DEPARTMENT OF BIOTECHNOLOGY

BT 3761-

DOWNSTREAM PROCESSING LABORATORY MANUAL

REGULATION 2021

IV YEAR & VII SEMESTER

MADHA ENGINEERING COLLEGE
MADHA NAGAR
CHENNAI- 600 069

BT 3761 DOWNSTREAM PROCESSING LAB

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EXPT NO : 1

CENTRIFUGATION

Aim: To separate different organelles of yeast cells by centrifugation

Principle: Centrifugation is used to separate materials of different densities when gravitational force is insufficient for separation. The basic principle involved in centrifugal separation of solids is the density difference between the solids and the surrounding fluid. Normally a suspension of solids on standing settles down slowly under the influence of gravity. The process is known sedimentation. In centrifugation, the process of settling is aided by centrifugal forces.

When a solid particle moves through a viscous medium, (infinite continuum) its velocity is affected by two opposing forces, the gravitational force and drag force. The particle is accelerated by the gravitational force resulting from the density difference between the particle and the surrounding fluid. The gravitational force Fg acting on spherical particles

$Fg= \pi/6 (d^3 (\rho s-\rho)) g$ where d is the diameter of the particle $\rho s, \rho$ are the densities of the sphere and fluid respectively and g is the gravity.

The drag force $Fd =3 \pi d \eta v$ where η is the viscosity of the medium and v is the velocity of the spherical particle. The solid concentrate produced by centrifugation differs from that produced by filtration. At best centrifugation produces a paste often it yields only a concentrated suspension, however many biological which can be filtered effectively can be centrifuged.

During the circular motions of a centrifuge, the centrifugal force

$Fc =ma =m\omega^2 r$ where $\omega^2 r$ = Acceleration due to centrifugal force.

The rotational speed of a centrifuge n is generally expressed in terms of number of revolutions per minute (rpm).The centrifugal force may be expressed in terms of revolutions of the centrifuge by substituting

$\omega=2\pi n/60$ $Fc = mr (2\pi n/60)^2$ $Fc = 0.01097\ mrn^2$

The centrifugal force may be expressed in terms of gravitational force

$Fg = mg$ $Fc/Fg = \omega^2 r/g$.

Thus the force developed in the centrifuge is $\omega^2 r/g$ times as large as the gravity force and is often equivalent to so many g forces. By increasing the speed rpm and hence the g value step by step , one can pellet down different components of the homogenate at each step. This type of centrifugation is known as differential centrifugation.

Apparatus Required:

Beakers, Sonicator, Centrifuge, Centrifuge tubes

Chemicals Required:

Yeast powder Potassium Hydroxide Potassium phosphate, distilled water.

Procedure: Prepared the yeast suspension by adding 5 gm of yeast powder into 500 ml of 0.2 M Potassium phosphate buffer at pH of 7.5 and kept overnight.

1. 7.80 g of Potassium dihydrogen phosphate was dissolved in 250 ml of distilled water . (Solution 1).
2. 2.80 g of Potassium hydroxide was dissolved in 250 ml of distilled water. (Solution 2).
3. 225 ml of solution 2 was added to 250ml of solution 1 and the mixture was adjustedto 500 ml by using distilled water and concentrated KOH solution to bring the pH to 7.5. (Phosphate Buffer)

1. Added 5 gm of Yeast powder into a beaker containing 50 ml of the Phosphate buffer.
2. Transferred the cell suspension into a beaker and sonicated the sample for a period of20 to 30 min on ice to prepare the homogenate.
3. The homogenate was then subjected to differential to fractionate the different organelles of the cell

   In this method the centrifuge was filled with the homogeneous solution and then centrifuged at required rpm for the required time. It resulted into two fractions a pellet at the bottom of the tube containing the sedimented material and a supernatant solution containing the unsedimented material.

   By increasing the speed (rpm) and hence the g value step by step one can pellet down different components of the homogenate at each step. This type of centrifugation is known as differential centrifugation.

   The different organelles of the homogenized yeast cell suspension were separated by centrifuging at first with 1500 rpm for 10 min to separate the cell debris and the supernatant from the first tube was transferred to another centrifuge tube and then centrifuged at 3000 rpm for 10 min to separate the nucleic acids then the supernatant from the second tube was transferred to another centrifuge tube and then centrifuged at 8000 rpm for 10 min to separate the proteins then the supernatant from the third tube was transferred to another centrifuge tube and then centrifuged at 10000 rpm for 10 min to separate the mitochondria

Result:

Thus the different organelles were separated from the cell suspension.

EXPT NO: 2

CELL DISRUPTIONS BY ULTRASONICATION

Aim: To disrupt Yeast cells using a Ultra Sonicator

Experiment Principle: The treatment of microbial cells in suspension with inaudible ultrasound greater than 18000Hz results in their inactivation and disruption . Ultrasonication utilizes the rapid sinusoidal movement of a probe within the liquid. It is characterized by high frequency (18KHz -1MHz) , small displacements (less than 50μm); moderate velocities a few m/s, steep transverse velocity gradient (up to $4000s^{-1}$) and very high acceleration (up to 80000g).

Ultrasonication phenomena takes place when acoustic power input is sufficiently high which allows for the multiple productions of micro bubbles at nucleation sites in the fluid. The bubbles grow during the rarefying phase of sound wave, and then are collapsed during the compression phase. On collapse a violent shock wave passes through the medium. The whole process of gas bubble nucleation, growth and collapse due to action of intense sound wave is called cavitation. The collapse of the bubble converts sonic energy into mechanical energy in the form of shock waves . This energy input imparts motions to parts of cells which disintegrate when their kinetic energy content exceeds the wall strength. An additional factor which increases cell breakage is the micro streaming (very high velocity gradient causing shear stress)

Equipment Principle: Ultrasonic homogenizer produces a high power ultrasound with high amplitudes The oscillations are transmitted and amplified along the length of horn and the probe into The aqueous sample to be treated. The HF generator converts the received power frequencies of 50 or 60 Hz into high frequency of 20 KHz. The ultrasonic convertor connected to the HF generator transforms the high frequency power from the HF generator to Ultrasound converting it to a form of mechanical energy. This is achieved through a robust PZT transducer system. Hence the tip of the probe also vibrates at a frequency of 20 KHz And transfers these vibrations with high power density to the sonicated sample.

Apparatus Required: Ultrasonic homogenizer, HF generator, Probe, microbial cell culture and beaker.

Chemicals Required: Yeast powder, Potassium hydroxide, Potassium Phosphate, Sodium chloride, Coomasie brilliant blue, Ethanol, Ortho phosphoric acid and distilled water.

Procedure:

Prepared the yeast suspension by adding 5 gm of yeast powder into a 500 ml of 0.2 M Potassium Phosphate buffer at a pH of 7.5 and kept it overnight.

Phosphate buffer:

1 Dissolved 6.80 g of Potassium dihydrogen phosphate in 250 ml of distilled water solution (1)

2 Dissolved 2.80 g of Potassium dihydrogen phosphate in 250 ml of distilled water solution (2)

3. Added 225 ml of solution (2) to 250 ml of solution (1)and adjusted the mixture to 500ml by using distilled water and concentrated KOH solution and brought the pH to 7.5.

4. 5 gm of the yeast powder was added into a beaker containing 50 ml of the phosphate buffer.

5. A small amount of the sample was taken for O.D measurement before sonication and After sonication.

6. The cell suspension was then transferred into a beaker and the sample was sonicated on ice using the procedure given below.

The following recommendations may be followed during the operation of the ultrasonicator:

1. Before connecting the power, compare with the mains voltage on the generator base and connect only the appropriate type to a grounded socket. 230v, 50/60 Hz

2. Place the HF generator on a solid , even dry base.

3. Place the ultrasonic convertor safely and properly into the support.

4. Do not touch the processing vessel with the vibrating probe.

5. When sonicating liquids, the probe should be inserted 10 mm deep in order to avoid air Intake and mixing in the liquid. If air mixing is wanted, the probe should only be inserted a few millimeters. The recommended immersion depth I of the probes is between 1 and 20 mm.

