



PITHAPUR RAJAH'S GOVERNMENT COLLEGE

Autonomous and NAAC Re-accredited with 'A' Grade (3.17/4.00 CGPA)

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LECTURER IN MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

P.R.G.C (A), KAKINADA

**microbial and analytical
techniques**

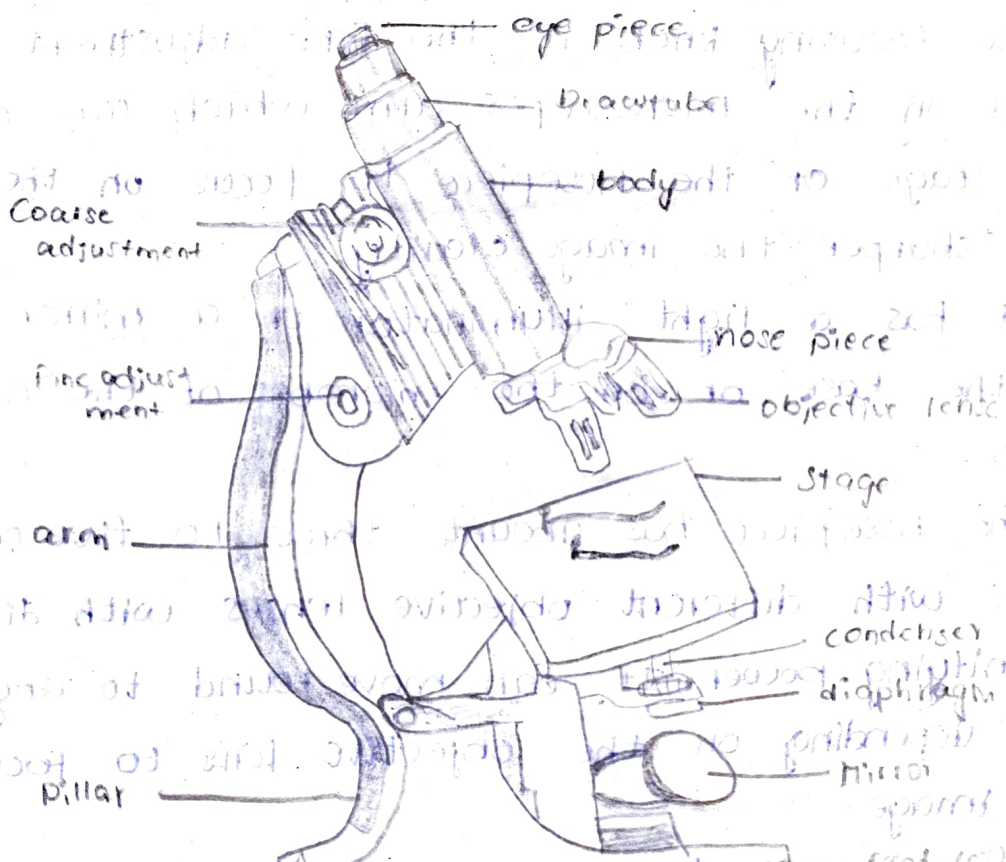
Unit-1 - Microscopy

MICROSCOPE :

Bright Field Light Microscope [Compound light microscope]

This is the most basic optical microscope used in microbiology laboratories which produces a dark image against a bright background. Made up of two lenses, it is widely used to view plant and animal cell organelles including some parasites such as paramecium after staining with basic stains. Its functionality is based on being able to provide a high-resolution image, which highly depends on the proper use of the microscope. This means that an adequate amount of light will enable sufficient focusing of the image to produce a quality image. It is also known as a compound light microscope.

Parts of a bright-field microscope



It is composed of:-

→ Two lenses which include the objective lens and the eyepiece or ocular lens.

Objective lens is made up of six or more glasses, which make the image clear, from the object.

→ The condenser is mounted below the stage which focuses a beam of light on to the specimen. It can be fixed or movable, to adjust the quality of light but this entirely depends on the microscope.

→ They are held together by a sturdy metallic curved back used as an "arm" and a stand at the bottom, known as the base, of the microscope. The arm and base hold all the parts of the microscope.

→ The stage where the specimen is placed, allowing movement of the specimen around for better viewing with the flexible knobs and it is where the light is focused on.

→ Two focusing knobs i.e. the fine adjustment knob, found on the microscope's arm which can move the stage or the nosepiece to focus on the image the sharper the image clarity.

→ It has a light illuminator or a mirror found at the base or on the microbes of the nose piece.

→ The nosepiece has about three to five objective lenses with different magnifying power. It can move round to any position depending on the objective lens to focus on the image.

→ An aperture diaphragm also is

of a lens to separate or distinguish between, small objects closely linked together.

Whereas the Eyepiece magnifies the image at the end of the viewing, its magnification range is lower than that of the objective lens at $8\times$ - $12\times$ ($10\times$ standard) and that of the objective lens at $40\times$ - $100\times$.

Magnification and resolution of the microscope is highly dependent on the objective lens.

APPLICATIONS OF THE BRIGHT FIELD MICROSCOPE

Vastly used in microbiology, this microscope is used to view fixed and live specimens that have been stained with basic stains. This gives contrast, for easy visibility under to identify basic bacteria cells and parasite (protozoans) such as paramecium.

TRANSMISSION ELECTRON MICROSCOPY [TEM]

→ The electron source is commonly a filament of 30-150 potential.

→ The electron beam passes through the center of a ring like magnetic coil.

→ After being transmitted through the specimen, the magnetic objective focuses the electrons into a first image of the object which is enlarged.

→ The final enlarged image can be reviewed by striking a fluorescent screen which is visible.

→ In electron microscopes, electrons replace the illuminating beam.

→ Electron microscopes have a practical resolution roughly 1000 times better than the light microscope. With many electron microscopes, points closer than

- A Heated tungsten filament in the electron gun generates a beam of electron that is focused on the specimen by the condenser.
- The column containing the lenses and specimen must be under high vacuum to obtain a clear image because electron are deflected by collisions with air molecules.
- The specimen scatters some electrons, but those that pass through are used to form an enlarged image of the specimen on a fluorescent screen.
- A denser region in the specimen scatter more electron and therefore appears darker in the image since fewer electrons strike that area of the screen these region, are said to be "electron dense".

SCANNING ELECTRON MICROSCOPE: [SEM]

- Transmission electron microscope from an image from radiation that has passed through a specimen.
- The scanning electron microscope (SEM) produces an image from electron released from atoms on an objects surface.
- The sem has been used to examine the surfaces of microorganisms in great detail, many SEMs have a resolution of 7nm or less.
- Specimen preparation for sem is relatively easy and in some cases, air-dried material can be examined directly.
- Microorganisms usually must first be fixed dehydrated and dried to preserve surface structure and prevent collapse of the cells when they are

→ STAINING TECHNIQUES :

There are different types of staining techniques.

- * Simple staining techniques
- * Differential staining techniques
- * Negative staining techniques

(1) SIMPLE STAINING

Aim : To stain the bacterial cells with simple basic stain and observe the morphology.

Requirements : 24 hours old bacterial culture of bacillus, 1% methylene blue and glass slide.

Procedure :

- (1) Take a clean glass slide, wash and dry them.
- (2) Place a loop full of broth of bacterial culture on the clean glass slide at the center.
- (3) Spread the bacterial suspension thinly on the slide with inoculating loop to prepare the smear.
- (4) Fix the smear by heating gently over the spirit lamp flame.
- (5) Stain the smear by adding few drops of methylene blue solution to the smear and allow it to stand for one to eight minutes.
- (6) Remove the stain and wash this smear gently with slowly running water tap.
- (7) Blot - dry the slide using blot paper but do not wipe the slide.
- (8) Examine the preparation under oil immersion objective of microscope.

