17MBU513A INSTRUMENTATION AND BIOTECHNIQUES – PRACTICAL (4H – 2C)

Instruction Hours / week: L: 0 T: 0 P: 4 **Marks:** Internal: **40** External: **60** Total: **100**

End Semester Exam: 3 Hours

SCOPE

The course offers the students with an opportunity to gain knowledge on the bioinstrumentation and concepts of principles and applications.

OBJECTIVES

To develop skills related to

- Understand the principles of various instruments used in the life sciences.
- Ability to operate the instruments.
- Data analysis and interpretations.

EXPERIMENTS

1. Study of fluorescent micrographs to visualize bacterial cells – Demonstration
2. Ray diagrams of phase contrast microscopy and Electron microscopy – Demonstration
3. Separation of mixtures by paper / thin layer chromatography.
4. Demonstration of column packing in any form of column chromatography.
5. Separation of protein mixtures by any form of chromatography.
6. Separation of protein mixtures by Polyacrylamide Gel Electrophoresis (PAGE).
7. Determination of λmax for an unknown sample and calculation of extinction coefficient.
8. Separation of components of a given mixture using a laboratory scale centrifuge.
9. Understanding density gradient centrifugation with the help of pictures.

SUGGESTED READINGS

1. Wilson K and Walker J. (2010). Principles and Techniques of Biochemistry and Molecular Biology. 7th edition, Cambridge University Press.
2. Nelson DL and Cox MM. (2008). Lehninger Principles of Biochemistry, 5th edition, W.H. Freeman and Company.
3. Willey MJ, Sherwood LM & Woolverton CJ. (2013). Prescott, Harley and Klein's Microbiology. 9th edition, McGraw Hill.
4. Karp G. (2010) Cell and Molecular Biology: Concepts and Experiments. 6th edition. John Wiley & Sons. Inc.
5. De Robertis EDP and De Robertis EMF. (2006). Cell and Molecular Biology. 8th edition. Lipincott
6. Williams and Wilkins, Philadelphia.
7. Cooper G.M. and Hausman R.E. (2009). The Cell: A Molecular Approach. 5th edition. ASM Press & Sunderland, Washington D.C., Sinauer Associates, MA.
8. Nigam A and Ayyagari A. (2007). Lab Manual in Biochemistry, Immunology and Biotechnology. Tata McGraw Hill.

1.Study of fluorescentmicrographs to visualise bacterial cells

Principles of fluorescence microscopy

Fluorescence

Fluorescence is a property of some atoms and molecules to emit light at longer wavelengths after absorbing light of a particular and shorter wavelength (Herman 1998). Each fluorescent atom or molecule can absorb only certain wavelengths of light. Upon absorbing a photon of energy,the electron of a fluorescent molecule is excited from the ground state to a higher electronic energy and vibrational state. The energized electron then returns to the ground energy state with a loss of vibrational energy to the environment. During the return of the molecule to the ground state, a photon of longer wavelength is emitted. This is referred to as fluorescence (Ploem and Tanke 1987; Herman 1998). The emitted light is always of a longer wavelength and of a lower intensity than that of the absorbed light because of the energy loss. The excitation (or absorption) spectrum can be obtained by scanning the absorption wavelengths at a fixed emission wavelength. Similarly, the emission spectrum can be obtained when the excitation wavelength is constant and emission wavelengths are measured. Some molecules are autofluorescent and emit fluorescence when excited. This phenomenon is called primary fluorescence. Secondary fluorescence represents the emission produced after a molecule is combined with a primary fluorescent molecule called a stain or fluorochrome (Altem_ller and van Vliet-Lanoe 1990).

Fluorescence microscopy

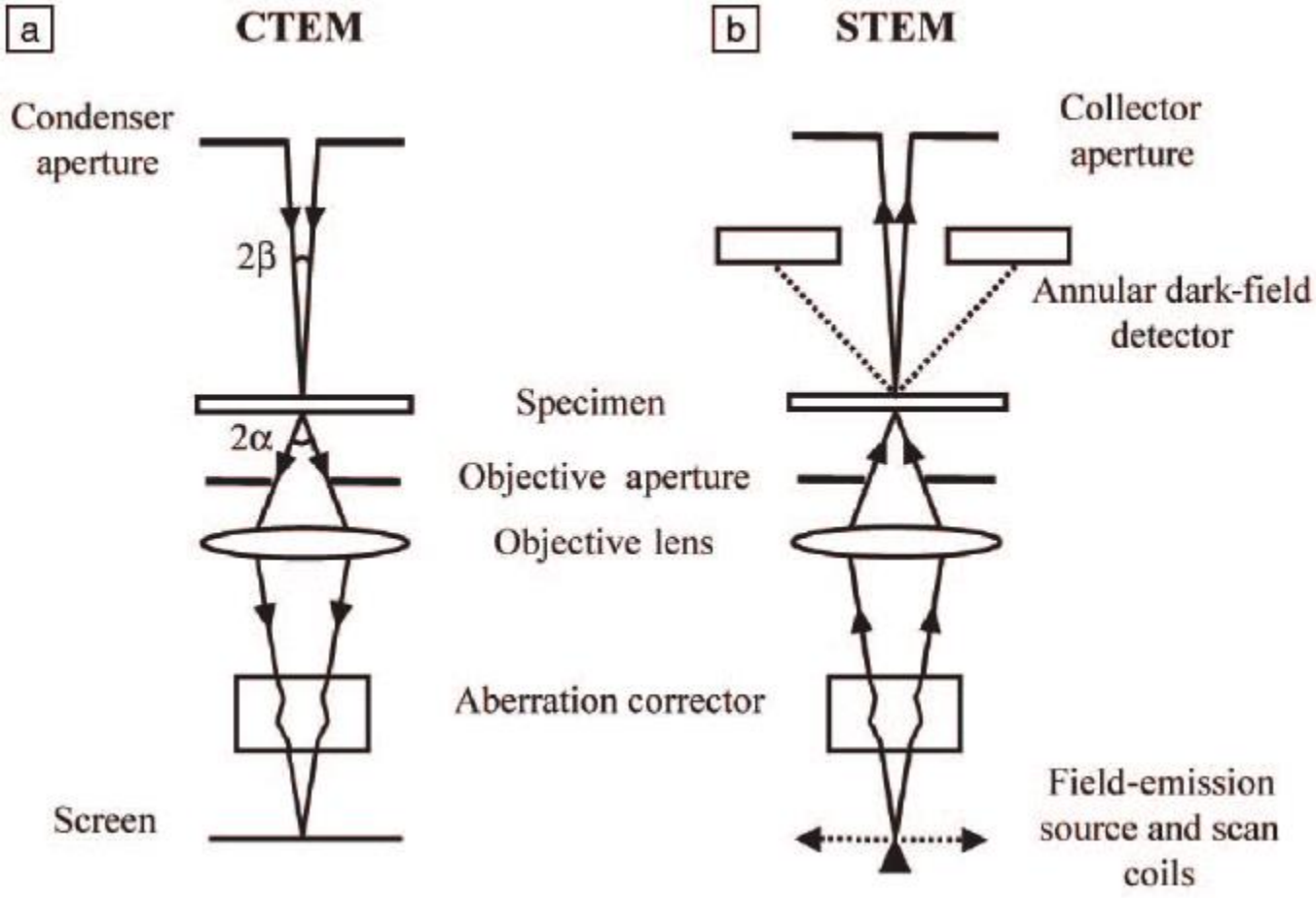
Fluorescence microscopes can be categorized into two types based on their optical paths: (1) transmitted-light fluorescence microscopes, and (2) incident-light or epiillumination fluorescence microscopes. Transmitted-light fluorescence microscopes require careful alignment of the condenser and objective lenses to avoid the excitation light interfering with the emitted fluorescence (Fig. 2). A barrier filter helps screen out the excitation light. Transmitted- light fluorescence microscopes cannot be used for simultaneous fluorescence/phase or fluorescence/differential interference contrast imaging. With the epi-illumination

