable cell count is the number of living cells while total cell count reflects all the cells in a sample.

Hemocytometer:

The hemocytometer has been an essential tool for hematologists, medical practitioners, biologists and now brewers and ethanol production researchers. Yeasts are an economically important organism used for ethanol production, in the beverage and alternative fuels industries as well as a leavening agent in the baking industry. Concentration and viability determinations are routinely performed for quality control purposes in yeast production, fermentation processes and fungicides research to monitor proliferation of pathogenic yeasts.

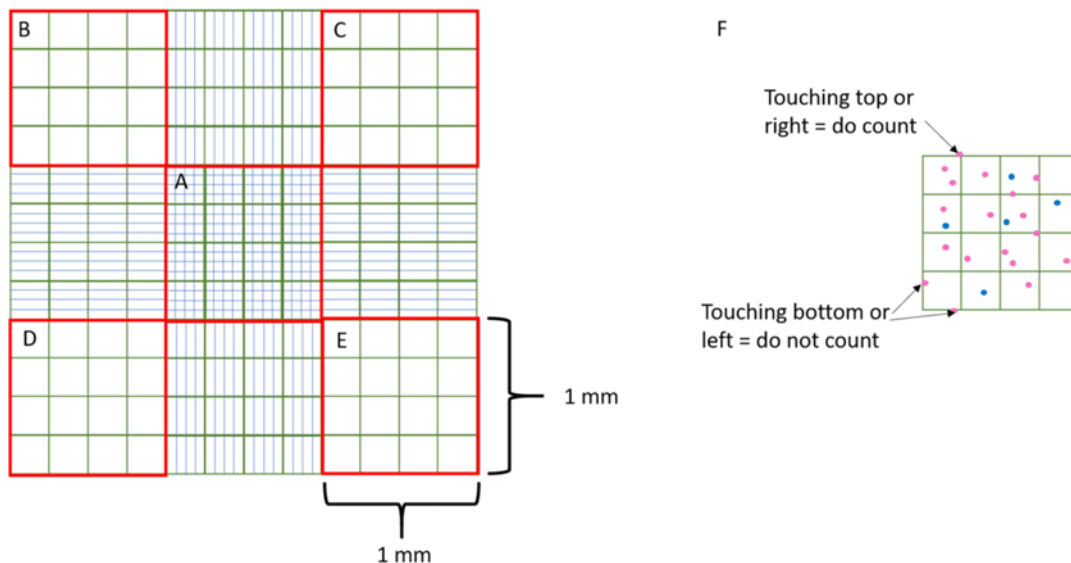
A hemocytometer consists of a thick glass microscope slide with a grid of perpendicular lines etched in the middle. The grid has specified dimensions so that the area covered by the lines is known, which makes it possible to count the number of cells in a specific volume of solution. The most common type of hemocytometer has an “H” shape engraved in the middle that encloses two separate mirror-like polished grid surfaces and provides the coverslip mounting area



A Hemocytometer

The full grid on a hemocytometer contains nine squares, each of which is 1 mm^2 . The central counting area of the hemocytometer contains 25 large squares and each large square has 16 smaller squares.

When counting cells that overlap an exterior line or ruling, count only those cells on the top or right-hand line of the large square to avoid counting cells twice. Suspensions should be dilute enough so that the cells or other particles do not overlap each other on the grid and should be uniformly distributed.



Cell counting with a hemocytometer

For large cells, you can simply count the cells inside the four large corner squares and the middle square. For a dense suspension of small cells, you may wish to count the cells in the four outer and middle squares of the central square or make a more dilute suspension.

Using Methylene Blue to Measure Yeast Cell Viability

In general, methylene blue is used to measure yeast viability/vitality. Methylene blue is a metachromatic stain that has a molecular mass of 319.85 g/mol . Metabolically active viable/vital yeast cells with dehydrogenase activity can convert the methylene blue to a colorless substance while the dead cells retain the blue color of the stain. Therefore, live and dead yeast cells can be manually counted using the hemocytometer to determine yeast cell concentration and viability.

Methylene Blue Preparation

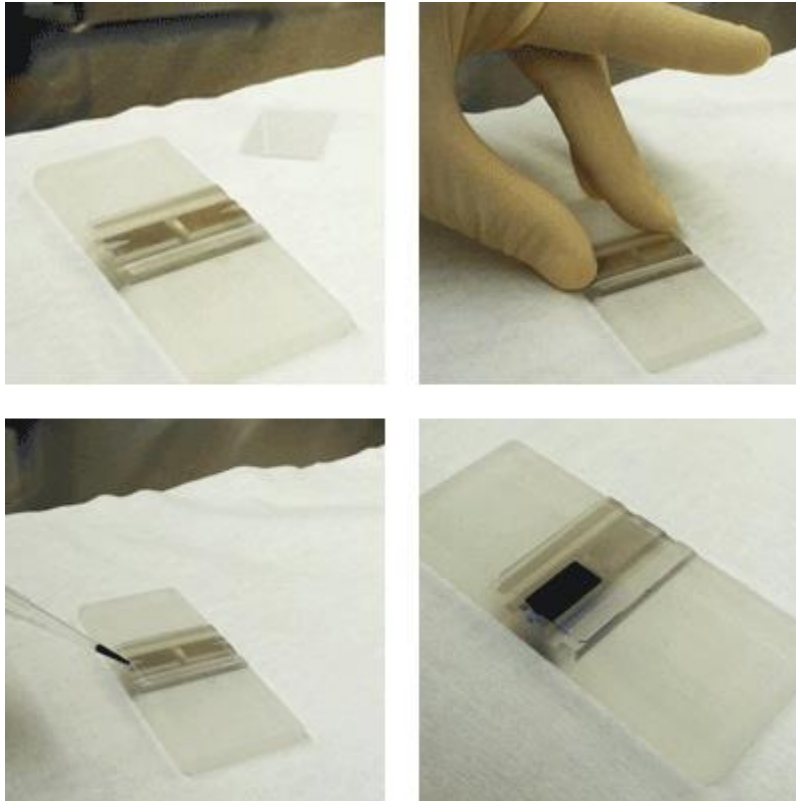
Step 1. Dissolve methylene blue in sodium citrate solution (2% w/v) to a final concentration 0.01% (w/v).