(2) DIFFERENTIAL STAINING

Aim: The staining differentiates bacteria into two classes. Gram positive bacteria and gram negative bacteria.

In 1884 Danish physician Christian Gram developed a staining technique called "Gram's staining" based on cell wall.

Requirements: Glass slide, Crystal violet, Iodine, Acetone, safranin, water and microscope.

Procedure:

- (1) Take a clean grease free slide and a loop full of suspension is placed on the center of the slide.
- (2) Heat fix the smear by flame heating.
- (3) Stain the smear by flame heating with basic dye like crystal violet for 30 sec. it acts as primary stain.
- (4) Rinse the slide with water for 2 sec.
- (5) It is followed by treatment with Iodine solution for 1 min it acts as mordant.
- (6) Rinse the smear with water.
- (7) The smear is decolorized by washing with ethanol or acetone. This step separates bacteria into gram positive and gram negative bacteria.
- (8) Gram positive bacteria retain crystal violet.
- (9) Gram negative bacteria lose the crystal violet and become colourless.
- (10) Finally the smear is counter stained with basic dye called safranin. Gram negative bacteria appear pink to red and gram positive bacteria appear purple color.

Gram positive

Gram negative



Fixation



Crystal Violet



Iodine treatment



Decolorization



Counter stain
Safranin



(3) NEGATIVE STAINING :

Aim: The size and shape of the bacterial cells can be determined by negative staining. Because it does not disturb the shape of its bacterial cell. The stain and the bacterial cells are negatively charged so there is repulsion action between the cells and dyes used are nigrocin or Indian ink.

Requirements: Nigrocin solution, sterile tooth pick, glass slide, marking pencil, inoculation loop, Bunsen burner.

Procedure :

- (1) Take a clean and grease free slide.
- (2) Take a loop full of bacterial suspension and place at one end of the slide
- (3) On to the bacterial suspension place a drop of nigrocin (or) Indian ink.
- (4) With the help of another glass slide spread out the dye and cells as a thin film

Unit-2 Sterilization and Disinfection techniques

(i) Sterilization :

The process of killing micro-organisms including their spores is known as "sterilization".

Sterilization is of two types:

- (1) Physical sterilization
- (2) Chemical sterilization

Physical sterilization:

In this sterilization by using instruments and sunrays physically we kill the micro-organisms.

Types : (1) Sunlight

(2) Drying

(3) Dry heat

a) ~~fanning~~ flaming

b) Incineration

c) Hot air oven

(4) Moist heat

a) Pasteurization

b) Boiling

c) Steam under pressure

(5) Filtration

a) candles

b) Asbestos pads

c) Membranes

(6) Radiation

(7) Ultrasonic and sonic vibration

(1) Sunlight:

* Sunlight possesses appreciable bacterial activity and plays an important role in the spontaneous

ous sterilization.

- * The action is primarily due to ultraviolet rays.
- * Direct sunlight has an active germicide effect due to the combine effect of ultraviolet and heat rays.
- * Bacteria suspended in water are readily destroyed by exposed of sunlight.

(2) Drying :

- * Moisture is essential for the growth of bacteria.
- * Drying in air has therefore, a deleterious effect on many bacteria.
- * However, this method is unreliable, and is only of ~~these~~ theoretical interest.
- * Spores are unaffected by drying.

(3) Dry Heat:

T.D.T [Thermal Death Time] The time required for sterilization is inversely proportional to the temperature of exposure and can be express as TDT.

a) Flaming :

Inoculating loop or wire, the tip of forceps and searing spatules are held in a bunsen burner flame they become red hot.

Inoculating loop corying infective material may be dipped in a disinfectant before flaming to prevent spottering.

b) Incineration :

This an excellent method for safety destroying materials such as contaminated cloth, animal

corcoses and pathological material plastics such as pvc and polystyrene can dealt with similar, but polystyrene can dealt with similar but polystyrene materials emit cloud of dense black smoke and hence should be autoclave in appropriate containers.

c) Hot air oven :

- * Many objects are best sterilized in the absence of water by dry heat sterilization.
- * Glassware should be perfectly dried and should be arranged in a manner that allow free circulation of hot air.
- * Test tubes, flasks should be plugged with cotton wool.
- * Other glassware such as petri dishes and pipettes should be wrapped.
- * Above 180°C cotton plugs would get charged

Temp	Time
160	45
170	80
180	7.5
190	1.9

(i) Tyndalization :

In this process media has to sterilize when the media is exposure to 100°C for 20 on three successive days.

1st day - 100°C for 20 to kill vegetative cells

2nd day - 100°C for 20 remove all spores

3rd day - 100°C for 20 to kill all microorganisms

It is also called fractional sterilization.

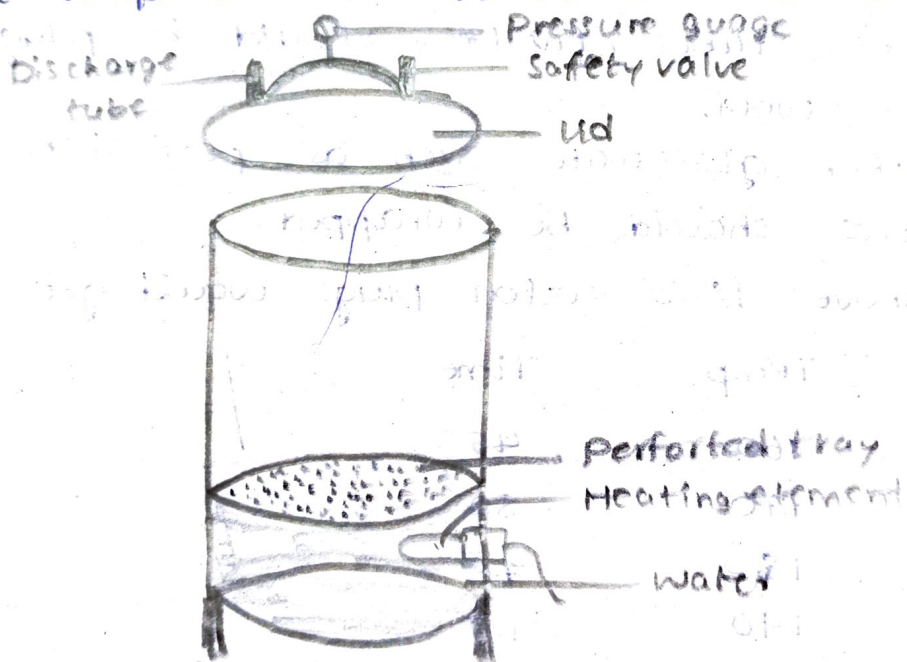
Autoclave

* The principle of the autoclave or steam sterilizer is that water boils when its vapour pressure equals that of the surrounding atmosphere.

* Autoclave is a cylindrical device made of steel or gunmetal.

* The vapour is equal to outside of the temperature (the visible temp and inside vapour temp)

* It kills the micro-organisms and vegetative spores at temp 121°C for 15 min at 15 lbs pressure.



Autoclave

Temp

Time

121

15

126

10

134

3

Pressure cooker

Pressure cooking is a method of cooking in a sealed vessel that does not permit air or

liquids to escape below a preset pressure.

At this pressure (boast relative to sea level atmospheric pressure), water boils at 45°C (125°F).

Filtration:

An especially valuable technique for sterilizing heat sensitive materials is filtration.

→ They are two types of filters:

(1) Depth filters

(2) Membrane filters.

(1) Depth filters:

One of the oldest types used is the depth filters.

On the depth filters microorganisms are retained either by electrostatic attraction.

(a) candle filters:

used in purification of water for industrial and drinking purpose.