set-up, the condenser is also the objective. Therefore, only one optical path to the detector exists (i.e., that of the emitted fluorescence) and perfect alignment is not required (Fig. 2). Other advantages of epifluorescence microscopy are the effective use of a dichromatic beam splitter and the easy change-over between fluorescence microscopy and transmitted light microscopy. All these advantages make incident-light fluorescence microscopy the more commonly employed system (Ploem 1993; Herman 1998).

The key components of epifluorescence microscopy include an excitation light source, wavelength selection devices (a set of well-balanced filter combinations), objectives, and detectors (Ploem 1993; Herman 1998).

Only the first two components will be discussed in this paper. A light source (e.g., tungsten halogen lamps, highpressure mercury, xenon, mercury/xenon combination arc lamps, or lasers) emits excitation energy of different wavelengths. The maximal excitation of fluorescence occurs when the light wavelength peak is close to the absorption peak of the fluorescent molecule (Herman 1998). Maximum excitation can be reached by using an excitation filter that only transmits light below a specific wavelength corresponding to the maximal absorbance of the molecule (Herman 1998). The excitation filter also reduces the intensity of background light that has the same wavelength range as the emitted fluorescent light. A second filter called a dichromatic beam splitter (also known as a dichromatic mirror) (Ploem 1993) is designed to reflect light of shorter wavelength while transmitting light of longer wavelength (Herman 1998). The excitation light is reflected by the dichromatic beam splitter and condensed by the objective before reaching the specimen.In theory, only the fluorescence emitted from the specimen passes through and reaches the detector (the eyepiece or a camera in the microscope). The development of the dichromatic beam splitter is one of the major advantages of epifluorescence microscopy over transmitted-light fluorescence microscopy (Ploem 1993). To further limit the unabsorbed excitation light from reach reaching the detector, a barrier filter is installed between the chromatic beam splitter and the detector.The various types of filters are defined by their wavelength selection characteristics: short pass filter, long pass filter, dichromatic beam splitter, wide pass filter, narrow band pass filter and short pass-long pass combination (Ploem and Tanke 1987; Herman 1998).Details of these filters are outside the scope of this review.

2 **Ray diagrams of phase contrast and electron microscopy**



Ray diagrams showing the important optical elements for (a) conventional transmission electron microscopy (CTEM) and (b) scanning transmission electron microscopy (STEM). The CTEM image is obtained in parallel; the STEM image is obtained pixel-by-pixel by scanning the probe. The diagrams are shown with the electron source at the top for CTEM and at the bottom for STEM, to show the reciprocal nature of the optical paths. STEM also provides simultaneous annular dark-field imaging. Actual microscopes have several additional lenses, and the beam-limiting aperture positions may differ.

Light passing from one object into another object of a slightly different refractive index or thickness undergoes a change in phase.

In a phase-contrast microscope, this difference in phase is translated into variation in brightness of the image and hence is detectable by eye. With a phase-contrast microscope, the differences among various cells with different refractive indices or thickness can be seen in unstained condition.

Microscopic objects can be seen in unstained condition, due to the difference in the refractive index of the object and its surrounding medium. Unstained structures within cells, not discernible by other microscopic methods can also be observed due to the slight differences in their refractive indices or thickness.

A phase-contrast microscope is a compound microscope fitted with a phase-contrast condenser and a phase-contrast objective (Figure 4.12). An annular aperture in the diaphragm placed in the focal plane of the sub-stage condenser controls the illumination of the object.