Step 2. Filter the methylene blue with 0.2-micron filter.

Hemocytometer Preparation

Step 3. Clean the hemocytometer and glass cover slip with 70% EtOH.

Step 4. Place the glass cover slip over the counting chambers.



Cell Counting Procedure

Step 5. Vortex the target yeast cell suspension and mix 1:1 with 0.01% methylene blue.

Step 6. Pipette 10 microliters of cell sample into the hemocytometer.

Step 7. Wait 60 seconds for the cells to settle.

Manually Count Cells in Sample

Step 8. Place the hemocytometer under a microscope with a typical magnification of 100

Step 9. Focus both onto the grid pattern and the cell particles, and count the total number of cells found in 4 large corner squares. If cells are touching the 4 perimeter sides of a corner square, only count cells on 2 sides, either the 2 outer sides or 2 inner sides.

Step 10. Count the live yeast cells (without methylene blue) and dead yeast cells (with methylene blue)

Cell Calculations & Disposal of Hemocytometer

Step 11. Multiply the dilution factor by the total number of cells, divide by the # of corner squares counted, and multiply by 10^4 to obtain cell concentration (cells/ml)

$$\text{Live Yeast Concentration } \left(\frac{\text{cells}}{\text{ml}} \right) = \frac{\text{live yeast count}}{\# \text{ of squares}} \times 10^4$$

$$\text{Viability (\%)} = \frac{\text{live yeast count}}{\text{live} + \text{dead yeast count}} \times 100$$

Step 12. Clean hemocytometer and glass cover slip with 70% EtOH.

Crystal Violet Protocol

Crystal Violet Preparation

Step 1. Prepare 0.1 M citric acid by dissolving 1.9212 g in 100 mL distilled water

Step 2. Prepare 0.1 M citric acid containing 0.01% (w/v) crystal violet by dissolving 0.005 g crystal violet (also known as basic violet 3 or gentian violet; C.I. 42555) in 50 ml of the 0.1 M citric acid prepared in Step 1.

Hemocytometer Preparation

Step 3. Clean the hemocytometer and glass cover slip with 70% EtOH

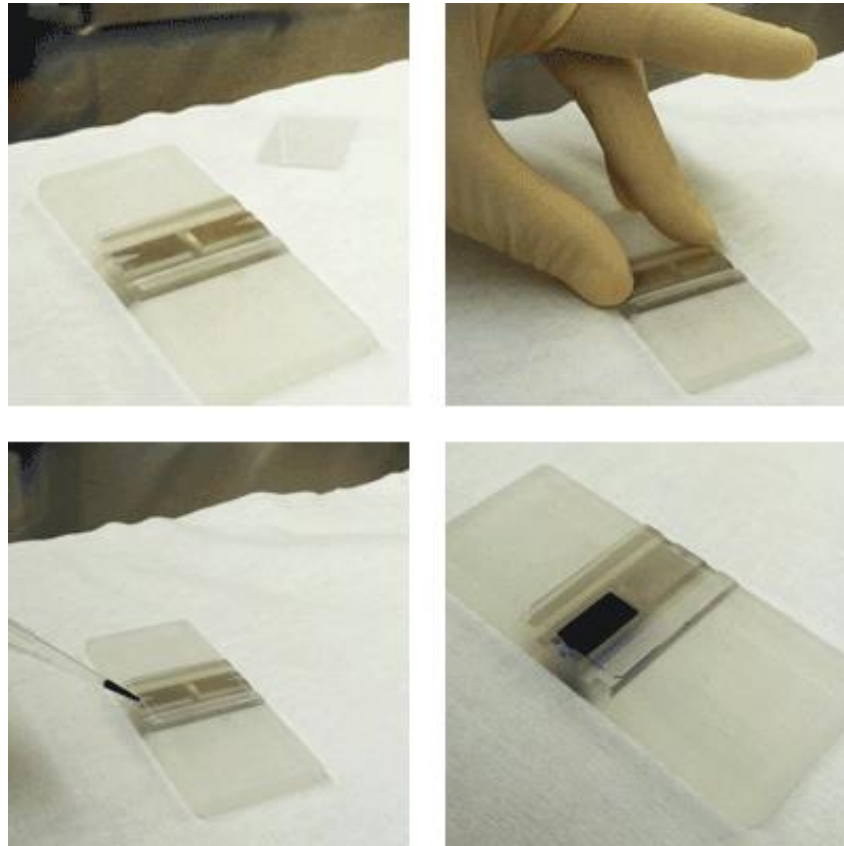
Step 4. Place the glass cover slip over the counting chambers.

Step 5. Centrifuge target cell suspension at 500 ± 50 g for 5 to 10 minutes.

Step 6. Decant supernatant. Add 1.0 mL 0.1M citric acid solution to the cell pellet. Mix well and incubate at 35° C for 1 to 2 hours.

Step 7. Separate nuclei by violent shaking followed by centrifugation at 1000 ± 100 g for

20 to 25 minutes



Cell Counting Procedure

Step 8. Discard supernatant. Re-suspend the cell pellet in 0.5 to 1.0 mL citric acid-crystal violet solution.