(b) Asbestos filters:

They are made from chrysotile type asbestos chemically composed of magnesium silicate.

(2) Membrane filters

The most common type of filter for sterilization in the field of microbiology is the membrane filters.

→ specific pore sizes from $25\mu\text{m}$ to less $0.25\mu\text{m}$

HEPA Filters: High efficiency particulate Air filters. (HEPA) can remove 99.7% of $0.3\mu\text{m}$ sized particles that is employed in laminar

Chemical sterilization:

* Disinfection is the process of killing inhibition or removal of microorganisms that may cause disease.

* The chemical agents called that are used for this disinfection are called as chemical disinfectants.

* Disinfectants are effective and toxic in destroying pathogenic microorganisms but not necessarily in killing the resistant spores.

(1) Phenol and phenolic compounds:-

* Phenol is the first disinfectant used by Joseph Lister, in 1880's.

* Phenol and phenolic compounds are very effective disinfectants.

* A 5% aqueous solution of phenol rapidly kills the vegetative cells of microorganisms.

(2) Alcohols:-

* Alcohols are one of the most widely used disinfectants and antiseptics.

* They are bactericidal and fungicidal but not sporicidal.

* The two most popular disinfectants are ethanol and isopropanol.

* They are effective when used in 70-80% concentration.

(3) Halogen:-

* The important halogens used as disinfectant agents are iodine and chlorine and their compounds.

* Iodine is one of the oldest and most

effective disinfectants.

- * At higher concentration it may even kill some spores

- * It may cause skin disease.

- * They are used in hospitals for preoperative skin degerming and in laboratories for disinfection.

(4) Aldehydes:

Several aldehydes having low molecule weight are used as disinfectants. the two important and widely aldehyde are formaldehyde and glutaraldehyde. formaldehyde and glutaraldehyde usually dissolved in water or alcohol before used.

Glutaraldehyde is an effective disinfectant it used as 2% buffered solution. Glutaraldehyde disinfectant the object with in 10 minutes of time.

(5) Hypochlorites:

Chlorine is the closely disinfectant for municipal water supplies and swimming pools. it is also employed in the dairy and food industries.

It may be applied to drinking water in a final concentration of 0.1 to 0.5 part per million.

It may be applied as chlorine gas, sodium hypochloride, calcium hypochlorite and hypochlorous acid.

Unit - 3 - Microbiological techniques

Isolation of pure cultures

* By definition, a pure culture is a population of cells that arises from a single cell and free from contamination with other species.

* A pure culture as species can be proposed by isolating the particular species from a mixture of cells or species.

Importance of pure culture:

Pure cultures are necessary and important for the following reasons.

- (1) Identification of the species
- (2) Analysis of food, water, industrial products.
- (3) Sterility testing of products destined for human eye.
- (4) Assay of antibiotics and vitamins
- (5) Determination of antibiotics sensitivities of pathogens isolated from patients
- (6) preparation of biological products or materials used for immunizations etc.

Isolation methods are of three types:

- (1) Plating techniques
- (2) Serial dilution technique
- (3) Micromanipulator technique

(1) plating techniques:

These are again divided into three types

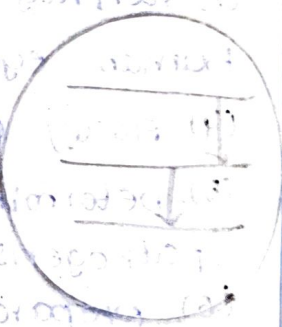
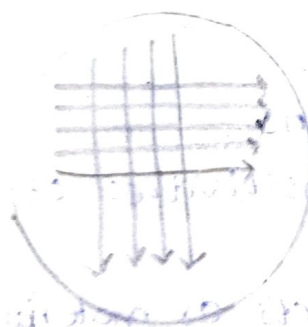
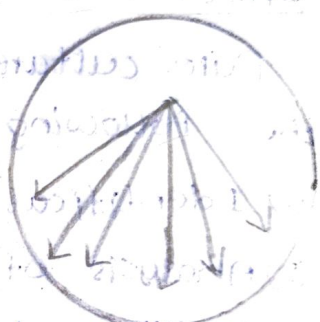
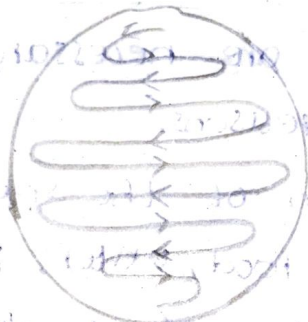
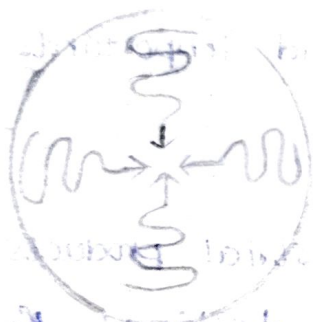
- (a) streak plate method
- (b) spread plate method
- (c) pour plate method

(a) streak plate method:

* This is the most practical method for the isolation of pure cultures.

* Depending on the mode of streaking there are different types of streak plate technique some of them are

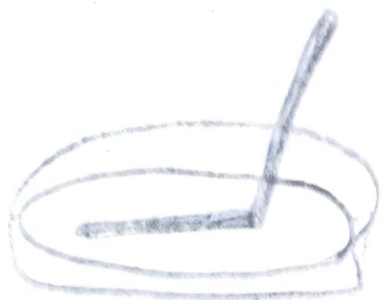
- (i) clock plate technique — explain brief with diagram.
- (ii) Radiant streak method — explain brief with diagram.
- (iii) Spiral streak method
- (iv) zig-zag streak method



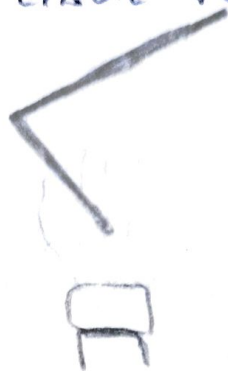
(b) Spread plate method:

It is an easy and direct method a loop full of technique containing a suspension of microorganisms is placed on the centre of the agar plate and it spread on the surface with the help of "L" shaped glass rod the glass rod is sterilized by dipping in alcohol when the suspension is spread bar the plate of individual micro-organisms are separated by one another

for this separation, it is necessary to dilute the suspension before application to prevent overgrowing growth of micro-organism. After incubation colonies are picked up and streak on to a fresh medium to ensure purity.



Dip the bent glass rod
in the dish and in
ethyl alcohol



Sterilize the glass rod
by the bunsen burner

(c) Pour plate method:

A small volume of diluted sample is mixed thoroughly with liquid agar medium which is cool about 45°C . Then the mixture is poured into sterile petri plates and allowed to solidify. As the medium solidifies the microbial cells get fixed in position. These trapped cells develop into individual colonies. On incubation growth of colonies occur both at the surface and sub surface areas of medium. The pour plate technique is used for qualitative studies to enumerate the microbial cells in the sample.

Note: The main disadvantage of this method is the difficulty of removing sub surface colonies for further colonies.

(d) Serial dilution technique:

The purpose of serial dilution is to reduce the number of microorganisms so that isolat

ed small amount is called the serial dilution. Serial dilution techniques is used in water, soil, milk and other industrial purposes.

