AVIT
AARUPADAI VEEDU INSTITUTE OF TECHNOLOGY
VINAYAKA MISSION'S RESEARCH FOUNDATION
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DEPARTMENT OF BIOTECHNOLOGY

B.TECH

DOWNSTREAM PROCESSING ENGINEERING LABORATORY MANUAL

(17BTCC92)

HOD

CONTENTS

LABORATORY RULES AND REGULATIONS

There are some rules which should be followed for the successful completion of the lab experiments, safety of the student and convenience of others working in the lab

(i) Upon entering the laboratory, place bags, books and other materials in specified locations. Never on bench tops
(ii) Always wear a lab coat before entering the laboratory for protecting clothes from Contamination or accidental discoloration by staining solutions
(iii) Keep the lab windows and doors closed during laboratory sessions to prevent contamination from air currents
(iv) Before and after each lab session wipe your work bench with a disinfectant like Lysol or ethanol
(v) Do not place contaminated instrument such as inoculation loop, needles and pipettes on bench top
(vi) Wash your hands with soap and water upon entering and prior to leaving the lab
(vii) Do not smoke eat or drink in the lab. These activities are absolutely prohibited
(viii) Tie back long hair to minimize its exposure to open flame
(ix) If a live culture is spilled, cover the area with a disinfectant solution for 15 min and then clean it
(x) In the event of personal injury such as cuts or burns, inform the instructor immediately. Open cuts and wounds should be covered
(xi) All microbial culture should be handled as potential pathogen
(xii) Never pipette any broth cultures or chemical reagents by mouth
(xiii) Always keep culture in an upright position in the test tube rack to carry culture when moving around the lab
(xiv) Familize with the exercise to be performed
(xv) Always perform the experiments to be sequence. Handle the apparatus and equipment carefully
(xvi) Label all plates tubes and cultures properly before starting the experiment
(xvii) As you perform the experiment, record your data in the notebook
(xviii) Materials such as stains, reagent bottle test tubes petriplates pipettes must be returned to the original location after use
(xix) Speak softly and avoid unnecessary movements around the lab to prevent distraction that may cause accidents
(xx) Always clean microscope stage, eyepiece and objective lens before and after use
(xxi) On completion of lab, session, place all the cultures and materials in disposal area as designated by the instructor

INTRODUCTION TO DOWNSTREAM PROCESSING IN BIOTECHNOLOGY

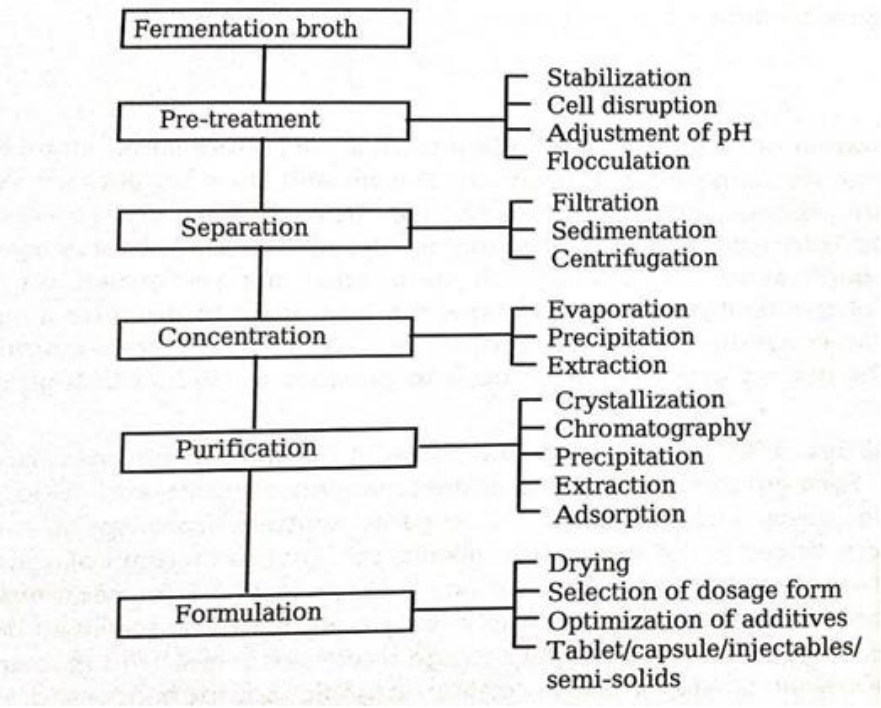
- Downstream processing refers to the recovery and purification of biologicals, biosynthetic products, particularly pharmaceuticals, from natural sources such as animal or plant tissue or fermentation broth

- It is an essential step in the manufacture of pharmaceuticals such as antibiotics, hormones (e.g. insulin and human growth hormone), antibodies (e.g. infliximab and abciximab) and vaccines; antibodies and enzymes used in diagnostics; industrial enzymes; and natural fragrance and flavor compounds.