Step 9. Pipette 10 microliters of cell sample into the hemocytometer.

Step 10. Wait 60 seconds for the cells to settle.

Manually Count Nuclei in Sample

Step 11. Place the hemocytometer under a microscope with a typical magnification of 100.

Step 12. Focus both onto the grid pattern and the cell particles, and count the total number of nuclei found in 4 large corner squares.

Step 13. If nuclei are touching the 4 perimeter sides of a corner square, only count cells on 2 sides, either the 2 outer sides or 2 inner sides

Step 14. Count the live nuclei (with crystal violet)

Nuclei Calculations & Cleaning of Hemocytometer

Step 15. Multiply the dilution factor by the total number of nuclei, divide by the # of corner squares counted, and multiply by 10^4 to obtain cell concentration (cells/ml).

$$\text{Cell Concentration } \left(\frac{\text{cells}}{\text{ml}} \right) = \frac{\text{nuclei}}{\# \text{ of squares}} \times 10^4$$

Step 16. Clean hemocytometer and glass cover slip with 70% EtOH

Practical No: 7
Preparation of washed bacterial cell suspension using centrifuge

Centrifugation is a method of separating molecules having different densities by spinning them in solution around an axis (in a centrifuge rotor) at high speed. It is one of the most useful and frequently employed techniques in the molecular biology laboratory. Centrifugation is used to collect cells, to precipitate DNA, to purify virus particles and to distinguish subtle variations in the conformation of molecules.

Centrifuges have two different units of measurement.

1. Revolutions Per Minute (RPM)

Revolutions Per Minute (RPM), with respect to centrifugation, is simply a measurement of how fast the centrifuge rotor does a full rotation in one minute. Basically, it tells us how fast the rotor is spinning. Centrifuges have a speed range that they are capable of achieving and varies depending on the type of centrifuge. A low speed centrifuge might spin at as low as 300 RPM, whilst a high speed centrifuge could spin up to 15000 RPM. Ultracentrifuges are also available and are the most powerful type of centrifuge, they can spin in excess of 150,000 RPM.

2. Relative Centrifugal Force (RCF)

Relative Centrifugal Force (RCF) or g-force (both are the same, RCF is expressed as units of gravity) is a measurement of the gravitational force that a sample is subjected to. The force is generated from the spinning of the rotor which, in turn, exerts this force outward on the centrifuge tube. Not only does RCF take into account the speed of rotation, it also measures the distance from the center of rotation to give us a g-force measurement. RCF is the preferred method of measurement as it will remain the same even if one is using a different centrifuge with a different rotor size.

Nutrient Media

Luria broth (LB) is a nutrient-rich media commonly used to culture bacteria in the lab. However, a liquid culture is capable of supporting a higher density of bacteria and is used to grow up sufficient numbers of bacteria necessary for an experimental use.

Protocol:

1. Prepare liquid LB. For example, to make 100 ml of LB, weigh out the followings into a 100 ml glass bottle:
 - 1.0g NaCl
 - 1.0g Tryptone
 - 0.5g Yeast Extract
2. Loosely close the cap on the bottle (do NOT close all the way or the bottle may explode) and then loosely cover the entire top of the bottle with aluminum foil. Autoclave and allow to cool to room temperature. Now screw on the top of the bottle and store the LB at room temperature.
3. Using a sterile pipette tip or toothpick, select a single colony from your LB agar plate.
4. Drop the tip or toothpick into the liquid LB.
5. Loosely cover the culture with sterile aluminum foil or a cap that is not air tight.
6. Incubate bacterial culture at 37°C for 12-18hr in a shaking incubator.
7. After incubation, check for growth which is characterized by a cloudy haze in the media.
8. Add cell suspension (1 ml) to an Eppendorf and centrifuge at 3000rpm for 10 minutes.
9. After centrifugation, remove upper layer (media) and wash the cell pellet with 1x PBS for 30 seconds.
10. Centrifuge the cells again for 5 minutes at 3000rpm.
11. Repeat step 10 and 11.
12. Remove upper layer (PBS) and store the pellet for further use.

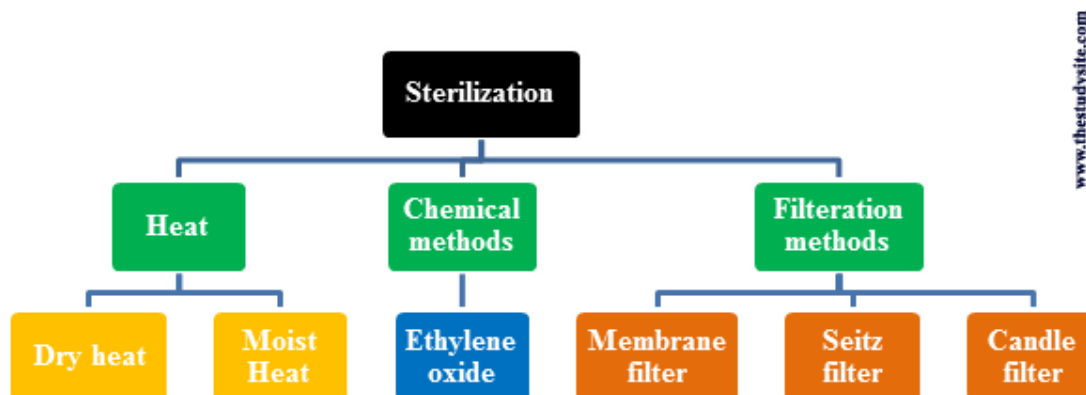
Practical No: 8

Demonstration of different sterilization techniques

Introduction

Sterilization is defined as total destruction of all microorganisms (whether or not pathogenic) and their spores, usually using drastic methods such as concentrated toxic chemicals (alcohols, chlorine, formaldehyde, glutar-aldehydes, etc.), very high temperature, or intense radiation. A sterilized item cannot support life in any form.