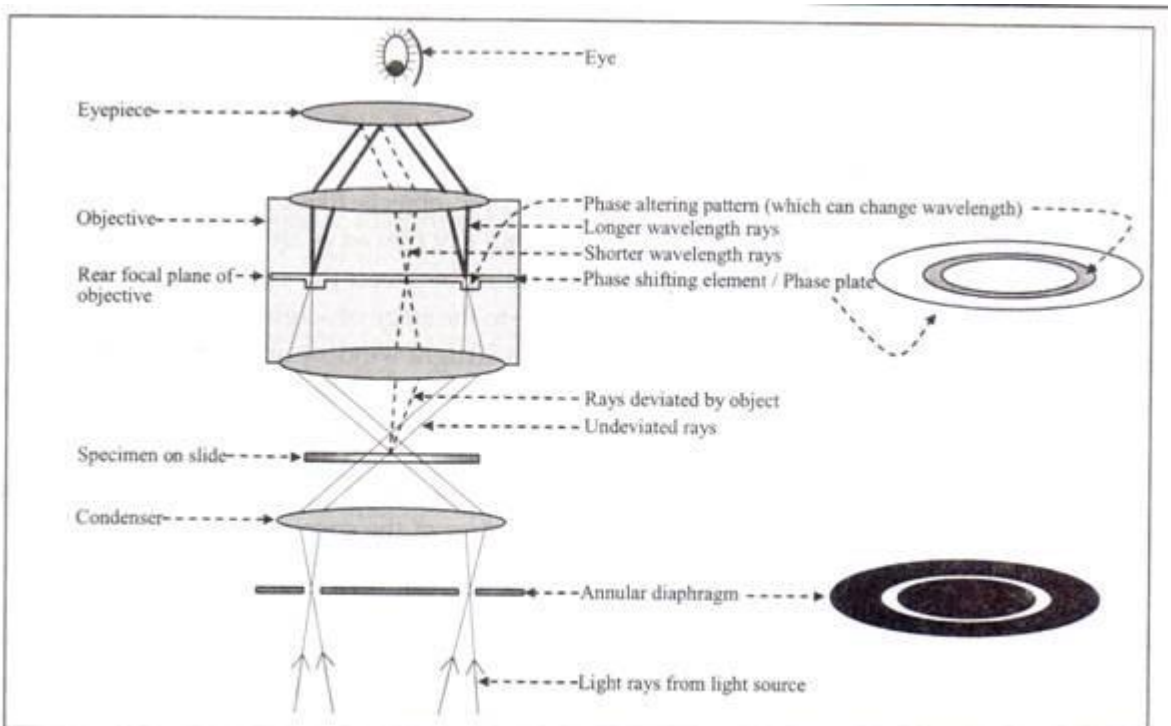


Figure 4.12: Ray diagram of a phase-contrast microscope

The image of the aperture is formed at the rear focal plane of the objective. In this plane, there is a phase-shifting element or phase- plate. The phase plate also has an annular ring of phase altering pattern, which can increase the wavelength of light passing through it. Light coming through the annular aperture of condenser passes through the object. Those rays, which are not deviated by the object (solid lines in figure), pass through the phase-altering pattern of the phase plate and acquire longer wavelength.

Those rays, which are deviated by the object structures (broken lines in figure), due to different refractive index, pass through the phase-plate not covered by the phase altering pattern. Thus, their wavelength remains unchanged. The difference in phase (wavelength) gives the contrast for clear visibility of the object.

3.Separation of mixtures by thinlayer chromatography

TLC is a type of planar chromatography.

- It is routinely used by researchers in the field of phyto-chemicals, biochemistry, and so forth, to identify the components in a compound mixture, like alkaloids, phospholipids, and amino acids.
- It is a semi quantitative method consisting of analysis.
- High performance thin layer chromatography (HPTLC) is the more sophisticated or more precise quantitative version.

Principle

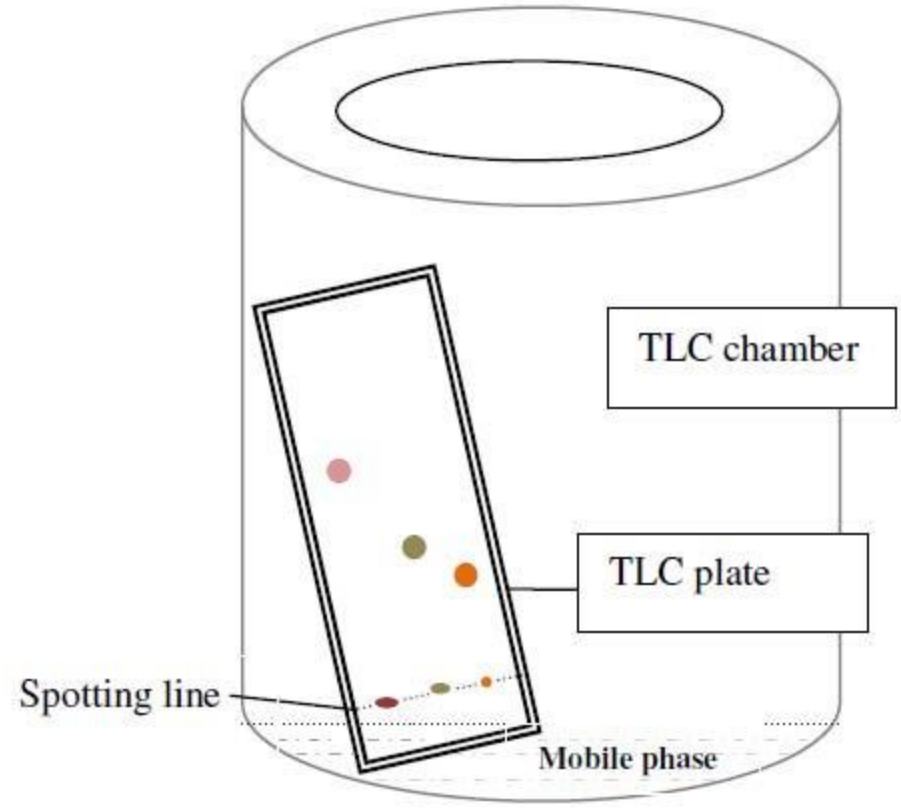
Similar to other chromatographic methods, thin layer chromatography is also based on the principle of separation.

1. The separation depends on the relative affinity of compounds towards stationary and the mobile phase.
2. The compounds under the influence of the mobile phase (driven by capillary action) travel over the surface of the stationary phase. During this movement, the compounds with higher affinity to stationary phase travel slowly while the others travel faster. Thus, separation of components in the mixture is achieved.
3. Once separation occurs, the individual components are visualized as spots at a respective level of travel on the plate. Their nature or character are identified by means of suitable detection techniques.