Count down operation mode:

In count down operation we can set the time and pulsed cycle. After starting the generator either by START/STOP KEY or the push button on top of the ultra sonic convertor. The maximum time setting is 99min and 59 sec.

Operating Guidelines:

1. Turned Power tuner to left stop

2. Pressed the main switch (Green lamp lights up), after 3 seconds the unit is ready to start LED display lights up.

3. The required settings were entered by means of mode key and +/- key Selection of time (minutes, Seconds) and pulsed cycle was done with by pressing the mode Key and after selection by pressing the +/- key the indicated value can be increased or decreased in the intervals of 12 digit.

4. After immersing the probe into the liquid the POWER tuner clockwise was set at the required value

5. Pressed the START/STOP at the generator or the push button on top of the ultrasonic convertor .The countdown program starts to run and stops after the countdown is over.

6. To switch OFF turn the power tuner to the left stop, Press the Mains Switch"

Determination of Optical Density:

Bradford reagent can be used to determine the concentration of protein in solution. The procedure is based on the formation of the complex between the dye brilliant blue and the protein in solution. The dye protein complex causes a shift in absorption maximum of the dye from 465 to 595 nm. The amount of absorption is directly proportional to the amount of protein present.

Bradford Reagent:

1. 100 mg of Coomasie brilliant blue dye was taken and dissolved in 50 ml of 95% ethanol.
2. 100 ml of Orthophosphoric acid was then added
3. The solution was made up to 200 ml with distilled water
4. The concentrated dye was diluted by taking 200 ml of dye solution and 800 ml of distilled water.

Phosphate Buffer Saline:

1. 7.80 g of Potassium dihydrogen phosphate was dissolved in 250 ml of distilled water. (Solution 1).
2. 2 2.80 g of Potassium hydroxide was dissolved in 250 ml of distilled water. (Solution 2).
3. The two solutions were mixed and to the resulting solution 4.38 g of sodium chloride was added to get 0.15 M NaCL in 0.2M Phosphate buffer.

Procedure for OD Measurement:

Standard BSA solution of concentration 2mg/ml was prepared by dissolving 0.2 g of BSA in 100 ml of PBS.

Note: The cell suspension was taken and centrifuged the cell debris was discarded and the supernatant liquid was then diluted to 10 times with PBS.

2ml of PBS and 5ml of Bradford reagent was used as blank.

1. 2 ml of the diluted cell suspension was taken and 5 ml of Bradford reagent was added and mixed by vortexing.
2. The mixture was allowed to stand at room temperature for 5 minutes.
3. The absorbance was measures at 595 nm after 10 min.

The Absorbance of the sample before Sonication = 0.45

The Absorbance of the sample after Sonication for 30 min = 0.74

Result: The absorbance values indicated that the proteins were released from cells due to cell disruption as a result of sonication.

EXPT NO : 3

CELL DISRUPTION IN DYNO MILL

Aim:

To study the cell disruption by mechanical methods and to find disruption constant of bakers yeast in dyno mill.

Principle:

With a bead mill it is possible to break open all types of microorganisms in **a** continuous process. If no data is available for the microorganism to be broken open it is done with the following conditions:

1. Glass beads (lead free) 0.5 mm dia
2. Volume to which the disruption vessel should be filled with the glass beads: 85%
3. Rotation velocity of the grinding discs: 10 m/s
4. Flow rate froe yeast: 10 x volume of grinding vessel /hr
5. Flow rate for bacteria: 5x volume of grinding vessel /hr
6. Cell concentration: unimportant

If the results of one run are unsatisfactory (i.e. less than 60% of the cells are broken), the first three variables can be optimized. For this purpose one determines the disruption constant k in batch cultures. During the experiment the release of the desired is measured with respect to time. If the breakage vessel has no openings to allow samples to be taken, the experiment can be performed using an externally fitted beaker. An increase in the value of k means that the correct choice for the value of a particular variable has been made for the organism in question. In batch culture, data can be obtained which allow the flow rate t o be optimized. If the time required for total breakage is 3 min, the time for continuous flow should be approximately 10 min because there is n o 'plug flow' in a bead mill. The working values obtained for specific microorganisms are valid only for the grinder and container used; i.e. Another model or even volume requires recalibration.

Cell breakage follows first order kinetics i.e. rate at which product is produced is at any time proportional to the amount of product available

$$\mathbf{dP/dt = - kP}$$

Where k is the disruption constant and P is the product concentration at time t; which can be solved to give

$$\mathbf{\ln (Pm / (Pm - P)) = kN = kt}$$

Pm is disruption amount of product released and N is the number of times the suspension has passed through the mill and is calculated as follows:

N = (Flow volume / Initial volume) t

Reagents Required:

Buffer: 100 mM Potassium phosphate buffer. pH 7.5

Lowry's Protein analysis reagents

Culture solution (Yeast or bacteria)

Procedure:

1. Suspended 1.6 kg of yeast in 100 mM Potassium phosphate buffer. pH 7.5 and made up to a final volume of 4:1 (40% w/v) . The pH of the suspension is determined and if necessary, readjusted to 7.5 by the addition of 100 mM potassium hydroxide.

2. Placed 850 ml dry glass beads into the mill vessel (volume , 1:1) and then filled with the cell suspension

3. Stirred the remaining yeast suspension continuously on ice connected to the input and the ouput valves of the mill vessel

4. Run the mill vessel at a speed of 2000 rpm and a flow volume of 10 liter per hour

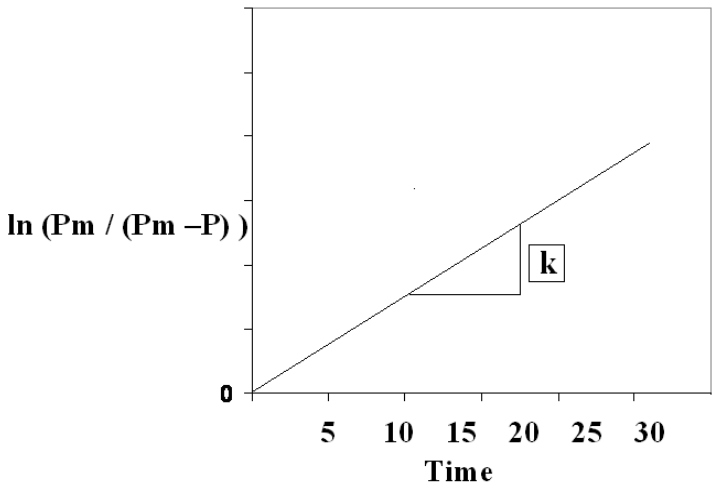
5. Throughout the experiment the grinding vessel should be cooled

6. The samples were taken at different intervals of time of 0,1,2,5,7.5,10,15,20,30,45,60 min. Throughout the experiment, the temperature, pH and conductivity of the yeast suspension was monitored

7. At the end of the experiment , water was passed through the mill vessel and subsequently emptying it to remove the remaining cell suspension.

8. Determined the maximum amount of protein released and the amounts released at time t, Plotted ln (Pm / (Pm –P)) versus t graph **.**

Note: The maximum protein concentration from the table is taken as Pm



The slope of the straight line is the breakage constant k.

1. In another graph plotted the values for temperature , pH and conductivity against time.

Observations and calculations:

S.No	Time in min	pH	Protei conc	Conduc	Pm -P	P/ Pm –P	ln (P / (Pm –P))	K = ln (P / (Pm –P) / t
1	01							
2	2							
3	5							
4	7							
5	10							
6	15							
7	20							
8	30							
9	45							
10	60							

Result:

The breakage constant for the given material in dyno mill is k= ---------- time $^{-1}$

EXPT NO: 4

EXTRACTION OF CASEIN FROM MILK BY AMMONIUM SULPHATE PRECIPITATION

Aim: To precipitate casein from milk using Ammonium Sulphate

Principle: Casein is a protein present in milk, which contains the entire essential Amino acids. It is in the solublised form because of its interactions with water, This keeps away hydrophobic interactions of the protein molecules. When Ammonium sulphate is added to milk the ammonium sulphate being more polar than Casein will attract the water molecules to itself and get solvated hence the water available for the solvation of protein is decreased, as a result the hydrophobic interactions of the protein molecules increases which leads to aggregation and subsequently precipitation.