Methods involved in sterilization



Flow sheet showing methods of sterilization

1. Heat sterilization

This is the most common method of sterilization, in which heat is used to kill microbes. The Temperature and duration of heating determines the extent of sterilization. As the temperature of heat raises the time span required for sterilization decreases. Further, the sterilization time increases with a decrease in temperature and vice-versa. But one needs to maintain minimum sterilization time or minimum contact time for the heat to be in touch with microbes or bacteria and thereby kill them. The heat method of sterilization is again of two types based on the type of heat used.

A) Moist heat methods**B) Dry heat methods****A. Moist heat method of sterilization**

In this method, heat is applied in the form of steam or by boiling. It includes following techniques;

1. Boiling 2. Pasteurization 3. Autoclaving (use of steam)

1. Boiling is preferred for metallic devices like surgical scissors, scalpels, needles, etc. Here substances are boiled to sterilize them.

Advantages

1. Heating water to a high temperature of 100°C kills most of the pathogenic organisms.
2. The simplest and easiest method of disinfecting.

2. Pasteurization is the process of heating the milk at a temperature of 60 °C or 72 °C for 3 to four times. Here alternative heating and cooling kills all the microbes and molds without boiling the milk.

Advantages

1. Pasteurized milk has a longer shelf-life than raw milk.
2. Pasteurization greatly reduces the risk of food poisoning.

Disadvantages

1. It affects the texture, flavor, and nutritional value of foods.

3. Autoclaving is the most common method used for drugs as it is powerful enough to kill bacterial spores. Bacterial spores are the forms of bacteria which are inert. They form a rigid cover over the cell wall during harsh climate. This cover prevents any damage to cell and drying of the cell. By steam sterilization, these forms of bacteria are also killed as steam destroys the cell wall.

Principle

In this method sterilization is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. The temperature of boiling depends on the surrounding atmospheric pressure. A higher temperature of steaming is obtained by employing a higher pressure. When the autoclave is closed and made air-tight, and water starts boiling, the inside pressures increases and now the water boils above 100°C. At 15 lb per sq. inch pressure, 121°C

temperatures is obtained. This temperature is maintained for 15 minutes in order to kill the bacterial spores. It works like a pressure cooker.

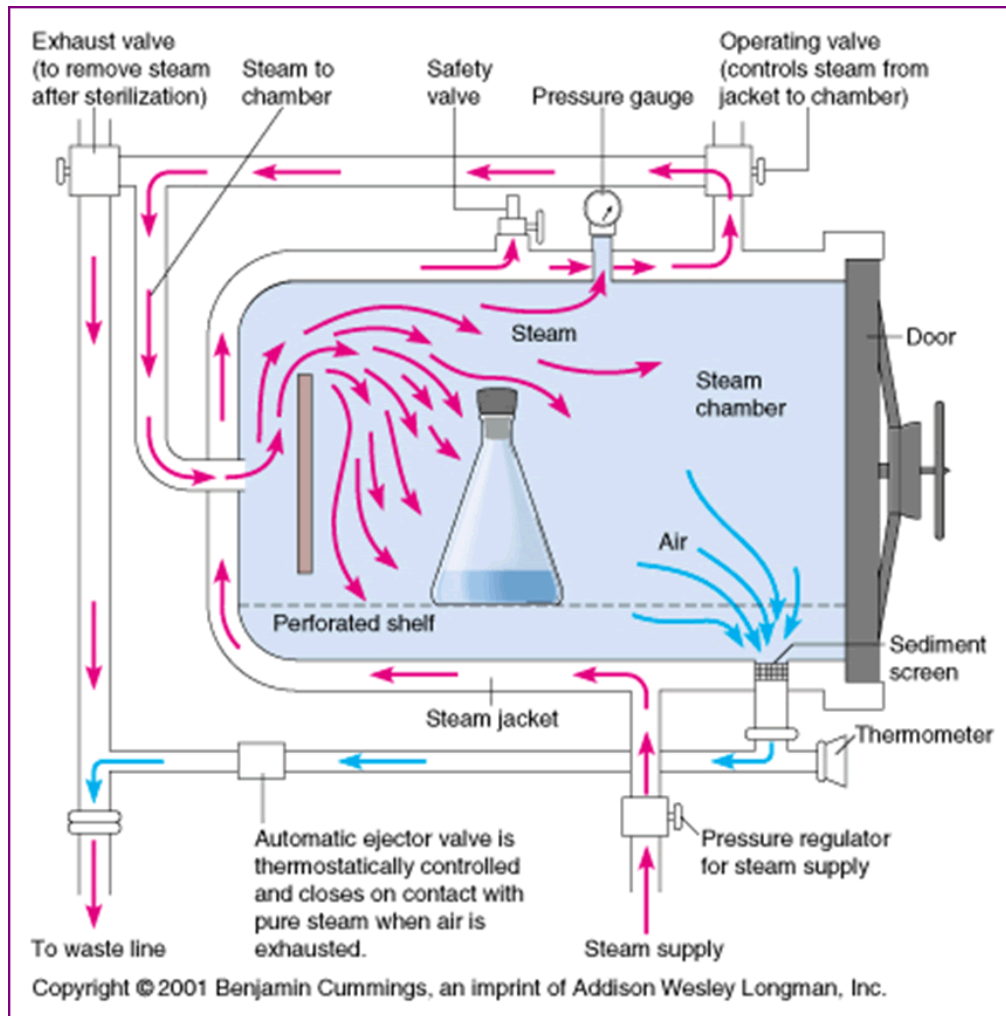
Construction of Autoclave

Autoclave is a metallic cylindrical vessel. On the lid, there are;

- (1) A gauge for indicating the pressure,
- (2) A safety valve, which can be set to blow off at any desired pressure, and
- (3) A stopcock to release the pressure. It is provided with a perforated diaphragm. Water is placed below the diaphragm and heated from below by electricity, gas or stove.