- Downstream processing is usually considered a specialized field in biochemical engineering, itself a specialization within chemical engineering, though many of the key technologies were developed by chemists and biologists for laboratory-scale separation of biological products.

- Downstream processing implies manufacture of a purified product fit for a specific use, generally in marketable quantities, while analytical bioseparation refers to purification for the sole purpose of measuring a component or components of a mixture, and may deal with sample sizes as small as a single cell.

STAGES IN DOWNSTREAM PROCESSING



EXP NO: **DATE:**

CELL DISRUPTION BY SONICATION

AIM

To disrupt microbial cells by sonication at different time intervals and to measure the amount of protein released

PRINCIPLE

Ultra sound waves of frequencies greater than 20kHz rupture the cell walls by a phenomenon known as cavitation. The passage of ultrasound waves in a liquid medium creates alternating areas of compression and rarefaction which change rapidly. The cavities formed in the areas of rarefaction rapidly collapse as the area changes to one of compression. The bubbles produced in the cavities are compressed to several thousand atmospheres. The collapse of bubbles creates shock waves which disrupt the cell walls in the surrounding region. The efficiency of the method depends on various factors such as the biological condition of the cells, pH, temperature, ionic strength and time of exposure. Ultrasonication leads to a rapid increase in the temperature and to avoid heat denaturation of the product it is necessary to cool the medium and also to limit the time of exposure.

The treatment of microbial cells in suspension with inaudible ultra sound (greater than 18000 Hz) results in their inactivation and disruption. Ultrasonication utilizes the rapid sinusoidal movement of a probe within the liquid. It is characterize by high frequency (18 kHz -1 MHz), small displacements (less than 50µm); moderate velocities (a few ms-1), steep transverse velocity gradient (up to 4000 s-1) and very high acceleration (upto 80,000g).

In ultrasonication phenomena, when acoustic power input is sufficiently high will allow 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 cavitations. The collapse of the bubble converts sonic energy to mechanical energy in the form of shock waves equivalent to several atmospheric (300MPa) pressures.

This energy input imparts motions to parts of cells which disintegrate when their kinetic energy content exceeds the cell wall strength. An additional factor which increases cell breakage is the

micro streaming (very high velocity gradient causing shear stress) which occur, near radically vibrating bubbles of gas, caused by the ultrasound.

Much of the energy absorbed by all suspensions is converted to heat effectively, so cooling is necessary. The rate of protein released by mechanical cell disruption, usually sound to the

$$\frac{dP}{dt} = -KP$$

proportional to the amount of releasable protein.

Where P = protein content remaining in associated cellst = time

K= release constant dependent on the system.

Integrating from P = P_m (maximum possible protein release at time zero) to P = P_t at time't' gives

$$\int_{P_m}^{P_t} \frac{dP}{P} = -K\int_0^t dt$$

$$\Rightarrow \ln\left(\frac{P_m}{P_t}\right) = Kt$$

As protein (Pr) released from the cells is given by Pr =P_m-P_t , the followingequation for cell breakage is obtained

$$\ln\left(\frac{P_m}{P_m - Pt}\right) = Kt$$

$$K = \ln (1\text{-}R)^{-1} / t$$

.

The constant (K) is independent of cell concentration up to high levels approximately proportional to the acoustic power above the threshold necessary for cavitations. Equipment for large-scale continuous use of ultrasonic has been available but not found extensive use in enzyme production. Reasons for this may be the conformational liability of some enzymes to sonication and the damage that they may realize through oxidation by free radicals, singlet oxygen, and hydrogen peroxide that may be concomitantly produced. Use of radical scavengers (eg.N_2O) has been shown to reduce this inactivation. As with most cell breakage methods, very fine cell debris may be produced which can hinderfurtherprocessing

MATERIALS REQUIRED

Overnight grown bacterial culture, sonicator, Eppendorf tubes, ice cubes, UV-visible spectrophotometer, Alkaline copper sulphate solution, Folin's reagent.