Materials Required:

Ammonium sulphate, Distilled water, Beaker, Conical flask, Filter paper, Milk, Buchner funnel, Watch glass and Folins reagent.

Procedure:

100 ml of milk was taken in a glass beaker and warmed slightly then either Ammonium Sulphate powder or saturated Ammonium sulphate solution was added slowly in Small increments with constant stirring. The amount of powder added or the amount of saturated solution added to the milk was noted. The addition and stirring was stopped when the precipitate started to form. The beaker was then kept aside for 10 min for the precipitation process to get completed. The precipitate was then collected by filtering. The precipitate was then washed several times with a small amount of water. The precipitate was then suspended in about 30 ml of ethanol for 10 min. The suspension was then filtered in a Buchner funnel and the precipitate was washed a second time with a mixture of equal volumes of ethanol and ether. Finally the precipitate was washed on a filter paper with 50 ml of ether and suction was applied to dryness. The powder was then removed and spread out on a watch glass to allow for evaporation of the ether. The final weight of casein was then noted.

Confirmation Test:

Confirmation for the presence of protein is as follows, protein reacts with Folin Cicoalateu Reagent to give a colored complex. The color so formed is due to the reaction of the Alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Reagents Required:

1. Alkaline sodium carbonate solution (20g/l of Na2CO3 in 0.1 mol /lit of NaOH)
2. Copper sulphate Sodium potassium tartarate solution. (5g of CuSO4.5H2O in 10g/lit of Na, K tartarate) was prepared fresh by mixing stock solutions.
3. Alkaline solution was prepared on the day of use by mixing 50 ml of (1) and 1mlof (2).
4. Folin Cicoalateu Reagent .Diluted the commercial reagent with an equal volume of water on the day of use.

Method:

5 ml of the alkaline solution was added to 1ml of the test solution of concentration 0.2 mg/ml. and mixed thoroughly. The solution was then allowed to stand at room temperature for 10 min or longer. 0.5 ml of diluted Folin Cicoalateu Reagent was added rapidly with immediate mixing. The appearance of Blue color indicated the presence of protein.

Calculation:

Note: Saturated Ammonium sulphate (AS) Solution was prepared by dissolving 750g of ammonium sulphate in 1000 ml of distilled water. The % saturation of Ammonium sulphate in the solution at which precipitation starts to take place can also be calculated. For instance when we add 100 ml of saturated solution to 100ml of milk Then the % Saturation of the mixture is 50% or for instance if we add 5g of ammonium Sulphate to cause the precipitation then the % saturation of the mixture is calculated by

Taking 75g/100 ml of milk as 100% saturation and hence for 5g/100 ml the % saturation is 6.6%.

Weight of Ammonium sulphate to cause the precipitation W = 22 gm

% Saturation of Ammonium sulphate in the mixture at onset of precipitation = W* 100/75 = 22* 100/75 =29.3%

Volume of saturated solution to cause the precipitation V = ml

% Saturation of Ammonium sulphate in the mixture at onset of precipitation

= V*100 / (V+100)

Weight of Watch glass W1 = 32.052g

Weight of Watch glass + Dry Casein W2 = 35.581g

Weight of Dry Casein W2 - W1 =3.53g

Result:

1. The weight of Casein precipitated in 100 ml of milk = 3.53g
2. The % saturation of Ammonium Sulphate in the solution at the onset of precipitation =29.3%

EXPT NO: 5

ULTRA FILTRATION

Aim:

To concentrate the given protein sample by ultra filtration.

Principle:

Ultra filtration involves a large flow across the membrane surface perpendicular to the flux through the membrane .Such a flow is essential when solid particles are being ultra filtered. The cross flow minimizes the development of filter cake which would retard the process. This lack of a cake is what distinguishes ultra filtration from conventional filtration process. When a macro molecular solution is being ultra filtered, the cross flow reduces macro molecular accumulation near the membrane surface. Such an accumulation is called concentration polarization which augments the osmotic pressure and hence reduces the flow through the membrane.

Apparatus Required:

A basic system consists of a pump, feed reservoir, and a permeate collection reservoir, pressure indicators, and valving. Components are connected by flexible tubing.

Chemicals Required:

Bovine Serum Albumin, Potassium hydroxide, Potassium phosphate, Sodium chloride, Commasie brilliant blue, Ethanol, Ortho phosphoric acid and distilled water.

Procedure:

Bradford Reagent:

1. 100 mg of Coomasie brilliant blue dye was taken and dissolved in 50 ml of 95% ethanol.
2. 100 ml of Orthophosphoric acid was then added
3. The solution was made up to 200 ml with distilled water
4. The concentrated dye was diluted by taking 200 ml of dye solution and 800 ml of distilled water.

Phosphate Buffer Saline:

1. 7.80 g of Potassium dihydrogen phosphate was dissolved in 250 ml of distilled water. (Solution 1).

2 2.80 g of Potassium hydroxide was dissolved in 250 ml of distilled water. (Solution 2).

3 The two solutions were mixed and to the resulting solution 4.38 g of sodium chloride

was added to get 0.15 M NaCL in 0.2M Phosphate buffer.

Standard BSA solution of concentration 2mg/ml was prepared by dissolving 0.2 g of BSA in 100 ml of PBS.

Assembling the System:

1. Loosened the saddle securing knob and swing it either direction to expose the saddle.
2. Selected the pump saddle which matches the tubing you are going to use.
   Saddle 1.6X1.6 3R for size 14 tubing
   Saddle 3.3X1.6 3R for size **14** tubing
3. Placed the pump in the saddle.
4. A long piece of tubing was taken and Luer Lock adaptors were attached to the tubing at both ends.
5. The tubing was placed under saddle then the knob was swung over the saddle until it engaged.
6. Secured the saddle.

Position the Reservoirs:

1. Taken one of the 120 ml reservoirs and cap down comers and Luer Lock fittings .
   Screwed the cap on the reservoir
2. Placed the reservoir securely in the left hand recess at the rear of the mounting platform.
3. Taken a 30 ml reservoir and positioned it in the right hand recess at the rear of the mounting platform. This reservoir acts as a collection vessel for the permeate.

Installation of Hollow fiber Cartridge:

Final tubing connections without Pressure Transducers

1. One of the permeate ports was capped
2. Attached the tubing from the pump inlet to the feed reservoir having the longest down comer.
3. Attached the tubing from the pump outlet to the cartridge feed (Inlet port)
4. 30 cm tubing was cut and Luer Lock fittings were installed at both ends. One of the ends was attached to the permeate port on the cartridge, the other end was attached to the permeate reservoir.
5. 40 cm tubing was cut. The retentate pinch valve was slid onto the tubing Luer Lock fittings were installed at both ends of the tubing. One end was attached to the reservoir port of the cartridge. The other end was attached to the feed reservoir port with the middle length down comer.

Initial Start up:

After making all the necessary connections we start up the Mid Jet system

1. The feed solution was added to the feed reservoir
2. The retentate pinch valve was fully opened
3. The pumping was started using the inline power switch.
4. The feed flow was adjusted by tightening the pump saddle securing knob.
5. Adjusted the pressure using the retentate line pinch valve.