Operating Procedure of Autoclave

- (a) Place materials inside,
- (b) Close the lid. Leave stopcock open,
- (c) Set the safety valve at the desired pressure,
- (d) Heat the autoclave. Air is forced out and eventually steam ensures out through the tap,
- (e) Close the tap. The inside pressure now rises until it reaches the set level (i.e. 15 Win), when the safety valve opens and the excess steam escapes,
- (f) Keep it for 15 minutes (holding time),
- (g) Stop heating,
- (h) Cool the autoclave below 100°C,
- (i) Open the stopcock slowly to allow air to enter the autoclave.



Procedure of autoclave

Advantages

The penetrating nature of steam makes it a great solution to destroy proteins in any microorganism after a certain period of time. It is environmentally safe having no toxic byproducts.

B. Dry heat methods

Here, substances are subjected to dry heat. These methods include;

1. Flaming
2. Incineration
3. Hot air oven.
4. Radiation sterilization

1. Flaming

It is the process of exposing metallic device like the needle, scalpels, and scissors to flame for a few minutes. The fire burns the microbes and other dust particles on the instrument directly.



Flaming

Advantages

1. Total destruction when needed.
2. Prevents reuse of materials.
3. Quick and simple method of killing microorganisms.

Disadvantages

1. It is relatively slow.
2. Many objects cannot withstand high temperatures.

2. Incineration

It is done specially to sterilize the inoculating loops used in microbial culturing technique. The metallic end of the loop is heated to red hot on the flame. This exposure kills all the germs.



Incineration

Advantages

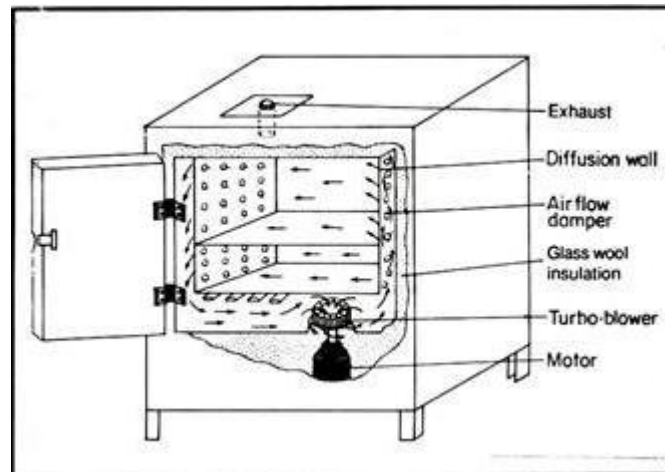
1. Total destruction when needed.
2. Prevents reuse of materials.
3. Quick and simple method of killing microorganisms.

3. Hot Air Oven

It is one of the most common methods used for sterilization. Glass wares, swab sticks, syringes, powder and oily substances are sterilized in hot air oven. For sterilization, a temperature of 160°C for 3 hours or 180°C for 2 hours is maintained. Spores are killed at this temperature. It leads to sterilization. Hot air oven is suitable for dry materials like powders, metal devices, and glassware etc.

Working principle

It is an apparatus with double metallic walls and a door. There is an air space between these walls. The apparatus is heated by electricity or gas at the bottom. On heating, air at the bottom becomes hot and passes between the two walls from below upwards, and then passes in the inner chamber through the holes of the apparatus. A thermostat is fitted to maintain a constant temperature of 160°C .



Hot air oven

Advantages

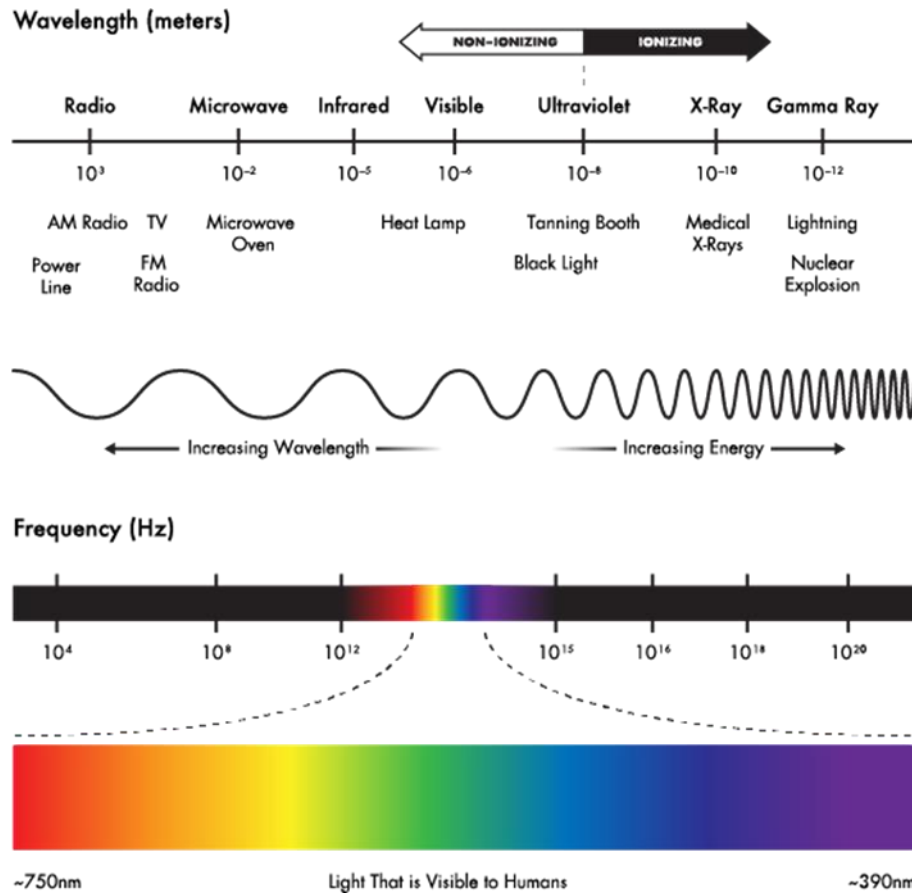
Dry heat sterilization is preferred for heat stable products that are sensitive to moisture.