Dilution plating technique

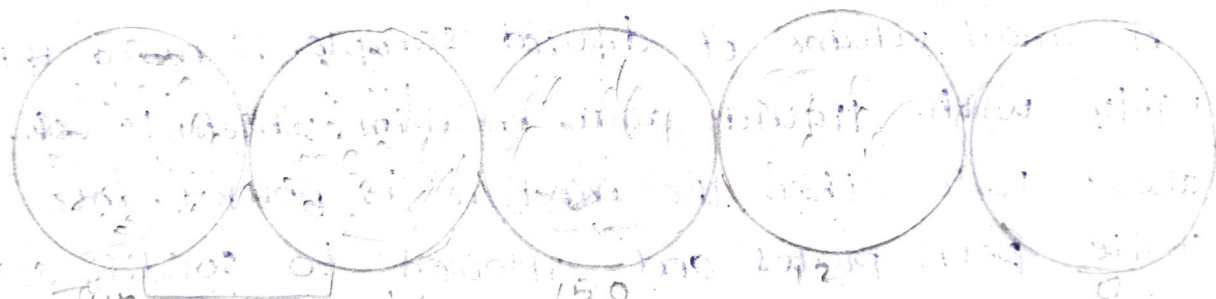
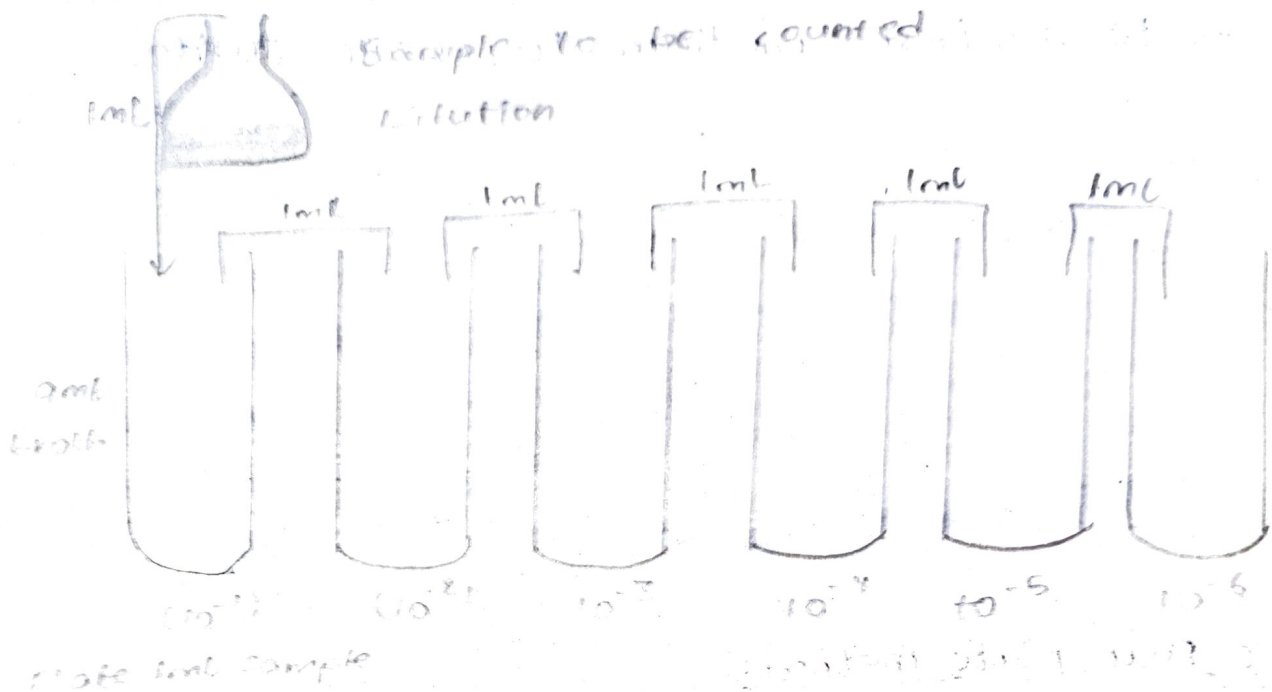


plate count

dilution factor cells colony forms units per milliliter original sample

Procedure

- (1) At first take some amount of inoculation in a sample test tube and take several test tubes with 9ml of distilled water in each.
- (2) After that transfer 1ml of inoculation with a 1ml sterile pipette to the first test tube and is marked as 10^{-1} as indication for dilution.
- (3) From the first tube

to the second test tube and marked as 10^{-2} diluting the original specimen to 100 times

(4) Repeat this dilution of the original sample of upto 10^{-7} thus using each time a fresh pipette

(5) After appropriate dilution [10^{-6} , 10^{-7}] transfer the 0.1 ml of diluted sample on to the petriplate and count for number of colonies are per the calculations.

A single dilution calculated as follows.

Dilution = volume of the sample / total volume of the sample and diluent

the number of microorganisms per ml calculated as follows.

No. of cell/ml = no. of colonies / dilution volume plated

Micro manipulator technique!

This technique involves the isolation of a single cell from the mixed microbial population.

* some times this method is also called as "single cell dilution" technique. for the isolation of a single cell by this method, a device namely micromanipulator is used.

* this method is more convenient to isolate large called microorganism such as algal and protozoa and harder to isolate bacteria.

preservation of microbial structure:

Microorganisms are usually grown or multiplied in laboratories.

By using artificial media to study various aspects of scientific and research interests

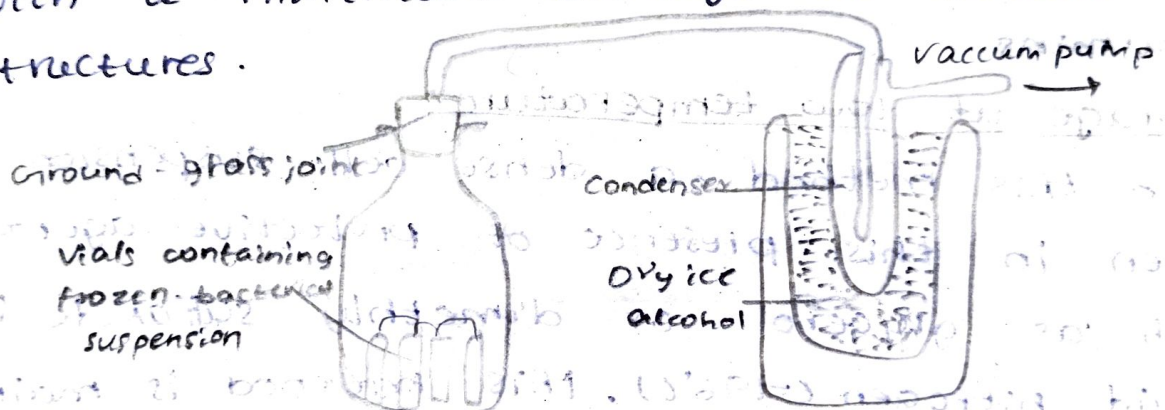
rates.

Lyophilization:

In this sublimation process occurs solid to vapour state.

Many bacterial species that are killed by ordinary drying can be preserved even more than 70 years by following the freeze drying process known as lyophilization.

The vials are connected to a high vacuum line and frozen to -60 to -70°C the ice in the frozen suspension sublimates under the vacuum these results in dehydration of the bacteria with a minimum damage to delicate cell structures.



Glass flask
for 10 to 100
Small Vials

Dewar flask

Seal

Asbestos packing

outer tube

inner vial

(Cotton plugged)

containing
Lyophilization
specimen

(3) can be time-consuming and labor-intensive
culture collection centers;

(1) ATCC (American type culture collection)

- * Private non-profit global biological research center and standard organization with the resources they need to conduct critical life science research.

- * It is needed for microbial growth and enumeration, differentiation and selection and colony purification.

- * Here testing methods are automated phenotypic and genotypic analysis.

- * More than four thousand species are available

(2) MTCC (Microbial type culture collection and gene)

- * It is an oldest culture collection of country. It is located in division of plant ecology / Indian method, and it is known for national apocitary for maintainance, supply and identification of fungal cultures.