System Components

TLC system components consists of

1. **TLC plates,** preferably ready made with a stationary phase: These are stable and chemically inert plates, where a thin layer of stationary phase is applied on its whole surface layer. The stationary phase on the plates is of uniform thickness and is in a fine particle size.
2. **TLC chamber.** This is used for the development of TLC plate. The chamber maintains a uniform environment inside for proper development of spots. It also prevents the evaporation of solvents, and keeps the process dust free.
3. **Mobile phase.** This comprises of a solvent or solvent mixture The mobile phase used should be particulate-free and of the highest purity for proper development of TLC spots. The solvents recommended are chemically inert with the sample, a stationary phase.
4. **A filter paper.** This is moistened in the mobile phase, to be placed inside the chamber. This helps develop a uniform rise in a mobile phase over the length of the stationary phase.



Procedure

The stationary phase is applied onto the plate uniformly and then allowed to dry and stabilize. These days, however, ready-made plates are preferred.

1. With a pencil, a thin mark is made at the bottom of the plate to apply the sample spots.
2. Then, samples solutions are applied on the spots marked on the line in equal distances.
3. The mobile phase is poured into the TLC chamber to a leveled few centimeters above the chamber bottom. A moistened filter paper in mobile phase is placed on the inner wall of the chamber to maintain equal humidity (and also thereby avoids edge effect this way).
4. Now, the plate prepared with sample spotting is placed in TLC chamber so that the side of the plate with the sample line is facing the mobile phase. Then the chamber is closed with a lid.
5. The plate is then immersed, such that the sample spots are well above the level of mobile phase (but not immersed in the solvent — as shown in the picture) for development.

Advantages

- It is a simple process with a short development time.
- It helps with the visualization of separated compound spots easily.
- The method helps to identify the individual compounds.
- It helps in isolating of most of the compounds.
- The separation process is faster and the selectivity for compounds is higher (even small differences in chemistry is enough for clear separation).
- The purity standards of the given sample can be assessed easily.
- It is a cheaper chromatographic technique.

Applications

1. To check the purity of given samples.
2. Identification of compounds like acids, alcohols, proteins, alkaloids, amines, antibiotics, and more.
3. To evaluate the reaction process by assessment of intermediates, reaction course, and so forth.
4. To purify samples, i.e for the purification process.
5. To keep a check on the performance of other separation processes.

Being a semi quantitative technique, TLC is used more for rapid qualitative measurements than for quantitative purposes. But due its rapidity of results, easy handling and inexpensive procedure, it finds its application as one of the most widely used chromatography techniques.

6. Allow sufficient time for the development of spots. Then remove the plates and allow them to dry. The sample spots can now be seen in a suitable UV light chamber, or any other methods as recommended for the said sample.

4.Demonstration of column packing in any form of column chromatography

Objective:

To separate Organic compounds with the help of Column Chromatographic technique.

Theory:

Chromatography:

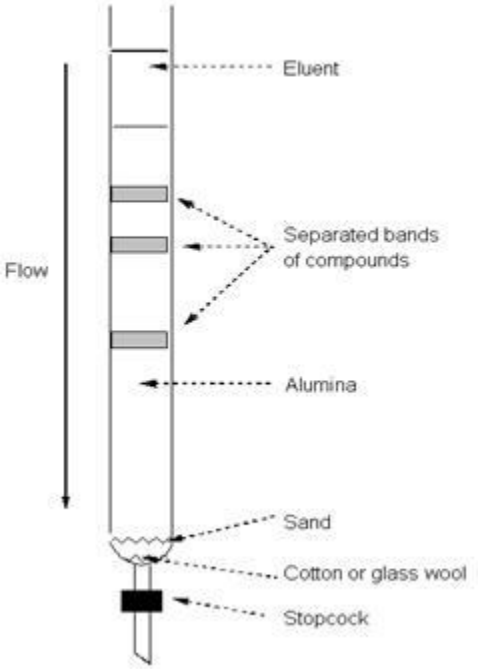
Chromatography has been developed into a new method of separation of mixture of substances mainly when they are available in small amounts. This method is very useful when the components of a mixture have almost the same physical and chemical properties and hence can't be separated by other usual methods of separations. The term chromatography means writing in colour (in Greek: Khromatos-colour, and graphos- written). It was discovered by Mikhail Tswett in 1906.

The methods of separation in chromatography are based on the distribution of the components in a mixture between a fixed (stationary) and a moving (mobile) phase. The stationary phase may be a column of adsorbent, a paper, a thin layer of adsorbent on a glass plate, etc., through which the mobile phase moves on. The mobile phase may be a liquid or a gas. When a solid stationary phase is taken as a column it is known as column chromatography.

Column Chromatography:

Column chromatography is one of the most useful methods for the separation and purification of both solids and liquids. This is a solid - liquid technique in which the stationary phase is a solid & mobile phase is a liquid. The principle of column chromatography is based on differential adsorption of substance by the adsorbent.

The usual adsorbents employed in column chromatography are silica, alumina, calcium carbonate, calcium phosphate, magnesia, starch, etc., selection of solvent is based on the nature of both the solvent and the adsorbent. The rate at which the components of a mixture are separated depends on the activity of the adsorbent and polarity of the solvent. If the activity of the adsorbent is very high and polarity of the solvent is very low, then the separation is very



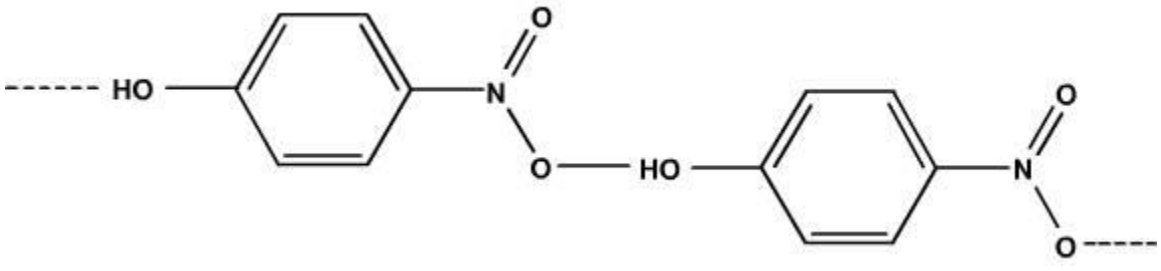
slow but gives a good separation. On the other hand, if the activity of adsorbent is low and polarity of the solvent is high the separation is rapid but gives only a poor separation, i.e., the components separated are not 100% pure.