4. Radiations

This method involves exposing the packed materials to radiation for sterilization. There are two types of radiations available for sterilization. i.e., non-ionic and ionic radiations.

□ **Non-ionic radiation sterilization** is safe to the operator e.g. ultra-violet radiations. These radiations can be used even at the door entrance to prevent entry of live microbes through the air. Non-ionizing radiation is longer wavelength/lower frequency lower energy radiation. So, it lacks the ability to penetrate substances and can only be used for **sterilizing surfaces**.

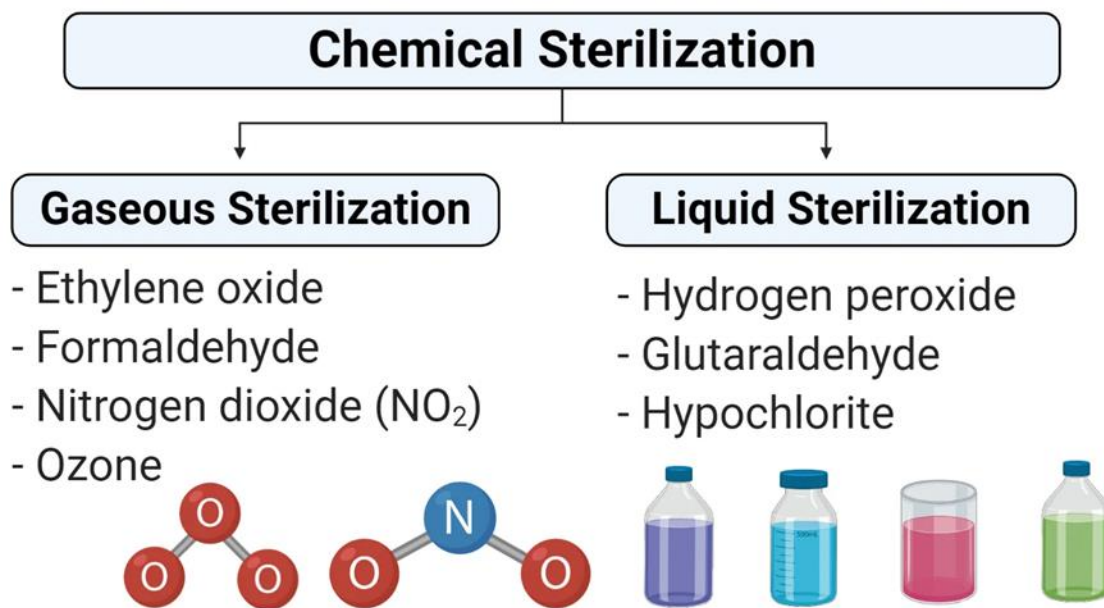
□ **Ionizing radiation sterilization:** These are powerful radiations which are very useful for sterilization. The operator needs to protect himself from exposure to these radiations using special clothing e.g. X-rays and γ -rays etc. Ionizing radiation is the use of short wavelength and high-intensity high energy radiations to destroy the microorganisms.



Ionizing and non-ionizing radiations used in sterilization methods

2. Chemical method of sterilization

Chemical Sterilization is the process of removal of microorganisms using chemical bactericidal agents. Although physical methods of sterilization are more appropriate for effective sterilization but it is not recommended to use them for heat-sensitive materials like plastics, fiber optics, and biological specimens. Under such conditions, chemicals either in liquid or gaseous state can be used for sterilization. However, it is crucial to ensure that materials undergoing sterilization are compatible with the chemicals being used. Besides, it is important to adopt safety measures in the workplace during the use of chemical agents. The chemical method of sterilization can be categorized as liquid and gaseous sterilization.



Types of chemical sterilization

1. Gaseous Sterilization

- Gaseous sterilization involves the process of exposing equipment or devices to different gases in a closed heated or pressurized chamber.
- Gaseous sterilization is a more effective technique as gases can pass through a tiny orifice and provide more effective results.