Operating parameters

Typical Recirculation rate: 50-140 ml/min

Typical inlet pressure 10 -20 psig

Determination of Optical Density:

4. 2ml of Standard BSA was taken and 5ml of Bradford reagent was added and mixed by vortexing.
5. The mixture was allowed to stand at room temperature for 5 min.
6. The absorbance was measured at 590 nm after 10 min

The absorbance of the solution of BSA was determined prior to ultra filtration and after ultra filtration.

The absorbance of the solution prior to ultra filtration = 0.778

The absorbance of the solution after ultra filtration = 1.178

Result:

The absorbance of the solution after ultra filtration was found to increase, thus the given sample of the protein was concentrated by ultra filtration.

EXPT NO: 6

EXTRACTION OF PROTEIN BY AQUEOUS TWO PHASE METHOD

Aim : To measure the amount of protein in the given sample using Aqueous Two Phase Extraction (ATPE)

Introduction:

Bio molecules and cellular particles can be separated and purified by partition between two immiscible aqueous polymer phases. In this method, macromolecules and particles are separated according to their surface properties. The separation can be based on size, electric charge, hydrophobicity or bio specific recognition. The method can be highly selective.

Theory

Aqueous Two Phase systems are generally composed of a water solution of structurally distinct hydrophilic polymers (e.g. Polyethylene glycol , dextran) or of one polymer and certain salts (e.g. Ammonium Sulphate , Potassium Sulphate) . Above critical concentrations of these components, spontaneous phase separation takes place with each of the two resulting phases enriched with respect to one of the components.

Aqueous Two Phase systems containing two polymers have found wide application for the separation of most biological materials and systems containing a single polymer and salts have also proved useful in the separation of macromolecules (Albertsons 1986)

Success with Aqueous Two Phase systems depends on the ability to manipulate phase composition so as to obtain appropriate partition coefficients K and selectivity for the material of interest.

There are several ways to manipulate system composition so as to give phases with appreciably different physical properties and the three below are relatively important

(a) Choice of polymers, polymer concentration ,polymer molecular weight ;

(b) Choice of Salts and salt concentration

(c) Chemical modification of one of the polymers by attaching a ligand for which receptors exist on the material of interest. In the last case the resulting procedure is called affinity partitioning. Aqueous solution of most hydrophilic polymers such as starch , gelatine, or agar slow incompatibility in binary mixtures and form two immiscible Aqueous phases , each containing primarily only one of the two phase forming polymers and a high proportion of water. At low concentrations of the polymers, homogeneous solutions (single phase) are obtained, but at discrete concentration ratios, measurable by cloud point method, phase

separation occurs. The mutual insolubility of the two hydrophilic polymers may be attributed to the molecular form of each polymer, which results in mutual repulsion.

1 Aqueous Polymer Two Phase systems

Aqueous liquid – liquid two phase system are formed when two polymers are dissolved together above certain concentrations. The most characteristic feature of these two phase systems is that both phases are aqueous (water content 85 to 99%), allowing partition of bio macromolecules and cellular particles of diverse origin under non denaturing conditions.

POLYMER	**POLYMER / LOW MOLECULAR WEIGHT SUBSTANCE**
1. Polymer – Polymer System	
Polyethylene glycol PEG	Dextran Polyvinyl alcohol PVA Poly vinyl pyrrolidone PVP
Polypropylene glycol PPG	Ficoll Dextran Polyvinyl alcohol PVA
Polyvinyl alcohol PVA	Poly vinyl pyrrolidone PVP
Poly vinyl pyrrolidone PVP	Dextran Methyl cellulose
2 **Polymer / Low Molecular Weight Substance Systems**	
PEG	Potassium Phosphate
Poly vinyl pyrrolidone	Potassium Phosphate
Polypropylene glycol PPG	Glucose
Dextran	Propyl alcohol
Sodium Dextran sulphate	Sodium Chloride
3. Poly electrolyte - Non ionic Polymer + Salt systems	
Sodium dextran sulphate	PEG and Sodium chloride PVA and Sodium chloride
Sodium Carboxy methyl cellulose	PEG PVA

Several pairs of the polymers can be used for forming aqueous two phase systems.

2. Polymer –Salt Two phase Systems:

Many polymers form two phase liquid liquid systems when combined with suitable salts (e.g Phosphates or sulphate). Because of their low costs and short setting time. Polyethylene glycol /salt systems have found use for technical extraction of enzymes.

3. Partition

The partition of a solute (e.g. a protein) between the phases is described by a partition coefficient K defined as the ratio between the concentrations of solute in the upper and lower phase.

Calculation:

$$\% \text{ PEG} = \frac{(\text{Weight of PEG Solution} \times \% \text{ of PEG in the solution})}{\text{(Total weight of the mixture)}}$$

$$\% \text{ Salt} = \frac{(\text{Weight of Salt Solution} \times \% \text{ of Salt in the solution})}{\text{(Total weight of the mixture)}}$$

SAMPLE	O.D at 540 nm	Protein concentration(µg/ml)
Blank		
Top Phase	0.20	80
Bottom Phase	0.37	145

Partition coefficient = $\frac{\text{Conc of protein in top phase}}{\text{Conc of protein in bottom phase}}$

Procedure:

1. Prepared 35% (w/w) Ammonium sulphate (i.e. 35 g of salt in 65 g of water)
2. Prepared 50% of PEG 400 (i.e 20g of PEG in 20ml of water)
3. Prepared 100ml of BSA solution of concentration 2mg/ml
4. Diluted the stock solution to get different concentrations of BSA and prepared a standard graph by plotting Absorbance vs concentration.

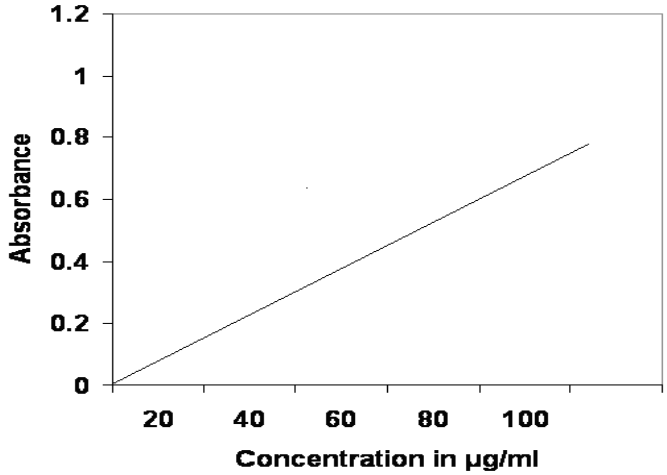


Table:

S.NO	Protein concentration(µg/ml)	O.D at 540 nm
1		
2		
3		
4		
5		

5. Created the aqueous two phase system by adding the salt solution into a separating funnel containing a known volume of PEG solution .The volume of the salt solution was noted
6. The mixture was shaken vigorously and allowed to settle down for some time to obtain the two phases in the separating funnel.
7. 2ml of the bottom phase was drained out and kept in a test tube and 2ml of the top phase was carefully decanted and kept in another test tube. These were used as blanks
8. Contacted the protein with the two phases by adding 10 ml of the stock solution into the separating funnel.
9. The separating funnel was left undisturbed for 10 minutes for the protein to partition between the two phases.

10. The optical density was measured for both the phases at 540 nm and the corresponding concentrations were obtained from the standard graph and the partition coefficient was calculated.

Result: The partition coefficient of the protein in the PEG/ Potassium phosphate system =0.55

EXPT NO: 7

AFFINITY CHROMATOGRAPHY

Aim:

To purify IgG from Human Serum

Principle:

The technique of affinity chromatography exploits the formation of specific and reversible complexes between a pair of biomolecules. One of the pair is called the ligand and usually Immobilized onto a stationary phase while the other, called a counter ligand, is adsorbed From the extract that is passing through the chromatographic column containing the immobilized ligand. The specificity or affinity exhibited by a ligand to the counter ligand is due to a combination of different types of interactions such as vanderwaals forces, hydrogen bonding and hydrophobic interactions.