PROCEDURE

1. Take 15 ml of bacterial culture in a centrifuge tube and centrifuge at10000 rpm for 5 minutes.
2. Discard the supernatant and resuspend the pellet in 10ml of distilled water.
3. Set the controller power 50% and frequency of 25 kHz.
4. Sonicate the sample.
5. At every 30 sec (30-180sec), transfer 1 ml of sample from the centrifuge to the 1.5 ml eppendorf tubes.
6. Centrifuge all the eppendorf tubes at 10000 rpm for 5 minutes.

PROTEIN ESTIMATION

1. Take 0.2 ml of supernatant and determine the amount of protein in the sample by Lowry's method
2. Plot the graph Conc (mg/ml) vs time (sec) and determine the maximum amount of protein released.

RESULT

Maximum amount of protein released (C_{max}) =…….

TABULATION

S.No	Sonication Time (sec)	OD at 595 nm	Conc of released protein (mg/ml)
1	30 sec		
2	60 sec		
3	90 sec		
4	120 sec		
5	150 sec		
6	180 sec		

CALCULATIONS

From standard graph, Conc (mg/ml) vs time (sec)

Maximum Concentration of protein released corresponding to highest absorbance value ……..

EXP NO: **DATE:**

CENTRIFUGATION STUDIES DURING SETTLING OF YEASTCELLS

AIM

To study the effect of:

i) Increasing speeds of centrifugation on the settling of the yeast cell particles.

ii) Increasing centrifugal times on the settling of yeast cell particles.

PRINCIPLE

Centrifugation is a basic separation technique used to separate material of different densities when gravitational force is insufficient for their separation. Normally a suspension of slowly under the influence of gravity. This process is called sedimentation. In centrifugation, the process of settling is added by centrifugal forces.

The centrifugal force, F is given by

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

Where m = mass of the particle on which it is acting, w = angular velocity, r = radius.

It has been observed that by fixing the type (density and size) of particles, to be settled, under the given study and also the type of rotor, the settling of a particle subjected to centrifugation depends on the speed of centrifugation as well as the time spent by the particles at that speed. Hence, varying these two parameters (keeping either constant) we can study centrifugation profiles of particles.

MATERIAL REQUIRED

Yeast, Centrifuge, UV-visible Spectrophotometer, Centrifuge tubes,

PROCEDURE

1. Prepare 100 ml of stock yeast solution by dissolving 2 g of yeast in 10 ml of warm water (distilled) and make it up to 100 ml using normal water (distilled) (Since yeast is not easily soluble in normal water use warm distilled water).
2. Pipette out 10 ml of the stock solution into 10 centrifuge tubes

3. The first 5 tubes are centrifuged for 5 minutes each, with varying centrifugal speeds of 1000, 1500, 2000, 2500, 3000 rpm. The O.D's of supernatant are recorded at 540 nm.
4. The next 5 tubes are centrifuged at 1500 rpm with varying times of centrifugation of 1, 2, 3, 4, 5 min respectively. Here also, supernatants are collected and optical densities are noted.
5. Graphs of OD_{540} vs rpm and OD_{540} vs Centrifugation time are plotted.
6. Based on these graphs, the perfect speed for centrifugation and duration for the same for efficient separation of all particles can be measured.

RESULT

The profiles of OD vs rpm and OD vs centrifugation time are plotted. the perfect speed for centrifugation and duration for the same for efficient separation of all particles was found to be…….

OBSERVATION AND TABULATION

Concentration	Speed (rpm)	Time(min)	Optical Density (540 nm)
2 g / 100 ml	1000	5	
	1500	5	
	2000	5	
	2500	5	
	3000	5	
	3500	5	

Concentration	Speed (rpm)	Time(min)	Optical Density (540 nm)
2 g / 100 ml	1500	1	
	1500	2	
	1500	3	
	1500	4	
	1500	5	

EXP NO: **DATE:**

FLOCCULATION

AIM

To clarify the given solution containing cell debris by using different flocculants and to determine the Debye radius of each flocculants.