The adsorbent is made into slurry with a suitable liquid and placed in a cylindrical tube that is plugged at the bottom by a piece of glass wool or porous disc. The mixture to be separated is dissolved in a suitable solvent and introduced at the top of the column and is allowed to pass through the column. As the mixture moves down through the column, the components are adsorbed at different regions depending on their ability for adsorption. The component with greater adsorption power will be adsorbed at the top and the other will be adsorbed at the bottom. The different components can be desorbed and collected separately by adding more solvent at the top and this process is known as *elution*. That is, the process of dissolving out of the components from the adsorbent is called elution and the solvent is called is called eluent. The weakly adsorbed component will be eluted more rapidly than the other. The different fractions are collected separately. Distillation or evaporation of the solvent from the different fractions gives the pure components.

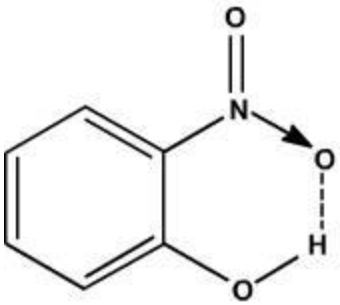
Intermolecular forces, which vary in strength according to their type, make organic molecules to bind to the stationary phase. The stronger the intermolecular force, the stronger the binding to the stationary phase, therefore the longer the compound takes to go through the column.

Intra-molecular hydrogen bonding is present in ortho- nitro phenol. This is due to the polar nature of the O-H bonds which can result in the formation of hydrogen bonds within the same molecule. But in para-nitro phenol, inter molecular hydrogen bonding (between H and O atoms of two different para-nitro phenol molecules) is possible. As result of inter molecular hydrogen bonding para-nitro phenol undergo association that increases the molecular weight, whereby decreasing volatility.

Para nitro phenol with inter molecular hydrogen bonding:



Ortho nitro phenol with inter molecular hydrogen bonding:



Procedure:

1. **Preparation of the Column:**

- Place the column in a ring stand in a vertical position.
- A plug of glass wool is pushed down to the bottom of the column.
- Prepare slurry of silica gel with a suitable solvent & pour gently into the column.
- Open the stop cock & allow some solvent to drain out. The layer of solvent should always cover the adsorbent; otherwise cracks will develop in the column.

2. **Adding the Sample to the Column:**

- Dissolve the sample mixture in a minimum amount of solvent (petroleum ether).
- Remove the solvent by placing the mixture in a rotary evaporator at a low temperature.
- Place the dry powder on a piece of weighing paper and transfer it to the top of the column through the funnel.

3. **Developing the Chromatogram:**

- Attach a dropping funnel filled with petroleum ether on to the column.
- Add petroleum ether continuously from the funnel to the top of the column.
- Open the stopcock carefully.
- The components of the mixture run down the column forming two separate yellow bands.

4. **Recovering the Constituents:**

- Continue running the petroleum ether till both the bands are eluted out separately.
- Collect the constituents in two different R.B flasks. (Ortho nitrophenol is obtained first, followed by para nitro phenol.).
- Evaporate the solvent by placing the mixture in a rotary evaporator.

5.Separation of protein mixtures in any form of chromatography

AIM

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. Proteins can be purified based on characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase. Four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion. Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography. Column chromatography is one of the most common methods of protein purification.

Principle

Chromatography is based on the principle where molecules in mixture applied onto the surface or into the solid, and fluid stationary phase (stable phase) is separating from each other while moving with the aid of a mobile phase. The factors effective on this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among their molecular weights [1, 2]. Because of these differences, some components of the mixture stay longer in the stationary phase, and they move slowly in the chromatography system, while others pass rapidly into mobile phase, and leave the system faster

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a “solid” phase or “a layer of a liquid adsorbed on the surface a solid support”.
- Mobile phase: This phase is always composed of “liquid” or a “gaseous component.”
- Separated molecules.

- The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (ie. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, esther, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses [4].
- Stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable, and non-volatile samples [5].
- The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achive a satisfactory separation within a suitable timeinterval. Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography

Column chromatography

Since proteins have difference characteristic features as size, shape, net charge, stationary phase used, and binding capacity, each one of these characteristic components can be purified using chromatographic methods. Among these methods, most frequently column chromatography is applied. This technique is used for the purification of biomolecules. On a column (stationary phase) firstly the sample to be separated, then wash buffer (mobile phase) are applied (Figure 1). Their flow through inside column material placed on a fiberglass support is ensured. The samples are accumulated at the bottom of the device in a tme-, and volume-dependent manner

6.SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDSPAGE)

AIM:

SDS-PAGE was performed to separate and observe the protein pattern of the sample by the method of Lammeli (1970)

PRINCIPLE:

SDS-PAGE was performed to accomplish the following:

a) To observe the protein pattern of the enzyme mixture.

b) To determine the homogeneity of the purified enzyme mixture.

c) To determine the molecular weight of the purified enzyme.

REAGENTS REQUIRED:

1. Preparation of stock solution and buffers:
   30% acrylamide a) Acrylamide: 29.2g b) N, N-methelyne–bis–acrylamide: 0.8g Added water, dissolved and made upto 100mL and filtered with Whatman no.1 filter paper.

2. Separating gel buffer:

a) Tris-HCl: 1.5M, pH 8.8 18.171g of Tris was dissolved in 60mL of water and adjusted the pH to 8.8 with HCl and finally made upto 100mL with water.

3. Stacking gel buffer:

a) Tris-HCl:

1M, pH 6.8 6.057g of Tris was dissolved in 60mL water and adjusted the pH to 6.8 with HCl and upto 100mL with water.