- Besides, gases are commonly used along with heat treatment which also facilitates the functioning of gases.
- However, there is an issue of release of some toxic gases during the process which needs to be removed regularly from the system.
- The mechanism of action is different for different types of gases.
- Some of the common gases used for gaseous sterilization are explained below:

Ethylene oxide

- Ethylene oxide (EO) gas is a common gas used for chemical treatment applied to sterilize, pasteurize, or disinfect different types of equipment and surfaces because of its wide range of compatibility with different materials.
- EO treatment often replaces other sterilization techniques like heat, radiation, and even chemicals in cases where the objects are sensitive to these techniques.
- This method is a widespread method used for almost 70% of all sterilizations and around 50% for disposable medical devices.
- The mechanism of antimicrobial action of this gas is assumed to be through the alkylation of sulphhydryl, amino, hydroxyl and carboxyl groups on proteins and amino groups of nucleic acids.
- EO treatment is usually conducted at the temperature range of 30-60°C for several hours which aids in the activity of the gas.
- The efficacy of gas depends on the concentration of gas available for each article which is greatly assisted by the good penetrating nature of the gas. It diffuses readily into many packaging materials including rubber, plastics, fabric, and paper.
- EO kills all known microorganisms such as bacteria (including spores), viruses and fungi (including yeasts and molds). It is compatible with almost all materials even when repeatedly applied.
- This process, however, is not without drawbacks as the level of gas in the sterilizer goes on decreasing due to absorption and the treated articles need to undergo a process of desorption to remove the toxic residual wastes.
- Organisms are more resistant to EO treatment in a dried state, as are those protected from the gas by inclusion in crystalline or dried organic deposits.

Formaldehyde

- Formaldehyde is another important highly reactive gas which is used for sterilization.
- This gas is obtained by heating formalin (37% w/v) to a temperature of 70-80°C.

- It possesses broad-spectrum biocidal activity and has found application in the sterilization of reusable surgical instruments, specific medical, diagnostic and electrical equipment and surface sterilization of powders.
- Formaldehyde doesn't have the same penetrating power of ethylene oxide but works on the same principle of modification of proteins and nucleic acids.
- As a result of low penetrating power, its use is often limited to paper and cotton fabrics.
- Formaldehyde can generally be detected by smell at concentrations lower than those permitted in the atmosphere and thus can be detected during leakage or other such accidents.

Nitrogen dioxide (NO₂)

- Nitrogen dioxide is a rapid and effective sterilant that can be used for the removal of common bacteria, fungi and even spores.
- NO₂ has a low boiling point (20°C) which allows a high vapor pressure at standard temperature.
- This property of NO₂ enables the use of gas at standard temperature and pressure.
- The biocidal action of this gas involves the degradation of DNA by nitration of phosphate backbone which results in lethal effects on the exposed organism as it absorbs NO₂.
- An advantage of this gas is that no condensation of the gas occurs on the surface of the devices because of the low level of gas used and the high vapor pressure. This avoids the need for direct aeration after the process of sterilization.

Ozone

- Ozone is a highly reactive industrial gas that is commonly used to sterilize air and water and as a disinfectant for surfaces.
- Ozone has potent oxidizing property that is capable of destroying a wide range of organisms including prions. Ozone is usually generated from medical-grade oxygen so its use does not involve hazardous chemicals.
- Similarly, the high reactivity of ozone allows removal of waste ozone by converting the ozone into oxygen by passing it through a simple catalyst.
- However, because ozone is an unstable and reactive gas, it has to be produced on-site, which limits the use of ozone in different settings.
- It is also very hazardous and thus only be used at a concentration of 5ppm, which is 160 times less than that of ethylene oxide.

2. Liquid Sterilization

- Liquid sterilization is the process of sterilization which involves the submerging of equipment in the liquid sterilant to kill all viable microorganisms and their spores.
- Although liquid sterilization is not as effective as gaseous sterilization, it is appropriate in conditions where a low level of contaminants is present.

Different liquid chemicals used for liquid sterilization includes the followings:

Hydrogen peroxide

- Hydrogen peroxide is a liquid chemical sterilizing agent which is a strong oxidant and can destroy a wide range of microorganisms.
- It is useful in the sterilization of heat or temperature-sensitive equipment like endoscopes. In medical applications, a higher concentration (35-90%) is used.
- H_2O_2 has a short sterilization cycle time. The cycles are as short as 28 minutes whereas, ethylene oxide has cycles as long as 10-12 hours.
- However, hydrogen peroxide has drawbacks like low material compatibility, lower capacity of penetration and associated health risks.
- Vaporized hydrogen peroxide (VHP) is used to sterilize largely enclosed and sealed areas, such as entire rooms and aircraft interiors.

Glutaraldehyde

- Glutaraldehyde is a liquid sterilizing agent which requires comparatively long immersion time. For the removal of all spores, it requires Immersion time of 22 hours
- The presence of solid particles further increases the immersion time.
- The penetration power is also meager as it takes hours to penetrate a block of tissues.
- Use of glutaraldehyde is thus limited to certain surfaces with less contamination.

Hypochlorite

- Hypochlorite solution commonly known as liquid bleach, is another liquid chemical that can be used as a disinfectant.
- Submerging devices for a short period in liquid bleach might kill some pathogenic organisms but to reach sterilization submersion for 20-24 hours is required.
- It is an oxidizing agent which acts by oxidizing organic compounds in the modification of proteins in microbes which might ultimately lead to death.
- Appropriate concentrations of hypochlorite can be used for the disinfection of workstations and even surfaces to clean blood spills and other liquids.

Modes of action of chemicals

- Protein coagulation
- Disruption of cell membrane resulting in exposure, damage or loss of the contents.
- Removal of free sulfhydryl groups essential for the functioning of enzymes.
- Substrate competition – a compound resembling the essential substrate of the enzyme diverts or misleads the enzymes necessary for the metabolism of the cell and causes cell death.

Advantages

- Proven reduction of most bacteria and viruses in water.
- Residual protection against recontamination.
- Ease-of-use and acceptability.
- Chemical cleaning is more effective as compared to non-chemical products.