PRINCIPLE

Flocculation is a process where colloids come out of suspension in the form of floc or flakes. The action differs from precipitation in that, prior to flocculation, colloids are merely suspended in a liquid and not actually dissolved in a solution. In the flocculated system there is no formation of a cake since all the flocs are in the suspension. Particles finer than 0.1 µm in water remain continuously in motion due to electrostatic charge often negative which causes them to repel each other. Once their electrostatic charge is neutralized by the use of coagulant chemical, the finer particles start to collide and agglomerate under the influence of Van der Waals's forces. These larger and heavier particles are called flocs.

Flocculants or flocculating agents are chemicals that promote flocculation by causing colloids and other suspended particles in liquids to aggregate, forming a floc. Flocculants are used in water treatment processes to improve the sedimentation or filterability of small particles. Many flocculants are multivalent cations such as aluminum, iron, calcium or magnesium. These positively charged molecules interact with negatively charged particles and molecules to reduce the barriers to aggregation. In addition, many of these chemicals, under appropriate pH and other conditions such as temperature and salinity, react with water to form insoluble hydroxides which, upon precipitating, link together to form long chains or meshes, physically trapping small particles into the larger floc.

MATERIALS REQUIRED

Flocculating agents: 0.02M NaCl, KCl. $CaCl_2$, and Al_2SO_4 Test tubes, cell debris solution, UV-Spectrophotometer.

PROCEDURE

1. 5 ml of bacterial culture was taken in 5 different test tubes
2. In the first test tube, add 2 ml of water and to the remaining test tube add 2ml flocculantswere added and incubate for 30 mins.
3. After settling of microflocs, the supernatant is collected.
4. Absorbance was measured at 600 nm in colorimeter/UV Visible spectrophotometer

RESULT: The best flocculant was found to befrom experimental (least OD) and theoretical methods (Least Debye radius, r DR)……

TABULATION

S.No	Flocculent	Volume of flocculant(ml)	O.D at 600 nm	Debye radius(m)(r_{DR})
1	Water			
2	Aluminum sulphate			
3	Calcium chloride			
4	Potassium chloride			
5	Sodium chloride			

CALCULATION:

$$r_{DR} = \frac{1}{\kappa} = \left(\frac{\varepsilon RT}{8\pi F^2 \sum c_i z_i^2} \right)^{1/2}$$

where ε = absolute dielectric constant of the liquid
R = gas constant
T = absolute temperature
F = Faraday number
c_i = bulk concentration of different ions
z_i = valence of different ions

$$\sum c_i z_i^2 = 2I$$

Where I is the ionic strength of the solution. The Debye radius is typically less than 1 nm for most bioprocessing situations

From graph, least OD obtained for …….

EXP NO: **DATE:**

ISOLATION OF CASEIN FROM MILK BY PRECIPITATION

AIM

To isolate casein from milk by isoelectric precipitation method

PRINCIPLE

Precipitation involves the conversion of the soluble solutes into insoluble solids which can be separated from the liquid by physical methods of separation such as a filtration or centrifugation

Precipitation serves primarily as a method for the recovery and concentration of the desired product and it is well established for the recovery of bulk proteins. Structurally proteins are the most complex biomacromolecules. Many different methods have been devised to separate proteins from other proteins or biomolecules.

Proteins differ in solubility at a given pH. Similar to amino acids that comprise protein, protein itself can be either positively or negatively charge overall due to the amine and carboxyl group and the groups on the side chain It is positively charged at low pH and negatively charged at high pH .By changing pH of the protein the ionization of the week acidic and basic amino acid side chain of a protein is affected resulting in a net zero charge on the protein. At a certain pH value called its isoelectric point (pI) or isoelectric pH.

When pH of the protein solution at low ionic strength is adjusted to a value equal to its isoelectir point solubility of the protein decreases drastically over a narrow pH range and it tends to precipate out. This method is called isoelectric precipitation

There are three kinds of proteins in milk: casein, lactalbumin and lactoglobulin. They are complete proteins because they contain all amino acids essential for building blood and tissue. Casein is the main protein in milk and is present at a concentration of about 35g/l. Casein is a phosphroprotein .It is not a single caseins which– κ,compound, it is a heterogeneous mixture of α, β, differ from each other in the molecular weight and the amount of phosphorus groups they contain. Casein exists in milk as the calcium salt, calcium caseinate. The white color of milk is due to this salt as well as to emulsified lipids.