- * Here provides attempt examples of organization of an microorganism (or) animal and plant cell cultures that can be grown (or) maintained in the laboratory.

- * Microbial type culture collection are in pune, chandigarh specially fungal cultures are also available.

(3) DSMZ (Deutsche Sammlung von Mikroorganismen and Zellkulturen)

- * DSMZ is a global service provider.
- * German collection of microorganism and cell cultures.
- * Global service provider with more than the 10,000 with more than 80 countries provides microorganisms and cell cultures for university, non university and industrial research in.
- * It also serves as a security deposited for biological material in accordance to the budhapest.
- * DSMZ only hold bioresearch and biosafety.
- * It provides variety of stains of prokaryotes and the bacterial diversity metadata base.
- * Microorganisms includes bacterial strains, arch-
ea stains, yeast and fungi strains human
and animal cell cultures, bacteria phages,
plasmids etc.

Cultivation of Anaerobic Bacteria:

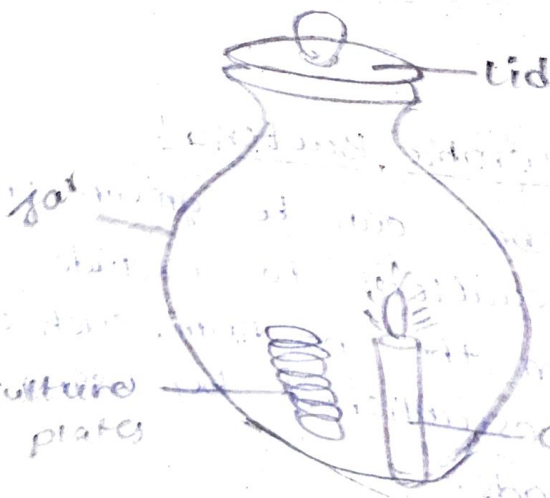
Stringent anaerobes can be grown only by taking special pre-cautions to exclude all atmospheric oxygen from the medium. Such an environment can be established by using one of the following methods.

1) Prereduced media: During preparation, the culture medium is boiled for several minutes to drive off most of the dissolved oxygen. A reducing agent Eg: cysteine, is added to further lower the oxygen content. Oxygen-free N_2 is bubbled through the medium to keep it anaerobic. the medium

is then dispensed into tubes which are being flushed with oxygen-free N_2 , stoppered tightly, and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen-free CO_2 by means of a cannula, restoppered, and incubated.

Candle Jar Method :

- * In this method, media plates with bacterial inoculum is kept inside the jar.
- * Seal the jar with the lit candle inside.
- * The candle flame will consume most of the oxygen in the jar and will produce an elevated level of carbon dioxide.
- * these conditions are ideal for the growth of anaerobic and microaerophilic organisms.



Candle jar method

→ Brewer jar

The Brewer jar method experiment is a laboratory technique used to separate and analyze proteins or other biomolecules using electrophoresis.

Setup:

(1) Prepare the brewer jar by filling it with a buffer solution.

(2) Add a gel or membrane support to the jar.

(3) Place the electrodes (anode and cathode) in the jar.

sample preparation:

Prepare the protein sample by mixing it with a loading buffer.

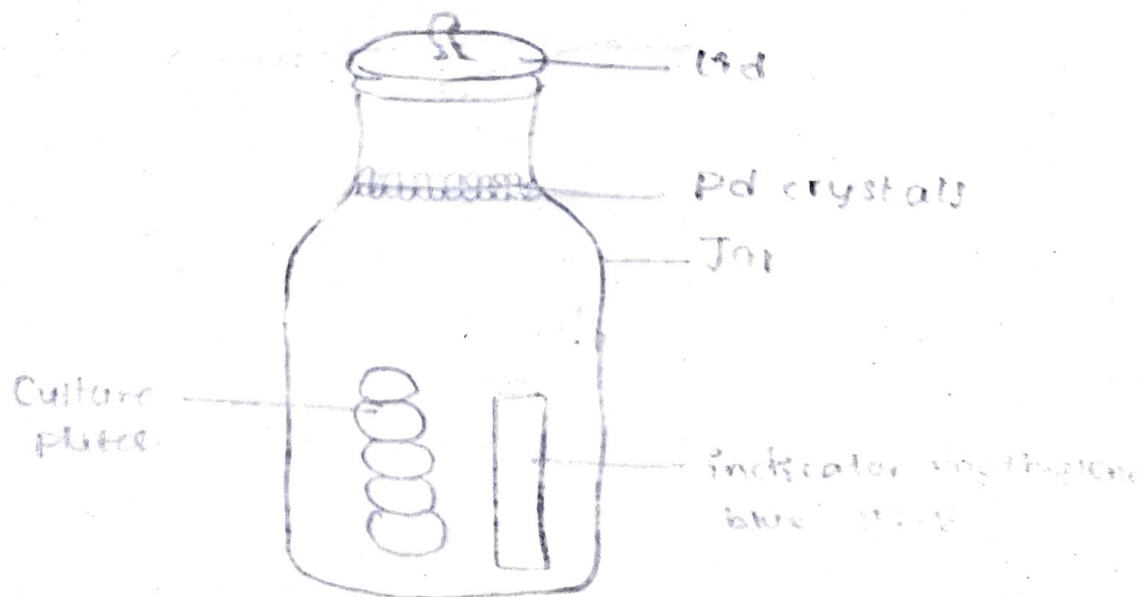
Load the sample onto the gel or membrane.

Electrophoresis:

(1) Apply an electric field to the jar using a power source.

(2) the proteins will migrate through the gel or membrane based on their charge and size.

(3) The separation process can take several hours, depending on the sample and settings.



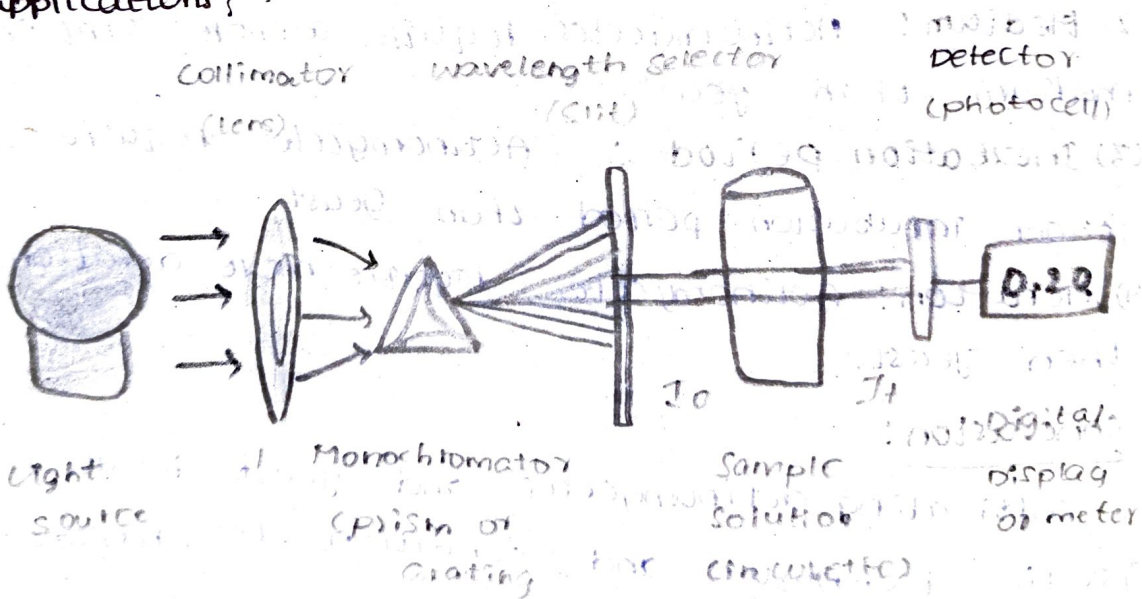
Brewer jar method

Unit-4 : Spectrophotometry and Chromatography

Spectrophotometer:

- * A spectrophotometer is an instrument that measure the amount of light absorbed by a sample.
- * Spectrophotometer techniques are mostly used to measure the concentration of solutes in solution by measuring the amount of the light that is absorbed by the solution in a cuvette placed in the spectrophotometer.
- * Scientist Arnold J. Beckman and his colleague at the National Technologies Laboratory (NTL) invented the Beckman DU spectrophotometer in 1940.