4. 10% SDS solution:

1g of SDS in 10mL of distilled water. 5. N,N,N'N'-Tetra methylene diammine(TEMED)

6.

10% Ammonium per sulphate (APS): 1g of APS in 10mL of distilled water.

7. Electrophoresis Buffer:

a) Tris: 25mM, pH 8.3 b) glycine: 250mM,pH 8.3 c) SDS: 0.1%: Dissolved in minimum amount of water (500mL) and then added SDS. Allowed to settle and dissolved. This was finally made upto 2.5liters.

8) Sample buffer 4x:

5.0mL a) Tris (1M, pH 6.8): 2.1mL b) 2% SDS: 100mg c) Glycerol (100%): 1.0mL d) b-mercaptoethanol: 0.5mL e) Bromophenol blue: 2.5mg f) Distilled water: 0.4mL

9) Staining solution (100mL):

a) Alcohol: 40%

b) Acetic acid: 10%

c) Commassie Brilliant Blue (CBB): 259mg d) Distilled water: 50%

10) Destaining solution (100mL)

a) Alcohol: 50%

b) Acetic acid: 10%

c) Distilled water: 40%

PROCEDURE

Preparation of gel:

The glass plates were washed in warm detergent solution, rinsed subsequently in tap water, deionised water and ethanol and dried. The unnotched outer plates were laid on the table and Vaseline (or grease) was coated. Spacer strips were arranged approximately at the sides and bottom of the plates. The notched inner plates were laid in position, resting on the spacer strips and the arrangement was mounted vertically. Sealing was done properly to avoid leakage.

APS and TEMED were added just prior to the pouring of gel. The solution was mixed well and poured into the space between the two plates leaving an inch of the upper space unfilled. Water was carefully laid over the surface of the poured gel mixture to avoid air contact, which reduces the polymerization reaction. The gel mixture was allowed to polymerize, undisturbed at room temperature for 60 minutes. In the mean time gel mixture for stacking gel was prepared. (The reagents in the following table yield 10mL

of solution after the addition of APS & TEMED) After the separating gel was polymerized the over laid water was removed carefully with filter paper and an appropriate comb was inserted between the plates. 0.1mL of 10% APS and 10 l of TEMED were added to the stacking gel mixture. It was mixed well and poured immediately (to the brim) over the separating gel. The stacking gel was allowed to polymerize. Additional gel mixture was added when gel retracted significantly.

Preparation of protein samples:

The required volume of sample buffer was added to protein samples and they were loaded (the final concentration of sample buffer in the prepared sample should come to 1x. If the protein was dried suspend it in 1x buffer). The samples were incubated for 2min in a boiling water bath prior to loading. When the polymerization was completed the comb was removed and the lower spacer strip was carefully removed. The Vaseline (or grease) from the bottom was removed with a piece of tissue paper. The gel was attached to the electrophoresis tank using appropriate clips/clamps. The lower reservoir was filled with 1x electrophoresis buffer, using a bent Pasteur pipette or syringe needle to remove any air bubble trapped beneath the bottom of the gel. The protein samples were loaded using a micropipette and the wells were completely and carefully filled with 1x electrophoresis buffer. The upper reservoir was also carefully filled with 1x electrophoresis buffer. The electrodes were connected to a power pack. The gel was run at constant current (20 milli ampere 100 volts) for 4-6 hrs at room temperature. Electrophoretic mobility of the samples was determined by bromophenol blue front. At the end of the run the power pack was switched off. The gel and plates were laid flat on the table and a corner of the upper glass plate was lifted up and the gel was carefully removed.

Staining of the gel:

After the completion of the electrophoresis, the gel was fixed with 10% trichloroacetic acid for 5minutes and stained with CBB. The CBB staining solution was prepared using methanol, acetic acid and double distilled water in the ratio of 4:1:5 and 0.25gm of CBB was added and the gel was stained over night. solution was prepared using methanol, acetic acid and double distilled water in the ratio of 4:1:5 and 0.25gm of CBB was added and the gel was stained over night.

Destaining of the gel:

The destaining of CBB stained gel was done by using methanol, acetic acid and double distilled water in the ratio of 5:1:4 till the appearance of clear bands on the gel.

RESULT:

The sample proteins are separated by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis. The proteins appeared as discrete bands in the gel. The relative molecular weights of the protein with respect to their bands were observed in Kilo Daltons.

7.Determinaton of λmax for an unknown sample and calculation of extinction coefficient

AIM:

(I) To determine λmax (wavelength of maximum absorption) of CuSO4– NH3 solution using a spectrophotometer

(II) To verify Beer-Lambert's law and apply it to find the strength of unknown solution

Theory:

When an electromagnetic radiation is passed through a sample, certain characteristic wavelengths are absorbed by the sample. As a result the intensity of the transmitted light is decreased. The measurement of the decrease in intensity of radiation is the basis of spectrophotometry. Thus the spectrophotometer compares the intensity of the transmitted light with that of incident lights. The absorption of light by a substance is governed by Beer's and Lambert's law. According to the Beer-Lambert's law: when a beam of monochromatic light of intensity I0 passes through a medium that contains an absorbing substance, the intensity of transmitted radiation I depends on the length of the absorbing medium and the concentration of the solution. Mathematically it can be represented as: Absorbance = log(I0/I) = εcl

Where, I0 = intensity of incident light I = intensity of transmitted light A = absorbance l = length of the absorbing medium c = concentration of the solution ε = molar absorption coefficient or molar extinction coefficient

The molar absorption coefficient or molar extinction coefficient is the absorbance of a solution having the unit concentration (c = 1M) placed in a cell of unit thickness (l = 1 cm). Absorbance is also called optical density (OD). Thus, for a particular wavelength λ, the absorbance or OD of a solution in a container of fixed path length is directly proportional to the concentration of a solution. i.e. A ∞ c A plot between absorbance and concentration is expected to be a straight line plot, passing through the origin, shows that Beer-Lambert's law is obeyed. This plot, known as calibration curve, can also be employed in finding the concentration (or strength) of a given solution.