Most proteins show minimum solubility at their isoelectric point and this principle is used to isolate the casein by adjusting the pH of milk to (4.5-4.8) its isoelectric point. Casein is also insoluble in

ethanol and this property is used to remove unwanted fat from the preparation. Milk is present at a high PH than the isoelectric point of casein, so to precipitate the casein acetic acid is added drop by drop until the isoelectric point is reached. Note that if excess CH_3COOH is added the precipitate redissolves. The isoelectric point of a protein (I.E.P.): is the pH at which the molecule is electrically neutral (net charge is zero). When a protein is charged e.g. with a –ve charge, the –ve molecules repel each other and thus remain in the solution and do not precipitate. But when these molecules are neutral at the I.E.P. they tend to precipitate due to the absence of the repelling forces and, the high molecular weight of protein. After the Precipitation of casein it is filtrated and washed with H_2O to remove excess acid.

MATERIALS USED

- Skimmed milk
- Acetic acid
- Beakers
- pH meter
- Isopropanol
- Filter paper
- Funnel
- Magnetic stirrer
- Conical flask

PROCEDURE

1. Take around 200mL of skimmed milk in a 500mL beaker and warm to 100^oF
2. Prepare acetic acid by dissolving 7 mL of glacial acetic acid in 50 mL of distilled water and add drop wise to the beaker containing milk, with constant stirring till the precipitation completes
3. Mass of casein is precipitated at its isoelectric point (pH 4.6). Since precipitation is not an instantaneous process, give a few minutes between acid additions to allow for stabilization. Allow the precipitate in the beaker by decantation of upper water layer
4. Wash the moist precipitate with 200 mL of distilled water thrice to remove the salts and filtered using Buchner funnel
5. Wash mass of casein obtained with isopropanol or ethanol and dry Calculate the amount of casein obtained

CONFIRMATION TEST

Dissolve the isolated casein in water and allow boiling for few minutes. Confirm the presence of protein by Lowry's method. The appearance of blue color indicates the presence of the protein casein

RESULT

The amount of casein obtained is……gram.

OBSERVATION

Weight of watch glass (W1) = gm

Weight of watch glass and dry casein (W2) = gm

Weight of dry casein = gm

EXP NO: **DATE:**

PRECIPITATION OF PROTEIN BY SALTING OUT (AMMONIUMSULPHATE PRECIPITATION)

AIM

To find % protein recovery from precipitation of proteins from the given solution by adding Ammonium sulfate

PRINCIPLE

Ammonium sulphate precipitation is a method of protein purification by altering solubility of protein. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed.

The solubility of protein varies according to the salt concentration. Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out).

Since proteins differ markedly in their solubilities at ionic strength, salting out is a very useful procedure to assist in the purification of a given protein. The commonly used salt is ammonium sulfate, as it is very water soluble and has no adverse effects upon enzyme activity. It is generally used as a saturated aqueous solution which is diluted to the requiredconcentration, expressed as a percentage concentration of the saturated solution (a 100 % solution)

MATERIALS REQUIRED

Test tubes, Graduated cylinder, Pipettes, UV spectrophotometer, Centrifuge

REAGENTS

- Prepare a known concentration protein solution(BSA – 2 mg / 10 ml) and raw hen white (collected)
- Ammonium sulphate

PHOSPHATE BUFFER SALINE (PBS) – 0.01M pH 7.2

- 28 mL of KH_2PO_4 (2.78 gm in 100 mL normal saline)
- 72 mL of Na_2HPO_4 (34.5 gm in 100 mL normal saline)

PROCEDURE

1. 10 ml of microbial cell culture/protein solution was pipette out in a test tube.
2. Weigh 6.97 gm of Ammonium sulphate salt (required saturation level) and add to the solution
3. Add the ammonium sulphate salt to the protein solution until the protein start precipitate.
4. Weigh the remaining amount of Ammonium sulphate salt and calculate the amount ofammonium sulphate added to the protein solution.
5. The solution was centrifuged for 10,000 rpm for 10 mins
6. The precipitate was collected carefully discarding as much supernatant as possible
7. To the precipitate 1 ml of PBS buffer (desired amount) was added to dissolve the pellet
8. The protein concentration measured by Lowry's method
9. Protein Recovery is calculated by using the formula

$$\% \text{ Protein Recovery} = \frac{\text{Final protein content}}{\text{Initial protein content}} 100$$