In practice, the ligand is usually attached covalently immobilized to a water insoluble Polymer stationary phase or gel such as agarose, dextran, cellulose, polyamide or porous Glass to form a tailor made chromatographic adsorbent material suitable to adsorb specifically the desired components from a mixture. The adsorbent is packed into a column and the Sample mixture is applied to the adsorbent bed facilitating the adsorption of the desired component.

AC separates proteins on the basis of a reversible interaction between a protein (or a group of proteins) and a specific ligand attached to a chromatographic matrix.

The technique is ideal for a capture or intermediate step and can be used whenever a suitable ligand is available for the protein (s) of interest. AC offers high selectivity, hence high resolution, and usually high capacity for the protein(s) of interest. The target protein(s) is specifically and reversibly bound by a complementary binding substance (ligand). Unbound material is washed away, and the bound target protein is recovered by changing Conditions to those favoring desorption. Desorption is performed specifically using a competitive ligand, or non specifically by changing the pH, ionic strength or polarity. Samples are concentrated during binding and protein is collected in purified, concentrated form. The key stages in separation are shown in figure. Affinity chromatography is also used to remove specific contaminants, for example Benzamidine Sepharose 6B removes serine proteases.

The purification of the protein may be confirmed by measuring their specific activity through kinetic assay.

Materials Required: FPLC system, HITRAP r protein affinity column. Human Blood sample, Beakers, distilled water, Sodium dihydrogen phosphate, Sodium hydroxide, Citric acid and trisodium citrate.

Procedure:

Buffer preparation:

Start buffer: (0.02 M of phosphate buffer at pH of 7.0)

1. 0.2 g of Sodium hydroxide was dissolved in 250 ml of distilled water to give 0.02 M NaOH
2. 0.78 g of Sodium dihydrogen phosphate was dissolved in 250 ml of distilled water to give 0.02 M NaH2PO4.
3. The two solutions were mixed in the ratio 3:7 to give 0.02 M of phosphate buffer at pH of 7.0.

Eluting buffer: (0.1 M Citrate buffer at pH of 3-4.)

1. 3.84 g of citric acid was dissolved in 200ml of distilled water to give a 0.1M Citric acid
2. 1.47 g of trisodium citrate was dissolved in 50 ml of distilled water to give 0.1 M trisodium Citrate.
3. The two solutions are mixed in the ratio 15:5 to give 0.1 M Citrate Buffer at a pH of 3.5.

Procedure:

Collection of Blood sample:

Using Sterile syringe blood sample of about 2.5 ml was collected from a healthy animal or Human source. To the sample an anticoagulant was added immediately say 3% Tri sodium citrate.

Serum Separation:

Centrifuged the above sample at 2500 -3000 rpm for 5 minutes at 4 degree centigrade.

Collected the supernatant in a sterile eppendorf. The samples can be stored at 4 degree centigrade.

Desalting of the sample:

The supernatant solution was desalted by passing through a desalting column. The desalted Supernatant solution containing the plasma proteins was then used for the purification of IgG Using a r protein A Affinity column which is a specific for IgG.

The following steps were carried out in the desalting of the plasma protein.

Procedure:

1. The column was equilibrated with the buffer solution-A or with distilled water. 5 column Volumes i.e. 25 ml of the buffer was used for equilibration. The flow rate was set at 5ml/min gradient off and concentration% of solution-B was set as zero.

fraction size was set as zero.

2. Nearly, twice the sample loop volume (1ml) of the sample with salt was injected manually,
3. Auto zero was then set.
4. The flow rate was reduced to1ml/min
5. Fraction size was set at 0.5 ml
6. The valve was then changed to injection mode

The protein sample gets eluted first separated from the salt and was collected in test tubes of the fraction collector, protein is indicated as the first peak followed by the salt which is shown as the second peak in the chromatogram.

Separation of IgG:

The following steps were carried out in the separation of IgG from the desired plasma proteins by Affinity chromatography.

1. Firstly the column was equilibrated with the buffer without the salt i.e.
   Start buffer 0.2 M phosphate buffer to 5 column volumes i.e. 5 ml by keeping the flow rate Set to 5ml/min. gradient off, conc% B (0) fraction size set to zero.
2. The sample was then injected with the salt manually approximately to twice the sample loop Volume (1ml)
3. Set auto Zero
4. Reduced the flow rate to 0.2ml/min
5. Set the fraction size to 0.5 ml
6. Changed the valve to injection mode and allowed it to flow for 5 min the sample was then injected into the column and the unbound proteins which do not interact specifically with the column gets washed away from the column by the start buffer.
7. Changed the valve to load mode.
8. Kept the tubes A and B in the start buffer and the elution buffer respectively and allowed it to flow.
9. Solvent programming was then done by setting the gradient
   Set gradient on, Set length to 30 ml, Set target conc %B to 100% Set flow rate 1ml/min.
10. As the % concentration of B increased the pH decreased and at a lower pH the interactions between the bound protein and the column got weakened and at an appropriate pH the bound protein (IgG) eluted from the column which was collected in a test tube in the fraction collector.

Result: Thus IgG was purified from Human Serum by Affinity Chromatography.

EXPT NO: 8

ION EXCHANGE CHROMATOGRAPHY USING CHROMATOGRAPHY SYSTEM

Aim: To purify Bovine Serum Albumin (BSA) by ion exchange chromatography using chromatography system.

Principle: Ion exchange chromatography is based on the differences in the binding capacity of charged sample molecules to oppositely charged groups attached to an insoluble matrix. This binding is electrostatic and reversible. The pH value at which a biomolecule carries no net charge is called isoelectric point (pI). When exposed to a pH below its pI the biomolecule will carry a net positive charge and will bind to a cation exchanger (SP and CM) .At a pH above its pI, the bio molecule will carry a net negative charge and will bind to an anion exchanger (Q, DEAE and ANX) .If the sample components are most stable below their isoelectric points an cation exchanger is used. If the sample components are most stable above their isoelectric points an anion exchanger is used. In Ion exchange chromatography the elution is based on gradient elution i.e. the composition of the eluant is changed by gradually increasing its salt concentration and when the salt concentration increases significantly the salt starts to replace The protein from its binding sites in the column and the protein gets eluted from the column.

In the purification of BSA we know that its pI is less than 6.0 and it is stable in the alkaline pH.So we choose a buffer whose pH is more than the pI of BSA and hence use an anion exchanger as column.

The purification of the protein may be confirmed by measuring their specific activity through kinetic assay.

Materials Required: FPLC system, HITRAP DEAE FF column or HITRAP Q FF column, Beakers.

Chemicals Required:

(1) Distilled water
(2) Bovine Serum Albumin (BSA)
(3) Sodium hydroxide
(4) Sodium chloride
(5) Sodium dihydrogen phosphate.

Preparation of necessary solutions:

1. Dissolved 2 g of Sodium hydroxide in 250 ml of distilled water to give (0.2M) solution.(A)
2. Dissolved 7.8 g of Sodium dihydrogen phosphate in 250 ml of distilled water to give (0.2.M) solution (B)

3. The two solutions A and B were mixed in proper proportions to give a resulting pH of 8.0 Solution C which was termed as the start buffer
4. Dissolved 17.53 g of Sodium chloride in 150 ml of distilled water to give 2M NaCL solution (D)
5. Dissolved 0.1 g of BSA in 50 ml of the start buffer this was the sample solution.
6. Mixed 150 ml of the start buffer with 150 ml of solution D to give the eluting buffer. The eluting buffer after mixing consisted of the start buffer plus 1M salt solution..

Procedure:

1. Firstly the column was equilibrated with the buffer without the salt i.e. Solution C to 5 column volumes i.e. 5 ml by keeping the flow rate Set to 1ml/min. gradient off, concentration% B (0) fraction size zero.
2. The sample was then injected with the salt manually approximately to twice the sample loop Volume (1ml)
3. Set auto Zero
4. Reduced the flow rate to1ml/min
5. The fraction size was set to 0.5 ml
6. The valve was changed to injection mode and flow was allowed for two column volumes the sample got injected into the column.
7. Changed the valve to load mode.
8. The tubes A and B were kept in the start buffer and the elution buffer respectively and Allowed to flow
9. Solvent programming was then done by setting the gradient Set gradient on, The gradient length was set to 30 ml, the target concentration was set %B to 100% The flow rate was set to2ml/min.