Apparatus:

Spectrophotometer, cuvette, beaker, pipette. Chemicals: CuSO4, NH4OH, tissue paper.

Procedure:

1. The spectrophotometer is calibrated for 100% transmittance in water.

2. The CuSO4 solutions of different strength are supplied, such as (N/60), (N/70), (N/80), (N/90) and (N/100).

3. Determination of λmax: Take 5 mL of CuSO4 solution and 5 mL of NH4OH solution in the cuvette and mix it well. Measure the absorbance or transmittance at different wavelengths. The minimum transmittance at a particular wavelength will correspond to maximum absorbance which will give λmax. [Important: Trnsmittance of the pure solvent (water) is always adjusted to 100% before each wavelength measurement. This is because the extinction coefficient of the blank (pure water) also changes with wavelength]

4. Verification of Beer-Lambert's law: Fix the wavelength at λmax value. Measure the absorbance or OD of different CuSO4-NH4OH solutions at that λmax. Plot a graph between OD and concentration (or strength) of the solution. [If a straight is obtained, then the BeerLambert's law is verified]

5. Now find out the absorbance or OD of the unknown solution and find out the strength of the solution form the graph.

8.Separation of components of a given mixture using a laboratory scale centrifuge

Aim:

To separate the components of a mixture using the following techniques:

- Separating funnel
- Chromatography
- Centrifugation
- Simple distillation
- Fractional distillation

How is a homogeneous mixture different from a heterogeneous mixture?

Most materials in our surroundings are mixtures of two or more components. Mixtures are either homogeneous or heterogeneous. Homogeneous mixtures are uniform in composition, but **heterogeneous mixtures are not uniform in composition.**

Air is a homogeneous mixture and oil in water is a heterogeneous mixture. Homogeneous and heterogeneous mixtures can be separated into their components by several physical methods. The choice of separation techniques is based on the type of mixture and difference in the chemical properties of the constituents of a mixture.

Centrifugation:

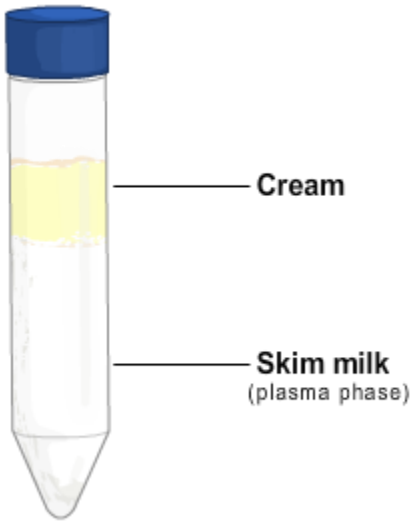
Sometimes the solid particles in a liquid are very small and can pass through a filter paper. For such particles, the filtration technique cannot be used for separation. Such mixtures are separated by centrifugation. So, **centrifugation is the process of separation of insoluble materials from a liquid where normal filtration does not work well.** The centrifugation is based on the size, shape, and density of the particles, viscosity of the medium, and the speed of rotation. The principle is that the denser particles are forced to the bottom and the lighter particles stay at the top when spun rapidly.

The apparatus used for centrifugation is called a centrifuge. The centrifuge consists of a centrifuge tube holder called rotor. The rotor holds balanced centrifugal tubes of equal amounts of the solid-liquid mixture. On rapid rotation of the rotor, the centrifuge tubes rotate horizontally and due to the centrifugal force, the denser insoluble particles separate from the liquid. When the rotation stops, the solid particles end up at the bottom of the centrifuge tube with liquid at the top.

Applications:

- Used in diagnostic laboratories for blood and urine tests.

- Used in dairies and home to separate butter from cream.
- Used in washing machines to squeeze water from wet clothes.



Materials required:


Centrifuge tube


Milk


Distilled water


Centrifuge machine

Procedure:

- Take a centrifuge tube and fill 3/4th of it with milk.
- Place the centrifuge tube in one of the holes of the rotor.
- Take another centrifuge tube and fill 3/4th of it with distilled water.
- Place it opposite the first centrifuge tube to balance the rotor in the centrifuge machine.
- Close the centrifuge machine and centrifuge the milk for 2 minutes.
- Stop the centrifuge machine and take the centrifuge tube containing milk from the machine.
- Observe the changes.

Observations:

- The fat rises to the top resulting in a cream layer at the top and skim milk (plasma phase of milk) at the bottom.

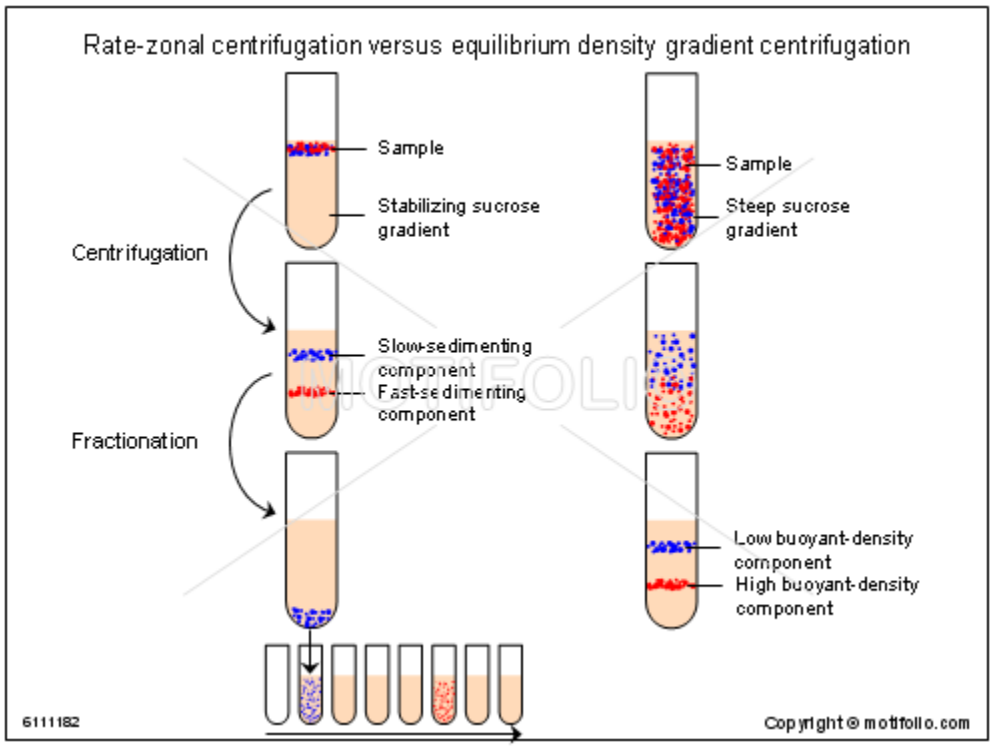
Inferences:

- The denser particles are forced to the bottom and the lighter particles stay at the top when spun rapidly.