RESULT

Maximum amount of protein was recovered by using ---------- % of ammonium sulphate

Percentage Protein recovery at 37 ^{0} C was found to be ------

CALCULATION

From standard graph, OD at 660nm = …… (BSA); OD at 660nm =.....(egg white)

Concentration of protein after precipitation (egg white) …….……

Concentration of protein after precipitation (stock) …….……

Protein Recovery is calculated by using the formula

$$\% \text{ Protein Recovery} = \frac{\text{Final protein content}}{\text{Initial protein content}} 100$$

EXP NO: **DATE:**

AQUEOUS TWO PHASE EXTRACTION

AIM

To isolate the given protein by aqueous two phase extraction and to find the partition coefficient

PRINCIPLE

Downstream processing is an integral part of any product development, and the final cost of the product depends largely on the cost incurred during extraction and purificationtechniques. The conventional techniques used for product recovery, for example precipitation and column chromatography, are not only expensive but also result in lower yields. Furthermore since solid– liquid separation by centrifugation or filtration results in some technical difficulties, for example filter fouling and viscous slurries, therefore, there is an ongoing need for new, fast, cost effective, ecofriendly simple separation techniques. Thus, for separation of biomolecules, aqueous two phase systems (ATPS) offer an attractive alternative that meets the abovementioned requirements as well as thecriteria for industrially compatible procedures. Hence, it is increasingly gaining importance in biotechnological industries. The advantage of using this technique is that it substantially reduces the number of initial downstream steps and clarification, concentration, and partial purification can be integrated in one unit. Furthermore, scale-up processes based on aqueous two phase systems are simple, and a continuous steady state is possible

An aqueous two-phase system is an aqueous, liquid–liquid, biphasic system which is obtained either by mixture of aqueous solution of two polymers, or a polymer and a salt. Generally, the former is comprised of PEG and polymers like dextran, starch, polyvinylalcohol, etc. In contrast, the latter is composed of PEG and phosphate or sulphate salts. This polymer-salt system results in higher selectivity in protein partitioning, leading to an enriched product with high yields in the first extraction step

Partitioning of the two phases is a complex phenomenon, taking into account the interaction between the partitioned substance and the component of each phase. A number of different chemical and physical interactions are involved, for example hydrogen bond, charge interaction,

van der Waals' forces, hydrophobic interaction and steric effects. Moreover the distribution of molecules between the two phases depends upon the molecular weight and chemical properties of the polymers and the partitioned molecules of both the phases.

Thus, the distribution of molecules between the two phases is characterized by the partition coefficient, K_{part}, defined as the ratio of the concentrate in the top (C_t) and bottom (C_b) phase, respectively.

$$K = Ct/Cb$$

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MATERIALS REQUIRED

PEG 6000,Sodium carbonate, Sodium phosphate, Sodium sulphate, Distilled water, Centrifuge tube (15 ml), Alkaline copper sulphate solution, Folin's reagent.

PROCEDURE

1. 35% (w/w) of Sodium phosphate (i.e. 35 g of salt in 65 g of water and 50% of PEG 6000 50 g of PEG 6000 in 50 g of water) was prepared Or 40% (w/w) of PEG 6000 and 25% (w/w) of Sodium salt solutions.
2. Take 5 ml of salt solution in test tube and add preweighed 30 mg of BSA and mix well.
3. Add 5 ml of PEG solution drop by drop. The contents were thoroughly mixed.
4. The mixture was allowed to stand for some time to obtain the two phases.
5. It was allowed to settle for 20 minutes and the two phases were separated by careful pipeting and the volumes were noted down.
6. For faster separation, Centrifuge the tube at 5000 rpm for 10 mins.
7. Carefully transfer the top phase solution to the test tube.
8. Estimate the amount of protein present in the top and bottom phase by Lowry's method.