As the % concentration of B increased the concentration of the salt increased and at a higher concentration of the salt in the eluting buffer the salt replaced the protein in the column and the protein got eluted from the column which was collected in a test tube in the fraction collector.

Result: Thus the protein was purified using Ion exchange chromatography.

EXPT NO: 9

ION EXCHANGE CHROMATOGRAPHY USING COLUMN

Aim: To estimate the amount of protein precipitate present in the sample (egg) and thereby purifying lysozyme using carboxy methyl cellulose.

Principle

The basic principle is that oppositely charged particles are attracted to each other. The stationery phase consists of fixed charges on a solid support. These charges can be either negative or positive charged molecules. Ex Carboxyl groups and attracts negatively charged molecules by DEAE Cellulose.

Proteins are complex ampholytes can be separated from a mixture of compounds on the basis of net positive or negative charge that they carry. Isoelectric point of a protein is the pH at which its net charge is zero. Therefore proteins will have a net charge depending on the pH of solution and its is possible to use either an anion exchanger or a cation exchanger.

In Ion exchange chromatography, solution contain portion of interest is applied to the ion exchanger. Protein binding to the ion exchanger is dependent on net charge of the protein at that particular pH and on the ionic strength of the mobile phase. Bound protein is then eluted out from the stationery phase by increasing the concentration of counter ions or by changing pH which alters the change on the protein. Weakly charged protein is displaced from the stationery phase with lesser concentration of counter ions than highly charged protein. Thus proteins are separated based on net charge.

The purification of the protein may be confirmed by measuring their specific activity through kinetic assay

Materials required

CM Cellulose, pH 7 10X equilibration buffer, ph 9 10x wash buffer. 5X elution buffer, Neutralsing solution, 5 M sodium chloride, pH 7, 0.5 M phosphate buffer. Micrococcus species, Lysozyme solution, standard column tube for mixing.

Procedure

1. Washed the column with 90oc hot water.
2. Fixed the column to the stand.
3. Removed the top cap of the column and packed the column with 2.5 ml of carboxy methyl cellulose.
4. Removed the bottom cap and equilibrated the column with 50 ml of 1 x equilibration buffer.
5. Broke an egg and collected the egg white separately.

6. To 6 ml of egg white an equal volume of distilled water was added and mixed in the tube provided to make a homogenous solution.

7. Adjusted the pH of the egg white to pH 7 by slowly adding neutralizing solution. The egg white turned slightly turbid.

8. Centrifuged the egg white solution at 6000 rpm for 10 min. and collected the supernatant.

9. Loaded the supernatant to equilibrate CMC column.

10. Replaced the top and bottom cap of the column and incubated for 1 hr at room temp with intermittent mixing.

11. After an hour, the column material was allowed to settle slowly.

12. The supernatant was decanted without disturbing the column.

13. The column was washed with approximately 30- 40 ml of 1X wash buffer.

14. Lysozyme was eluted from the column using 15 ml of 1X elution buffer.

15. The eluate was collected in test tubes as 2 ml fractions.

15. Read the OD at 280 nm. Pooled the fraction that shows a280= 0.5,

16. Washed the column with 10 ml of 1M NacL.

17. Replaced the top and bottom cap and stored the column at 4°c for next use.

Result

Lysozyme was purified

EXPT NO: 10

DESALTING OF PROTEIN BY GEL FILTRATION USING CHROMATOGRAPHY SYSTEM

Aim: To separate protein from salt using chromatography system

Principle: The process of separation of salt from protein is termed as desalting. In Gel filtration the separation of species is based on the size of the species. It is essentially a process of molecular sieving. The proteins have higher molecular weight than the salt and hence their sizes are larger than the size of the salt. This difference is utilized in the separation of the protein from the salt. In this process the mixture of protein and salt is introduced into a column and then eluted. Inside the column the larger protein molecules will not able to get inside the pores of the column and hence gets eluted through the interspaces between the column molecules. On the other hand the smaller salt molecules can pass through the pores of the column material and travels for a longer time. Hence when the elution process begins, the higher molecular weight proteins which are present outside the pores get eluted first by the eluant, while the low molecular weight salts which are inside the pores are eluted after.

The purification of the protein may be confirmed by measuring their specific activity through kinetic assay..

Materials Required: FPLC system, HITRAP Desalting column, Beakers,

Chemicals Required:

(1) Distilled water
(2) Bovine Serum Albumin (BSA)
(3) Sodium hydroxide
(4) Sodium chloride
(5) Sodium dihydrogen phosphate.

Preparation of necessary solutions:

Solution A:

0.36 g of Sodium hydroxide was dissolved in 90 ml of distilled water (0.1M) solution.

3.27 g of Sodium dihydrogen phosphate was dissolved in 210 ml of distilled water (0.1.M)

The two solutions were mixed to get 300 ml of 0.1M Buffer solution-A

Solution B:

17.53 g of Sodium chloride was dissolved in 300 ml of distilled water (1M NaCL).

Solution C:

150 ml of solution-A and 150 ml of solution-B were mixed to give 300 ml of the binding buffer (0.05 M Phosphate buffer and 0.5M NaCL).

The solution-A was used as the start buffer for column equilibration and column elution.

Note: Distilled water can also be used for this purpose.

Sample solution:

100 ml of the solution-C was mixed with 0.2 g of BSA to give 2mg/ml of solution of BSA.

Procedure:

1. The column was equilibrated with the buffer solution-A or with distilled water. 5 column Volumes i.e. 25 ml of the buffer was used for equilibiration. The flow rate was set at 5ml/min gradient off and concentration% of solution-B was set as zero. fraction size was set as zero.
2. Nearly, twice the sample loop volume (1ml) of the sample with salt was injected manually,
3. Auto zero was then set.
4. The flow rate was reduced to1ml/min
5. Fraction size was set at 0.5 ml
6. The valve was then changed to injection mode

The protein sample got eluted first separated from the salt and was collected in the test tubes of the fraction collector. The protein is indicated as the first peak and the salt as the second peak in the chromatogram.

Result: The protein was thus separated from the salt.

EXPT NO: 11

GEL FILTRATION CHROMATOGRAPHY USING COLUMN

Aim: To separate biomolecules on the basis of their size by gel filtration chromatography.

Principle:

Gel Filtration chromatography or Size exclusion chromatography or Gel permeation chromatography separates molecules based on the difference in their size. The sample is applied on the top of the column containing porous beads. As the molecules pass through the column of porous beads of cross linked agarose, they get separated. Large molecules cannot enter the pores and elute as a first peak in the chromatogram. They elute first and this is called "total Exclusion" Intermediate particles may enter the pores and may have an average residence time in the particles depending on their size and shape. Different molecules have different total transit times through the column. This portion of a chromatogram is called the "Selective permeation region" Small molecules enter the pores and have the longest residence time in the column and elute together at last in the chromatogram. This is called "total permeation limit".

The purification of the protein may be confirmed by measuring their specific activity through kinetic assay.

Materials required:

Gel filtration column Gel filtration buffer, sample.

Procedure:

1. Fixed the column vertically to a stand.
2. Equilibrated the column with 4 ml of Gel filtration buffer.
3. Drained out the buffer completely.
4. Loaded 0.2 ml of sample along the sides and onto the column.
5. Allowed the sample to sink completely and then 2 ml of buffer was added.

6 Allowed the buffer to flow out completely.

7. Kept filling the column with buffer till all the colored biomolecules were eluted out.
8. Collected the colored fractions in different tubes.

Result:

Noted down the order in which various molecules were collected and interpreted the result.