Precautions:

1. Close lids at all times during operation.
2. Always cap tubes before centrifugation.
3. Do not operate the centrifuge without the rotor properly balanced.
4. Do not open the lid while the rotor is moving. Allow the centrifuge to come to a complete stop before opening.
5. Stop the centrifuge immediately if an unusual condition (noise or vibration) begins and check load balances.

9.understanding density gradient centrifugation with the help of pictures



Abstract

Centrifuges are used in cell and molecular biology departments, in industrial laboratory settings and medical laboratories. They are the workhorses for separating the components of heterogeneous mixtures or for purifying biological particles. Fundamental to centrifuges is the increase in the effective force of gravity = relative centrifugal force (rcf) ⇒ expressed in multiples of Earth's gravity (1g). This is accomplished by spinning the sample to be disaggregated in the single cavity or the tubes of a rotor as the 'mobile part' of a centrifuge. Subjected to the rcf thus applied, the constituents of a sample will differentially sediment according to their physical properties, like size and molecular mass as well as density and viscosity of the sample solution.

Modern ultracentrifuges accelerate an appropriate rotor to speeds up to >50 000 revolutions per minute (rpm), generating thereby an rcf up to the millionfold of the Earth's gravity. Efficiency and range of activities of these latest machines reflect the technological progress during the past 3–4 decades with the upcoming microchip technology, the construction of new heavy-duty drive systems, the installation of a sophisticated control equipment and last but not least the access to the novel carbon-fibre material in the design of rotors. While the classic floor-standing ultracentrifuge has been revolutionised by these groundbreaking changes to its design, with the benchtop version, a novel model matching with the demands on flexibility and versatility of experimental working has been launched in the meantime.

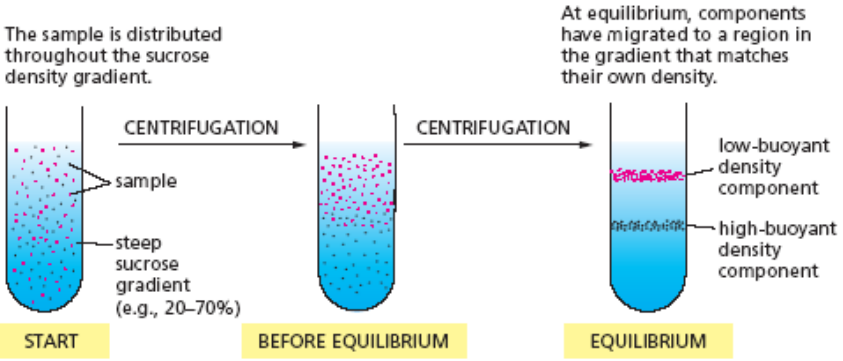
Basically, there are two kinds of ultracentrifuges, preparative and analytical ones. The former is widely used to fractionate tissue homogenates or cell suspensions, aiming to isolate and purify the distinct classes of biological material: intact cells, subcellular organelles, macromolecules and corresponding complexes, bacteria or viruses. By means of analytical centrifuges, the physico-chemical properties of a sedimenting particle or molecular interactions of the subunits of multiprotein complexes can be unravelled.

Key Concepts

- Centrifugation is widely used in studying fundamental cellular processes such as the inter- and intracellular transport of metabolites, the biosynthesis and breakdown of proteins or the molecular interactions of the components of multiprotein complexes.
- Ultracentrifugation is basically carried out in two ways: preparative and analytical centrifugation.
- The former aims to isolate and purify subcellular organelles or multiprotein complexes; the latter allows to analyse the mutual interactions between the subunits of multiprotein complexes and to unravel physico-chemical properties like the mass and size of macromolecules.
- Preparative centrifugation may be performed as batch-type (conventional) centrifugation or alternatively in the continuous-flow mode.
- The former is mostly used to separate and enrich organelles out of complex biological mixtures commonly limited in their quantity.
- Continuous-flow centrifugation is particularly useful for the large-scale collection of particles out of diluted solutions or suspensions (bacteria, viruses) as it combines high centrifugal forces with high throughput.
- Theoretically, two kinds of preparative centrifugation have to be distinguished: differential centrifugation and density gradient centrifugation.
- Differential centrifugation fractionates organelles according to their size, mass and shape yet leads only to an enriched rather than a highly purified preparation of a particular organelle.
- To get such a preparation genuinely purified, contaminants have to be removed by density gradient centrifugation.
- Rate zonal and isopycnic density gradient centrifugation differ in their basic concepts and the types of density gradients employed.

EQUILIBRIUM SEDIMENTATION

The ultracentrifuge can also be used to separate cell components on the basis of their buoyant density, independently of their size or shape. The sample is usually either layered on top of, or dispersed within, a steep density gradient that contains a very high concentration of sucrose or cesium chloride. Each subcellular component will move up or down when centrifuged until it reaches a position where its density matches its surroundings and then will move no further. A series of distinct bands will eventually be produced, with those nearest the bottom of the tube containing the components of highest buoyant density. The method is also called density gradient centrifugation.



A sucrose gradient is shown here, but denser gradients can be formed with cesium chloride that are particularly useful for separating the nucleic acids (DNA and RNA).

The final bands can be collected from the base of the tube, as shown above.