RESULTS: The partition coefficient and Yield of protein ……

OBSERVATIONS

Volume of top phase =
Volume of bottom phase =

From graph, OD at 660 nm, = …….(top); OD at 660 nm =.....(Bottom);

Concentration of protein in top phase =
Concentration of protein in bottom phase =

CALCULATIONS

Estimation of volume ratio

The volume ratio of the top and bottom phases can be obtained by the inverse lever arm rule, as given by:

$Vr = V_A/V_B$ = Volume of top phase/Volume of Bottom phase

Estimation of partition coefficient

The partition coefficient (K) is calculated as the ratio of the equilibrium concentration of the protein or enzyme in top phase (C_T) to that in bottom phase (C_B) .

$$K = \frac{C_T}{C_B} \qquad \text{…….. (2)}$$

Estimation of yield

Yield (%) of protein or enzyme in top phase is calculated by using the following equation:

$$Yield = \frac{KV_r}{1+KV_r} \times 100 \qquad \text{…….. (3)}$$

EXP NO: **DATE:**

ULTRAFILTRATION

AIM

To separate bovine serum albumin from the given solution suing ultrafilation membrane and to estimate the degree of separation

PRINCIPLE

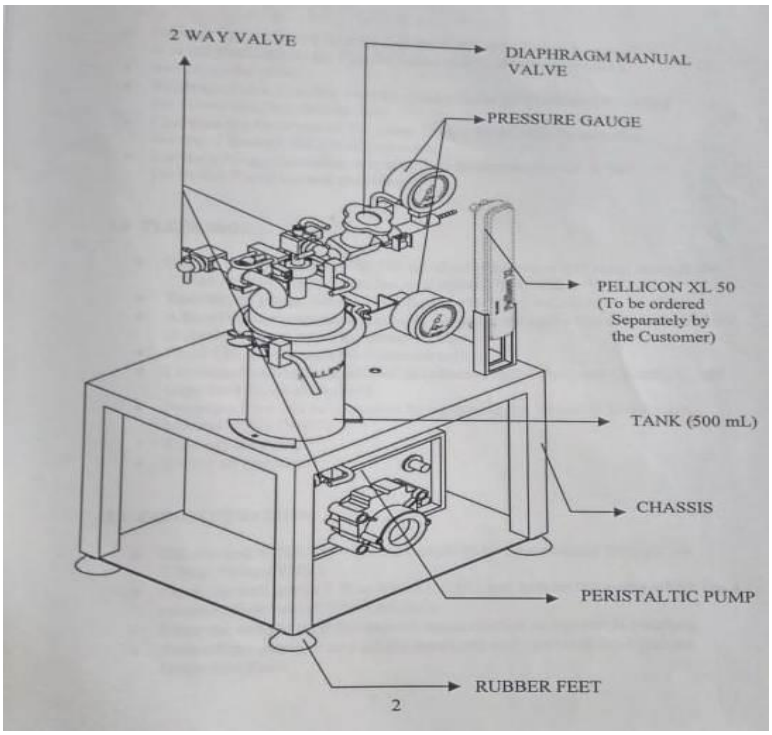
Fraction of protein is an important aspect of biotechnology especially in tissue engineering where the fermentation medium contains several peptides, growth factors ad proteins and the products like antibiotics is also present in the same medium. It is necessary to fractionate the proteins and the desired product from the rest of the components present in the medium. The separation of the components may be based on the size difference or the properties of the proteins.

Normally the separation of the solutes is considered effective if the size difference is around or more but it has been demonstrated that by controlling the operating conditions namely cross flow velocity, module geometry and Transmembrane pressure (TMP) and chemical environment like pH, ionic strength of the medium, proteins of smaller size variation can also be separated using membrane effectively.

In this experiment, the feed solution containing protein is separated out by passing it through the ultra filtration membrane and the degree of separation is estimated.

MATERIALS REQUIRED

- Ultrafiltration unit
- Measuring cylinder
- Protein solution
- Alkaline copper reagent
- Folins reagent
- Bovine serum albumin
- RO water
- Test tubes
- NaOH



PROCEDURE

1. Set the unit with required accessories like peristaltic pump, holding device, filter membrane and connecting tube
2. Filter membrane of about 30kDa is used for filtration
3. Connect the pump with respective reservoir by suing silicon tubes
4. Measure the permeate flux at TMP of 1 kgf/cm^2 with RO water (30-50 mL/min)
5. Pump about 500 mL of given protein solution through the membrane at a constant TMP of 1 kgf/cm^2 with retentate recycle to the feed flash and permeate collect in a separate flask
6. Concentrate the proteins by repeated filtration and continue till the feed is almost exhausted
7. Measure the quantity of permeate and retenate collected and record
8. Store the used membrane with 0.1 N NaOH for further use
9. Rinse the system with RO water before next use

MEASUREMENT OF PROTEIN CONCENTRATION (mg/mL)