Spectrophotometer: (Principle, Instrumentation, applications;



Spectrophotometer

Principle:

The spectrophotometer technique is to measure light intensity as a function of wavelength. It does this by diffracting the light beam into

a spectrum of wavelengths, detecting the intensities with a charge-coupled device, and displaying the results as a graph on the detector and then on the display device.

(1) In the spectrophotometer, a prism (or) grating is used to split the incident beam into different wavelengths.

(2) By suitable mechanisms, waves of specific wavelengths can be manipulated to fall on the test solution. the range of the wavelengths of the incident light can be as low as 1 to 2 nm.

(3) the spectrophotometer is useful for measuring the absorption of light by a solution at each wavelength.

Instrumentation of Spectrophotometer

The essential components of spectrophotometer instrumentation include:

(1) A stable and cheap radiant energy source

- Materials that can be excited to high energy states by a high voltage electric discharge (or) by electrical heating serve as excellent radiant energy sources

(2) A monochromator to break the polychromatic radiation into component wavelength (or) bands of wavelengths

- A monochromator resolves polychromatic radiation into its individual wavelengths and isolates these wavelengths into very narrow bands.

Prisms:

- A prism disperses polychromatic light from the source into its constituent wavelengths by virtue of its ability to reflect different wavelengths to a different extent.
- Two types of prisms are usually employed in commercial instruments. Namely, 60° corner quartz prism and 30° Littrow prism.

Grating:

- Gratings are often used in the monochromators of spectrophotometers operating ultraviolet, visible and infrared regions.

(3) Transport vessels: (cuvettes) to hold the sample

- Samples to be studied in the ultraviolet (or) visible region are usually glasses (or) solutions and are put in cells known as "cuvettes".

- Cuvettes meant for the visible region are made up of either ordinary glass (or) sometimes quartz.

(4) A photosensitive detector and an associated readout system.

- Most detectors depend on the photoelectric effect. the current is then proportional to the light intensity and therefore a measure of it.
- Radiation detectors generate electronic signals which are proportional to the transmitter light.
- These signals need to be translated into a form that is easy to interpret.

• This is accomplished by using amplifiers, ammeters, potentiometers and potentiometric recorders.

Applications:

• Some of the major applications of spectrophotometers include the following:

- Detection of concentration of substances
- Detection of impurities
- Structure elucidation of organic compounds
- Monitoring dissolved oxygen content in fresh water and marine ecosystems.
- Characterization of proteins.
- Detection of functional groups
- Respiratory gas analysis in hospitals
- Molecular weight determination of compounds
- The visible and UV Spectrophotometer may be used to identify classes of compounds in both the pure state and in biological preparations

Gas chromatography

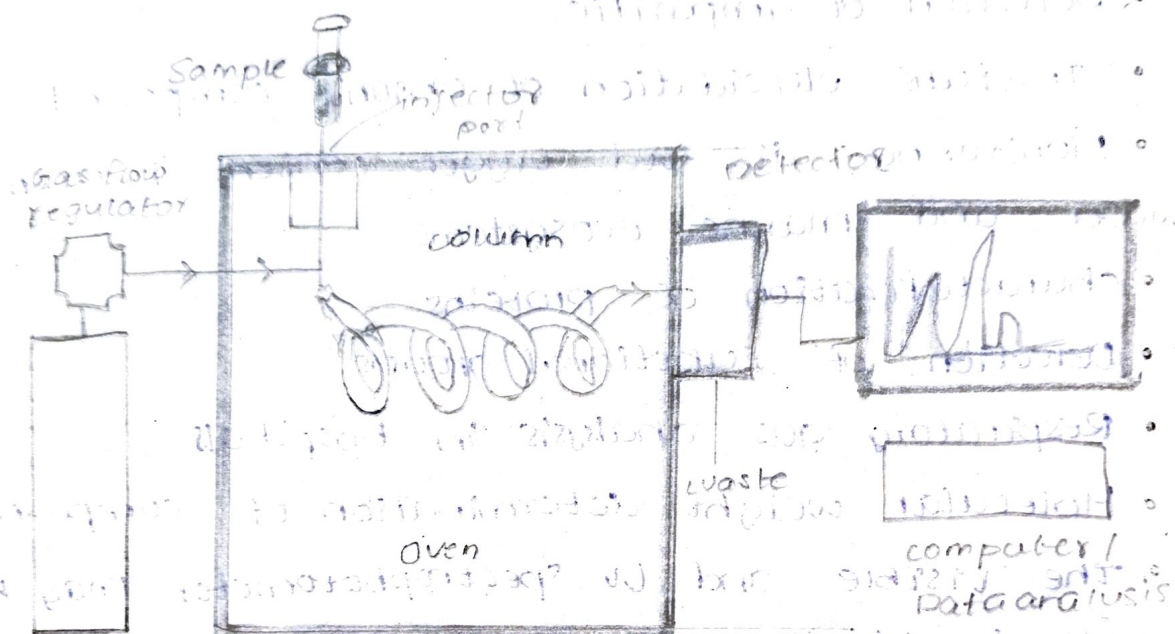
(Principle, Parts, Steps, Procedure, Uses)

Gas chromatography differs from other forms of chromatography in that the mobile phase is a gas and the components are separated as vapours.

- It is thus used to separate and detect small molecular weight compounds in the gas phase.
- The sample is either a gas or a liquid that is vaporized in the injection port. The

mobile phase for gas chromatography is a carrier gas, typically helium, because of its low molecular weight and being chemically inert.

- The pressure is applied and the mobile phase moves the analyte through the column. the separation is accomplished using a column coated with a stationary phase.



Principle:

The equilibrium for gas chromatography is partitioning and the components of the sample will partition (i.e. distribute) between the two phases.

Compounds that have a greater affinity for the stationary phase spend more time in the column and thus elute later and have a longer retention time (R_t) than samples that have a higher affinity for the mobile phase.

Affinity for the stationary phase is driven mainly by intermolecular interactions and the

of elution on the recorder as a plot of time versus the composition of carrier gas stream.

* The appearance time, height, width and area of these peaks can be measured to yield quantitative data.

Parts of gas chromatography

Gas chromatography is mainly composed of the following parts:

(1) Carrier gas in a high-pressure cylinder with attendant pressure regulators and flow meters.

- Helium, N_2 , H, Argon are used as carrier gases.

- Helium is preferred for thermal conductivity detectors because of its high thermal conductivity relative to that of most organic vapours.

- N_2 is preferable, when a large consumption of carrier gas is employed.

- Carrier gas from the tank passes through a toggle valve, a flow meter (1-1000 ml/min), capillary restrictors and a pressure gauge (1-4 atm).

- Flow rate is adjusted by means of a needle valve mounted on the base of the flow meter and controlled by capillary restrictors.

- The operating efficiency of the gas chromatography is directly dependant on the maintenance of constant gas flow.

(2) Sample injection system

- Liquid samples are injected by a microsyringe.

with a needle inserted through a self-sealing, silicon-rubber septum into a heated metal block by a resistance heater.