Interpretation:

The brownish red color component collected as the second component was Hemoglobin having a molecular weight of 64.5 kDa. The Blue colored component was blue dextran having a very

large molecular weight, molecular weight of 2000 kDa and hence exited fast from the column and was collected as the first fraction. The Pink Colored component was vitamin B12 having molecular weight of 376 Da. They are relatively small molecules and hence took a long time to exit the column and were collected as third fraction.

EXPT NO : 12

LYOPHILIZATION (FREEZE DRYING OF CULTURE)

Aim: To study the principles and applications of lyophilization

Principle: Lyophilization can be defined as a stabilizing process, in which a substance is first frozen then the quantity of solvent is reduced, first by sublimation and then by desorption to values that can no longer support biological or chemical activity.

Lyophilization is typically used to preserve biological product. It eliminates the need for refrigerated storage and reduces the product weight to lower shipping costs, and produces a dry substance that reconstitutes readily. This method is used for drying food, blood plasma and pharmaceuticals without destroying their physical structure.

In the lab, this process enables the drying of aqueous preparations by judicious equilibrium between pressure and temperature while maintaining the integrity of living cells such as bacteria, complex delicate structures such as enzymes, proteins or unstable chemical molecules. New biotechnology products will continue to increase the demand for freeze drying equipment when lyophilization becomes an alternative for cryopreservation.

Procedure:

There are three steps in the Lyophilization process

1. Freezing

2. Primary drying

3.Secondary drying

Freezing: The products or samples are cooled to a temperature below its eutectic point in a temperature controlled shelf within the freeze dryer. After complete freezing the pressure in the dryer is lowered to a defined pressure to initiate primary drying

Primary drying: (Sublimation): During this stage, water vapor is progressively removed from the frozen mass by sublimation where the shelf temperature is maintained at a constant low temperature. During the primary drying, heat is transferred from the shelf and the chamber walls to the drying front mainly by conduction and radiation. Ice sublimes leaving behind, the porous matrix of the initial structure. The water vapors formed pass through the dried cake to the surface and then through the dried chamber to the condenser. A high drying temperature results in a higher saturated vapor pressure, and hence a higher driving force for the flow of water vapor.

Accordingly, this shortens the cycle of lyophilization. The upper limit to the drying temperature during the primary drying stage is to ensure that the product temperature is maintained below the system collapse temperature.

Secondary drying (desorption): This stage is initiated by an increase in the shelf temperature and reducing the chamber pressure so that water absorbed to the semi dried mass can be removed until the residual water content decreases to the desired level. Secondary drying involves the removal of water that did not separate as ice during freezing. It is difficult to transfer energy into a porous matrix placed under a vacuum since it behaves like a perfect insulator. Therefore the secondary drying stage takes just as long as a primary stage, even though much lesser water is removed

Result: The principle and operation of lyophilization was studied.

EXPT NO: 13

EXTRACTION OF CASEIN FROM MILK BY DIELECTRIC PRECIPITATION

Aim: To precipitate casein from milk using Acetone

Principle:

The precipitation of casein from milk using an organic solvent like acetone is known as solvent mediated precipitation. In this type of precipitation, the dielectric constant of the solvent medium is changed because of the addition of acetone. Water (Solvent) has a high dielectric constant, and it can keep the oppositely charged groups separated efficiently. When a water soluble organic solvent like acetone of low dielectric constant is added, the overall dielectric constant of the solvent medium gets decreased.As a result the electrostatic interactions between the oppositely charged groups increases, leading to agglomeration and hence precipitation of casein. .

Materials Required:

Acetone, Distilled water, Beaker, Conical flask, Filter paper, Milk, Ethanol, Ether, Buchner funnel, Watch glass and Folins reagent.

Procedure:

100 ml of milk was taken in a glass beaker and warmed slightly then acetone was added to the milk slowly in small increments with constant stirring by keeping the beaker in ice. The volume of acetone added after each addition was noted. The addition and stirring was stopped when the precipitate started to form. The beaker was then kept aside for 10 min for the precipitation process to get completed. The precipitate was then collected by filtering. The precipitate was then washed several times with a small amount of water. The precipitate was then suspended in about 30 ml of ethanol for 10 min. The suspension was then filtered on a buchner funnel and the precipitate was washed a second time with a mixture of equal volumes of ethanol and ether. Finally the precipitate was washed on a filter paper with 50 ml of ether and suction was applied to dryness. The powder was removed and spread out on a watch glass to allow for the evaporation of the ether. The final weight of Casein was noted

Confirmation Test:

Confirmation for the presence of protein is as follows, protein reacts with Folin Cicoalateu Reagent to give a colored complex. The color so formed is due to the reaction of the Alkaline copper with the protein and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Reagents Required:

1. Alkaline sodium carbonate solution (20g/l of Na2CO3 in 0.1 mol /lit of NaOH0
2. Copper sulphate Sodium potassium tartarate solution. (5g of CuSO4.5H2O in 10g/lit of Na, K tartarate) Prepared fresh by mixing stock solutions.
3. Alkaline solution Prepared on day of use by mixing 50 ml of (1) and 1mlof (2).
4. Folin Cicoalateu Reagent .Diluted the commercial reagent with an equal volume of Water on the day of use.

Method:

5 ml of the Alkaline solution was added to 1ml of the test solution of concentration 0.2 mg/ml. And mixed thoroughly. The solution was then allowed to stand at room temperature for 10 min or longer. 0.5 ml of diluted Folin Cicoalateu Reagent was added rapidly with immediate mixing. The appearance of Blue color indicated the presence of protein.

Calculation:

Weight of Watch glass W1 =31.02g

Weight of Watch glass + Dry Casein W2 = 34.83g

Weight of Dry Casein W2 - W1 = 3.81g

Result:

1. The weight of Casein precipitated in 100 ml of milk = 3.81 g
2. The volume of acetone added to cause the onset of precipitation 60 ml

EXPT NO: 14

EXTRACTION OF CASEIN FROM MILK BY ISO ELECTRIC PRECIPITATION.

Aim: To extract Casein from milk by Iso electric precipitation

Principle: The solubility of most proteins, especially the globular proteins is influenced greatly by the pH of the aqueous medium. The pH at which a protein is least soluble is at its isoelectric pH wherein the net charge on the protein molecule is zero .Under these conditions there is no electrostatic repulsion between neighboring protein molecules and they tend to coalesce and precipitate. However it should be noted at pH values above or below the isoelectric point, all protein molecules have a net charge of the same sign, thus they repel each other, preventing the coalescence of single molecules to insoluble aggregates. Casein the principle phosphoprotein of milk is separated from other proteins by isoelectric precipitation, i.e. By adjusting the pH of milk to its isoelectric pH (4.8).

Reagents:	**Equipments**
(i) 0.5 N HCL	(i) pH meter
(ii) Ethanol	(ii) Filtration unit
(iii) Diethyl ether	(iii) Top loading Balance
(iv) Cow's milk	(iv) Centrifuge

Procedure:

(i) Centrifuged 100 ml of milk at 4000rpm for 20-25 min at room temperature and carefully Removed the cream /fat from the surface.

(ii) Transferred this skimmed milk into a 500ml beaker. An equal volume of distilled water was then added and stirred. Its pH was then noted.

(iii) 0.5 N HCL was added drop wise to the diluted milk and the pH was brought to 4.8 with constant stirring at this pH, casein started to precipitate. The precipitate was allowed to settle at room temperature for 30 min.

(iv) The supernatant was carefully decanted and the suspension filtered using a filtration unit connected to a suction pump with a Whatman NO.1 filter paper disc.

(v) The moist precipitate was washed thrice with 100 ml of distilled water to remove the salts. This was followed by two washes each with 100 ml of ethanol and diethyl ether.

(vi) The cake was then transferred from the funnel onto a watch glass, the material was spread uniformly and dried.

(vii) The weight of the dried casein sample was recorded.