Disadvantages

- They need to be used within a short duration of time as they have a relatively short shelf life.
- Generally, not heat stable.
- Can be quite corrosive to skin and metal substances.

3. Mechanical/Filtration methods

This method is used for sterilizing thermolabile solutions which will otherwise be degraded by other conventional heating methods. The drug solutions are passed through the sterile bacteria proof filter unit and subsequently transferring the product aseptically into the sterile containers which are then sealed.

Procedure

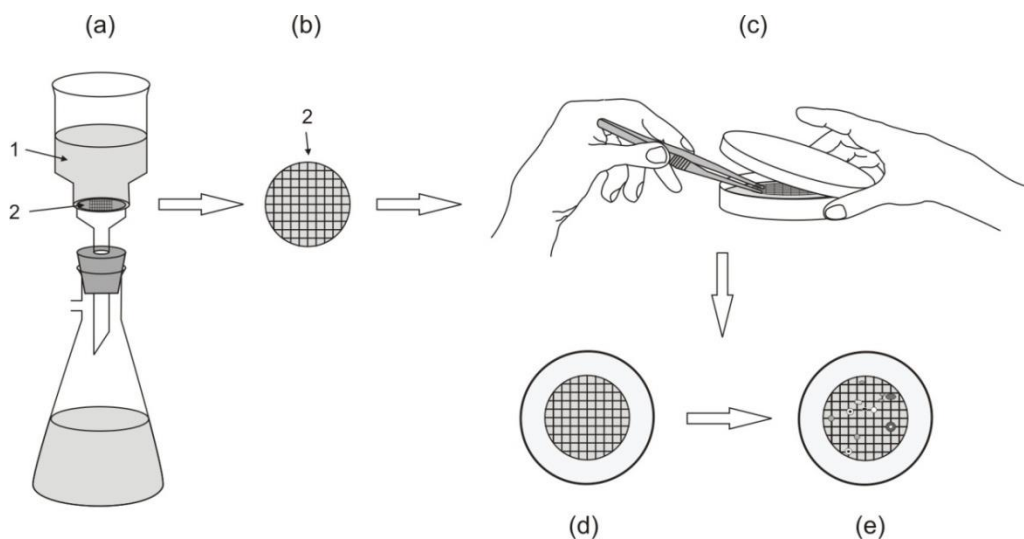
The solutions to be sterilized are passed through the filter and collected in the sterile receiver by the application of positive pressure to the non-sterile compartment or negative pressure to the sterile side.

Membrane filters

- Membrane filters are made of cellulose-derivative (acetate or nitrate). They are very fine and fixed in some suitable holders.
- Nominal pore size is 0.22 ± 0.02 mm or less is required.
- The membranes are brittle when dry. In this condition they can be stored for years together. They become very tough when dipped in water.
- They are sterilized by autoclaving or by ethylene oxide gas. They cannot be sterilized by dry heat as they decompose above 120°C .
- They are suitable for sterilizing aqueous and oily solutions but not for organic solvents such as alcohol and chloroform etc.
- Membrane filters are generally blocked by dirt particles and organisms. Pre-filtration (through glass-fiber paper prefilter) reduces the risks of blockage of the final filter.

Examples of membrane filters:

MF-Millipore – it is a mixture of cellulose esters

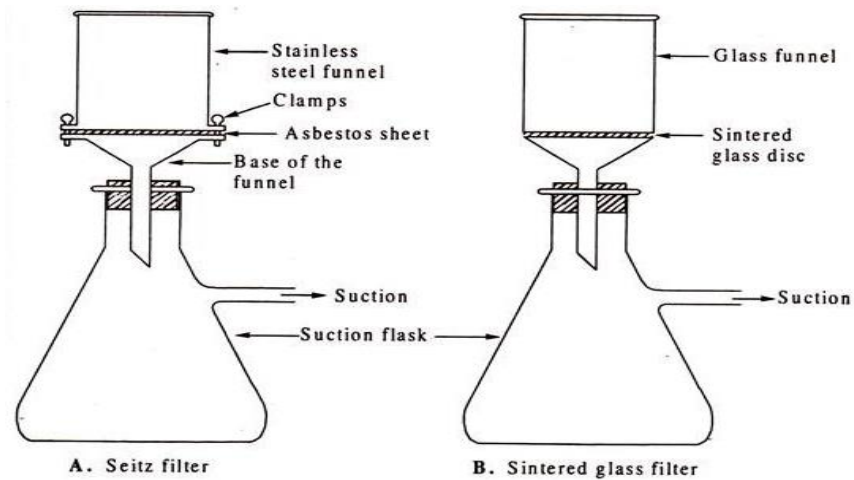


Sintered (or fritted) glass filters

Borosilicate glass is finely powdered in a ball-mill and the particles of required size are separated. This is packed into disc mounted and heated till the particles get fused. The discs thus made have pore size of 2 mm and are used for filtration. As these are made of glass and hence do not absorb liquids during filtration. The disadvantage is that they are very brittle and break easily. They are cleaned with the help of sulfuric acid.

Seitz filter:

These are made of asbestos or other material. They are pad like and thicker than membrane filters. They do not rupture during filtration. But the solution might get absorbed by the filter pad itself.



Examples of membrane filters

Advantages of sterilization by filtration

1. Thermolabile solutions can be sterilized.
2. It removes all the living microorganisms.

Disadvantages of sterilization by filtration

1. Filters may break down suddenly or gradually on use.
2. Sterility testing is obligatory on the filtered solution.
3. Filter media may be absorbed on the filter surface.
4. Viruses are not removed by filtration.
5. Suspensions and oils cannot be sterilized by this method due to their heavy load of particulate matters and viscosity.

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