- Gaseous samples are injected by a gas-tight syringe or through a by-pass loop and valves.
- Typical sample volumes range from 0.1 to 0.2 ml.

(3) The separation column

• The heart of the gas chromatography is the column which is made of metals bent in U shape or coiled into an open spiral or a flat pancake shape.

- copper is useful up to 250°

- Swage lock fittings make column insertion easy.

- several sizes of columns are used depending upon the requirements.

(4) Liquid phases

- An infinite variety of liquid phases are available limited only by their volatility, thermal stability and ability to wet the support.

- No single phase will serve for all separation problems at all temperatures.

Non-polar: Paraffin, squalane, silicone greases,

Apiezon L, silicone gum rubber, these materials separate the components in order of their boiling points.

Intermediate polarity: these materials contain

∴ polar or polarizable group on a long non-polar skeleton which can dissolve both polar and non-polar solutes - for example diethyl hexyl phthalate is used for the separation of high boiling alcohols.

polar: carbowaxes - liquid phases with a large proportion of polar groups. separation of polar and non-polar substances.

Hydrogen bonding: polar liquid phases with high hydrogen bonding e.g. Glycol.

Specific purpose phases: Relaying on a chemical reaction with solute to achieve separations e.g. AgNO_3 in glycol separates unsaturated hydrocarbons.

(5) Supports:

- the structure and surface characteristics of the support materials are important, parameters which determine the efficiency of the support and the degree of separation respectively.

- The support should be inert but capable of immobilizing a large volume of liquid phase as a thin film over its surface.

- The surface area should be large to ensure the rapid attainment of equilibrium between stationary and mobile phases.

- Support should be strong enough to resist

packed into a uniform bed.

- Diatomaceous earth, kieselguhr treated with Na_2CO_3 for 900°C causes the particle fusion into coarser aggregates.

- Glass beads with a low surface area and low porosity can be used to coat up to 3% stationary phases.

~~Glass beads with a low surface area and low porosity can be used to coat up~~

- Porous polymer beads differing in the degree of cross-linking of styrene with alkyl-vinyl benzene are also used which are stable upto 250° .

(6) Detector

- Detectors sense the arrival of the separated components and provide a signal.

- These are either concentration-dependent or mass dependant.

- The detector should be close to the column exit and the correct temperature to prevent decomposition.

(7) Recorder:

- The recorder should be generally 10 mV full scale fitted with a fast response pen (1 sec or less) the recorder should be connected with a series of good quality resistances connected across the input to attenuate the large signals.

Unit-5 : Centrifugation, Electrophoresis & Radio Isotopes.

Principles of centrifugation:

Centrifugation is a process that uses centrifugal force to separate components of a mixture based on their density. The principle relies on the fact that when a sample is spun at high speeds in a centrifuge, the components with higher density will move outward, toward the bottom of the centrifuge tube, while those with lower density will remain closer to the top.

Key principles:

(1) **Centrifugal Force:** When the centrifuge spins, it creates a force that is proportional to the speed of rotation and the distance from the center of rotation. This force acts on the particles in the mixture, causing them to move outward.

$$F = m \cdot r \cdot \omega^2$$

Where F = centrifugal force

m = mass of the particle

r = Radius of rotation

ω = angular velocity

(2) **Sedimentation:** As particles experience centrifugal force, they sediment (settle) at different rates depending on their size, shape and density. Larger or denser particles sediment faster than smaller or less dense ones.

(3) Sedimentation Rate: The rate at which particles sediment is determined by their size, shape and density, as well as the density of the medium in which they are suspended. This relationship is often described by the sedimentation coefficient in sedimentation equations.

Types of centrifuges

Centrifuges can be categorized based on their design, speed and intended application.

(1) Bench-top centrifuges:

Refrigerated centrifuges: Equipped with a cooling system to prevent overheating of samples.

Non-Refrigerated centrifuges: Suitable for samples that do not require temperature control.

(2) Microcentrifuges:

Designed for small volume samples, typically ranging from 0.2 to 2ml. Commonly used in molecular biology for tasks such as DNA/RNA extraction and small-scale protein separation.

(3) Clinical Centrifuges:

Used in medical laboratories for blood separation such as separating plasma or serum from blood cells.

(4) Ultracentrifuges:

Capable of achieving very high speeds (over 100,000 RPM) used for separating very

small particles or high-resolution separation.

(5) Industrial centrifuges:

larger and designed for high-volume processing, often used in food processing, chemical manufacturing and wastewater treatment.

(16) Swinging - Bucket centrifuges:

The buckets swing out to a horizontal position during centrifugation, providing better separation of particles with large volume samples.

(17) Fixed - Angle centrifuges:

The tubes are held at a fixed angle relative to the rotor axis, useful for separation smaller volumes and for applications.

(18) Tandem centrifuges:

Incorporate multiple rotors or a series of steps to achieve multiple levels of separation.

Applications of centrifuges:

1) Medical and clinical Applications.

- Blood separation: For obtaining serum or plasma from blood samples.
- Urinalysis: To separate cellular components from urine.
- Clinical chemistry: For processing samples before analysis.

2. Biological Research:

- Cell Fractionation: To isolate organelles

stable configuration

Electrophoresis of Proteins:

Sodium dodecyl sulphate (SDS) - polyacrylamide gel electrophoresis.

SDS - polyacrylamide gel electrophoresis is the most widely used method for analysing protein mixtures qualitatively.

SDS - PAGE are firstly boiled for 5 min in sample buffer containing β -mercaptoethanol and SDS.

It is particularly useful for monitoring protein purification and, because the method is based on the separation of proteins according to size, it can also be used to determine the relative molecular mass of proteins.

The mercaptoethanol reduces any disulphide bridges present that are holding together the protein tertiary structure, each protein forms a rod shaped structure with a series of negatively charged SDS molecules along the polypeptide chain. One SDS molecule is therefore completely swamped by the negatively charged SDS molecules. The rod-like structure remains, as any rotation that tends to fold up the protein's chain would result in repulsion between negative charges on different parts of the protein chain.

returning the conformation back to rod shape. The sample buffer also contains an ionisable tracking dye, usually bromophenol blue, that allows the electrophoretic run to be monitored and sucrose or glycerol, which gives the sample solution density thus allowing the sample to settle easily through the electrophoresis buffer to the bottom when injected into the loading well. Once the sample are all loaded a current is passed through the gel.

The sample to be separated are not in fact loaded directly into the main separating gel. When the main separating gel (normally about 5cm long) has been poured between the glass plates and allowed to set, a shorter (approx 0.8cm) stacking gel is poured on top of the separating gel and it is into this gel that the wells are formed and the proteins loaded.

When the current is switched on, all the ionic species have to migrate at the same speed otherwise that would be a break in the electrical circuit.

The smaller the protein the more easily it can pass through the pores of the gel, whereas large proteins are successively retarded by frictional resistance due to the sieving effect of the gels. Being a small

can only just enter the pores of this gel gels of 15% polyacrylamide are therefore useful for separating proteins.