Confirmation Test:

The confirmatory test was carried out similar to Ammonium sulphate precipitation

Calculation:

Weight of Watch glass W1 = 31.02 g

Weight of Watch glass + Dry Casein W2 = 34.53 g

Weight of Dry Casein W2 - W1 = 3.51 g

Result:

1. The weight of Casein precipitated in 100 ml of milk = 3.4g
2. The isoelectric point of casein = 4.8 pH

EXPT NO: 15

ISOLATION OF CHOLESTEROL AND LECITHIN FROM EGG YOLK

Aim : To isolate Cholesterol and Lecithin from egg yolk

Principle: Egg yolk is a good source of Cholesterol and phospholipids eg .Lecithin. These lipids can be isolated from yolk by fractionation with organic solvents.

Sample: Hens egg

Reagents:

1) Acetone, 2) Petroleum Ether

Procedure:

(i) A boiled egg was taken and the egg yolk (yellow colored) was collected carefully into a 150 ml glass beaker. 40 ml of acetone was added and the contents were stirred with a glass rod. The solids were allowed to settle. The acetone extract was decanted into a 100 ml glass beaker. Repeated the acetone extraction Once again. The acetone extracts containing Cholesterol were pooled into a glass container and stored.

(ii) Removed the acetone present in solid residual fractions by evaporation over a steam bath (under a fume hood).Cooled the container and added 30 ml of cold petroleum ether extract containing Lecithin in a glass container. Repeated the extraction procedure once again and pooled the extracts.

(iii) Evaporated individually both the extracts over a steam bath, using a China dish In small volumes in a fume hood Crude Cholesterol precipitated as light yellowish crystals, while Lecithin separated as yellowish brown waxy liquid.

Confirmatory test for Cholesterol:

Libermann Burchard Test:

To 2ml of Cholesterol (0.5%w/v) in chloroform taken in a test tube an equal volume of Acetic anhydride was added followed by addition of 2-3 drops of concentrated sulphuric acid. Vortexed the contents. The development of green color confirmed the presence of Cholesterol.

Result: Thus Cholesterol and Lecithin were isolated from egg yolk

EXPT NO : 16

BATCH ADSORPTION

Aim : To separate the product by means of adsorption on the given adsorbent material in a simple adsorption column system.

Principle:

Adsorption is a reversible phenomenon occurring at the surface of a solid. The forces of adsorption are mainly physical and are not strong. Hence desorption of the adsorbate is feasible in physisorption. In contrast chemisorptions lead to irreversible adsorption and practically have no application in bioseparations.

The advantages of adsorption process in bioseparations are several. It may be used for primary isolation as well as for concentration of desired products. Since adsorption is highly selective, the desired product may be directly from fermentation broths without the use of preliminary filtration or centrifugation step. Adsorption does not denature sensitive biomolecules and hence is preferred for the isolation of proteins. However, adsorption capacity of any adsorbent is generally small and in addition the design of adsorption process is complicated by non linear equilibrium and sometimes by strong adsorbent – adsorbate interactions.

Solid adsorbents conventionally used in bioseparations include activated carbon of vegetable origin, clay minerals, natural and synthetic zeolites, molecular sieves, alumina, silica gel and ion exchange resins based on synthetic polymers made from styrene and cross linked divinyl benzene. Typically the adsorbents are in the form of small pellets, beads or granules in the range of 0.1-12 mm in size with larger sized particles used in packed beads. The chosen adsorbent must be mechanically stable with relatively a large surface area and chemically inert towards the solute as well as the solvent.

Batch adsorption is used to adsorb solutes from the liquid phase when the quantities treated are relatively in small amounts.

The freundlich adsorption isotherm is given by the equation $\mathbf{C_\alpha = Ks^n}$

Where $C\alpha$ = Amount of solute adsorbed /Amount of adsorbent used

S = concentration of feed solution

K and n are freundlich constants.

The Congo red –Charcoal system follows the freundlich isotherm

Materials Required:

1. Congo red 2. Charcoal 3. Conical flasks 4. Shaker 5. Spectrophotometer

Procedure:

Preparing the standard curve for Congo red solution.

Stock solution:

Weighed 500mg of Congo red mixed it with 1000 ml of distilled water to obtain a concentration of 500 ppm.

Working Standard solution:

Took 5 test tubes and filled it with 2,4,6,8, and 10 ml of stock solution respectively. Made up the test tubes up to 10 ml with distilled water. The concentrations are 100, 200, 300, 400 and 500 ppm respectively.

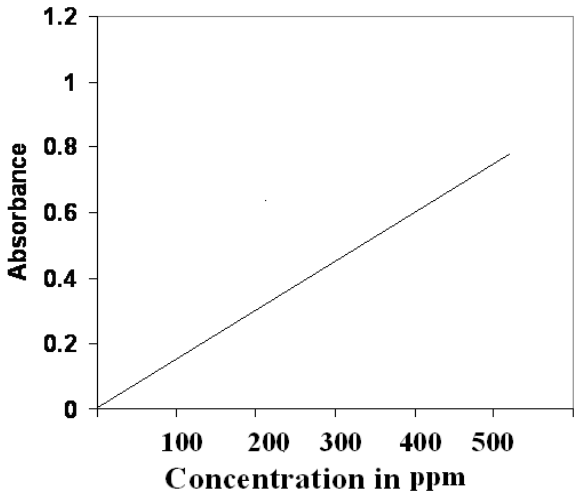
Absorbance Measurement:

Distilled water was used as blank and the absorbance for all the solutions were measured at 620 nm using a Spectrophotometer

Table:

S.NO	Concentration in ppm	Absorbance at 620 nm
1		
2		
3		
4		
5		

Plotted the graph between Absorbance and concentration for the Congo red solution



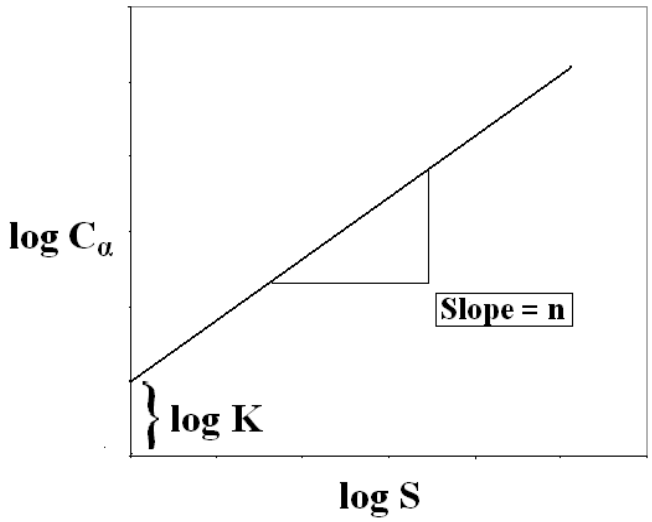
Congo red- Charcoal System :

1. Prepared 50 ml solutions of concentration (100, 200, 300,400,500 ppm) of Conge red.
2. Weighed 0.1 g of Charcoal and added inside 5 conical flasks separately.
3. The prepared solutions of Congo red were then added into these conical flasks and closed.
4. The flasks were kept in the shaker for 30 minutes
5. After the shaking process the solutions were filtered using a filter paper.
6. The absorbance values of the filtrate were measured at 620 nm in the spectrophotometer.

Table:

Initial Conc. C1ppm	O.D of filtrate At 620 nm	Final Conc. C2 ppm	Amount of adsorbent A g	$C_\alpha = \frac{(C1-C2)}{A}$	S ppm	log S	log C_α
100							
200							
300							
400							
500							

Plotted the graph between log $C\alpha$ **and** **log S**



The intercept in the graph gives log K and the slope of the graph give n

Thus the Freundlich Constants can be determined K = 7.41, n = 1

Result: The Freundlich isotherm for the Congo red Charcoal system is $C\alpha = 7.41\ S^{1}$