Acrylamide
concentration

Protein fractionation
range ($M_r \times 10^{-3}$)

5

60 - 350

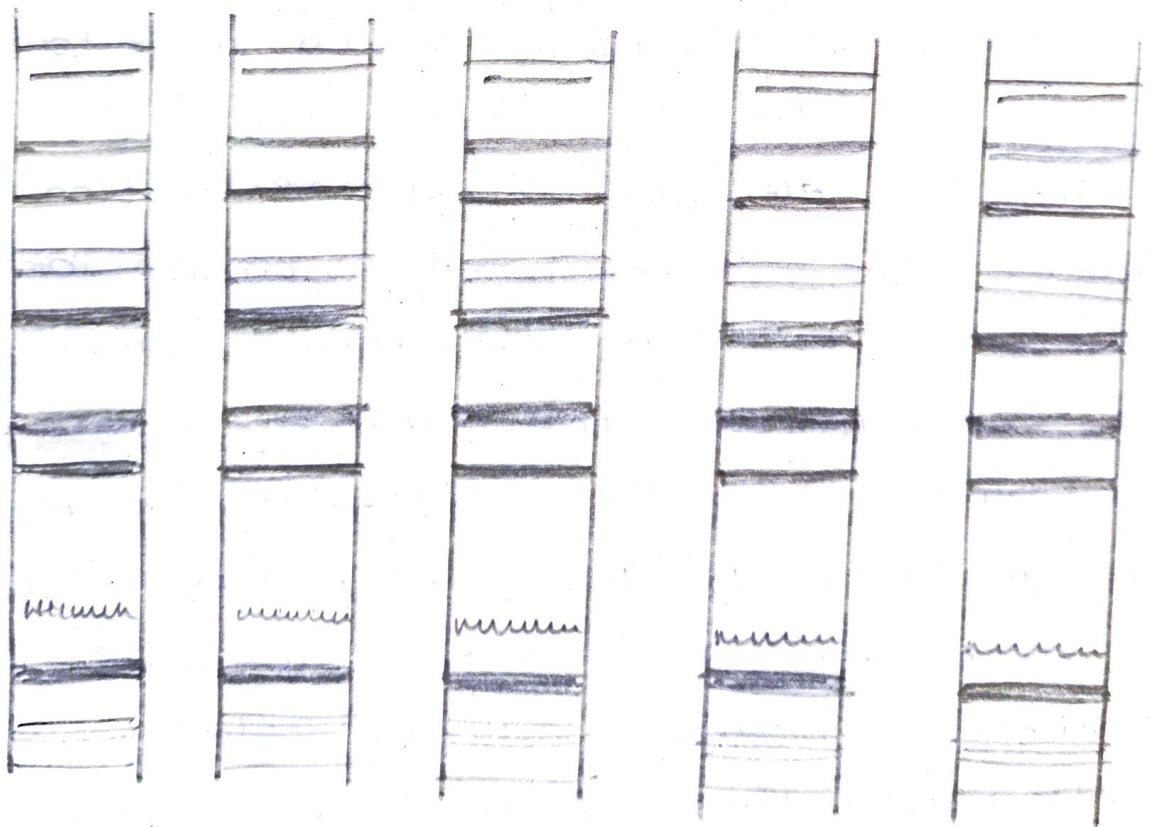
10

15 - 200

15

10 - 100

A relationship between acrylamide gel concentration and protein fractionation range



Characteristics of Radioisotopes:

(1) - Instability

- * Radioisotopes have an excess of energy or mass or both, making them unstable.
- * this instability leads to spontaneous radioactive decay, where the nucleus releases energy to achieve a more stable configuration.

(2) Types of Radiations:

Alpha particles:

- * consist of 2 protons and 2 neutrons
- * Heavy and positively charged
- * low penetration power, can be stopped by a sheet of paper or the outer layer of human skin.
- * Hazardous if ingested or inhaled, as they can cause significant internal damage.

Beta Particles:

- * High-Speed electrons (beta-minus) or positrons (beta-plus).
- * Moderate penetration power, can penetrate skin but are stopped by a few millimeters of plastic or aluminum.

* can cause burns and tissues damage with high exposure.

Gamma Rays:

* Electromagnetic radiation of high frequency and energy.

* High penetration power, can pass through the human body and require dense materials like lead or thick concrete for shielding.

* can cause deep tissue damage and increase the risk of cancer.

(5) Half-life :

* The Half-life is the time required for half the atoms in a radioactive substance to decay.

* It is a fixed property of each radio-isotope and does not change regardless of the physical or chemical state of the isotope.

Example - Half lives :

* Uranium - 238 : 4.5 billion years

* Carbon - 14 : 5730 years

* Iodine 131 : 8 days

* Polonium - 210 : 138 days

(4) Energy Release:

* The decay process releases energy, which can be in the form of kinetic energy of emitted particles or electromagnetic radiation (gamma rays).

* This energy can be detected and measured using radiation detectors such as Geiger-muller counters, ~~Ge~~ Scintillation detectors and semiconductor detectors.

(5) Transmutation:

Radioactive decay ~~see~~ results in the transformation of the original unstable isotope (Parent) into a different element or isotope (daughter).

Types of decay:

Alpha Decay: Reduces the atomic number by 2 and the mass number by 4 (e.g. Uranium-238 to thorium-234).

Beta-minus Decay: Increases the atomic number by 1 without changing the mass number (e.g. carbon-14 to Nitrogen-14).

Beta-plus Decay: Decreases the atomic number by 1 without changing the mass number (e.g. potassium-40 - Argon-40).

Gamma Decay: often accompanies alpha or beta decay, releasing excess energy without changing the number of protons or neutrons.

(6) Applications:

Medicine:

- * **Diagnostic imaging:** Radioisotopes like technetium - 99m are used in nuclear medicine to diagnose conditions (e.g. bone scan, cardiac imaging).
- * **Radiotherapy:** Cobalt - 60 and Iodine - 131 are used to treat cancer by damaging the DNA of cancer cells.

Industry:

- * **Radiography:** used to inspect the integrity of welds and materials in pipelines and structures.
- * **Tracer studies:** Radioisotopes are used to trace the flow of materials through systems such as tracking leaks in pipelines.

Scientific Research:

- * **Radiometric dating:** Carbon 14 dating is used to determine the age of archaeological and geological samples.
- * **Biological studies:** Radioisotopes are used to trace biochemical pathways and study metabolic processes.

Principles of Autoradiography:

(1) Labeling:

* A sample is exposed to a radioactively labeled compound, which gets incorporated into specific molecules or regions of the sample.

Common radioisotopes for labelling:

* Tritium (^3H): used for labeling proteins, nucleic acids, and lipids.

* Carbon-14 (^{14}C): used in metabolic studies and for tracing carbon in biochemical processes.

* Sulphur-35 (^{35}S): commonly used for labeling proteins and nucleic acids.

* Phosphorous-32 (^{32}P): used in studying nucleic acids and ATP.

(2) Exposure:

* the labeled sample is placed in close contact with a photographic emulsion or x-ray film.

* The emitted radiation from the radioactive decay exposes the emulsion or film, creating a latent image.

(3) Development:

* The exposed photographic emulsion or

Film is developed using standard photographic techniques.

- * Development reveals the pattern of radiation exposure as dark spots or bands on the film.
- * The intensity of the darkening is proportional to the amount of radioactivity present.

(4). Analysis

- * The developed autoradiograph is examined to determine the distribution and concentration of the radiolabeled compounds.
- * Quantitative analysis can be performed using densitometry or other imaging techniques.
- * Autoradiographs can provide information about the spatial distribution of labeled compounds within the sample.

Applications of Autoradiography:

(1) Biological Research:

- * Gene expression studies: Autoradiography can visualize the distribution of mRNA or protein products in tissues or cells.

* Protein localization: Used to determine the cellular or subcellular localization of specific proteins by labelling them with radioisotopes.

* Metabolic pathways: Tracing the incorporation of labeled substrates into metabolic products.

(2) Medicines:

* Drug Distribution Studies: Autoradiography can track the distribution of radiolabeled drugs within the body, helping to understand pharmacokinetics and pharmacodynamics.

* Pathology: Identifying the distribution of disease markers or therapeutic agents in tissues.

(3) Molecular Biology:

* DNA / RNA Hybridization: Used to detect specific nucleotide sequences within gels or tissue sections through labeled probes.

* Protein-DNA interactions: Studying interactions by labeling proteins or nucleic acids.