1. The amount of protein present in the permeate , retenate and original feed are determined by Lowry's method
2. Mix about 100 microlitre of protein sample each from permeate, retenate and feed collected with 90mL of distilled water separately

3. About 0.1 mL of protein sample from each of the above was made up to 1 mL with distilled water separately in test tube
4. Add to each test tube 5 mL of alkaline copper reagent, mix well and keep for 20 min.Add 0.5 mL of Folin's reagent with vortexing and incubate for 20 min
5. Note the OD at 660nm from the standard graph of BSA, Calculate the amount of protein in the given sample (feed, permeate and retenate)

RESULT

The protein from a given solution mixture is separated using ultrafiltation membranes and the degree of separation is estimated

OBSERVATIONS

Original feed A_{660nm}

Retenate A_{660nm}

Permeate A_{660nm}

Volume of feed =…… ….mL
Volume of retenate = …….mL
Volume of permeate collected = …….mL

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CALCUALTION

PROTEIN ESTIMATION(MG/ML)

From the standard graph(BSA)

Amount of Feed =

Permeate =

Retenate =

Degree of separation = weight fraction of BSA in permeate/weight fraction of BSA in reten

EXP NO: **DATE:**

SEPARATION OF PLANT PIGMENTS BY THIN LAYER CHROMATOGRAPHY

AIM

To separate plant pigments by Thin layer chromatography

PRINCIPLE

Thin layer chromatography is a chromatography technique used to separate chemical compounds such as amino acids, food dyes etc. It involves a stationary phase consisting of a thin layer of adsorbent material usually silica gel, aluminum oxide or cellulose immobilized onto a flat inert carrier sheet. A liquid phase consisting of the solution to be separated dissolved in an appropriate solvent is drawn through the plate via capillary action, separating the experimental solution. The separation of component in TLC is based on differential adsorption as well as positioning of the analyzte between the liquid stationary phase and solvent mobile phase. This technique is rapid as compared to paper chromatography. Molecules get separate between the hydrated stationary pahse and non polar mobile phase .Hydrophilic analytes have more affinity to the polar stationary matrix, less hydrophilic molecules tend to have more affinity toward mobile phase resulting in its faster movement and separation. The separated anlaytes are identified by comparing their R_f values to that Rf standards. Commonly used stationary matrix for TLC Include silica gel-G, Silica gel H, micro porous cellulose, alumina, flurosil, and polyamide. Its wide range of uses Included determination of the plant pigments, detection of pesticides or insecticides in food analysis, the Dye composition of fibers in forensics, separation of amino acids or identification of compounds present in a given substance

MATERILAS REQUIRED

- Petroleum ether
- Acetone
- Isopropanol
- NaCl
- $CaCO_3$
- Na_2SO_4
- Fresh leaves

- TLC Silica gel plate
- Mortar and pestle
- Separating funnel
- Measuring cylinder
- Round Bottom flask

PROCEDURE

EXTRACTION OF THE LEAF PIGMENTS

Take fresh leaves and grind in a mortar containing 22 mL of acetone, 3 mL of petrol ether and a spatula tip full of $CaCO_3$.Filter the pigment extract. Transfer the filtrate into a separating funnel and mix with 20mL of Petrol ether and 20mL of 10% aqueous NaCl solution. Shake the separating funnel carefully. When the two layers formed, allow the lower layer to drain into a beaker and thrown away. Wash the upper layer 3-4 time with 5 mL of distilled water. Afterward place the extract in a beaker and dry with about 4 spatula tips of Na_2SO_4. The liquid was carefully decanted into a round bottom flask. The leaf extract was concentrated to a final volume of about 3 mL.

PREPARATON OF DEVELOPING SOLVENT (MOBILE PHASE)

100mL of Petroleum ether, 11 mL of Isopropanol and 5 drops of distilled water

PREPARATON OF THE TLC CHAMBER

The developing solvent is placed into a TLC chamber. The solvent should completely cover the bottom of the chamber to a depth of approximately 0.5 cm. the chamber is closed and shaken. It is kept covered so that evaporation does not change the composition of the developing solvent mixture. After 15 min the chamber gets saturated with the solvent vapor

APPLICATION OF THE SAMPLE TO THE TLC PLATE

With a pencil a line was drawn approximately 1.5 cm from the bottom of the plate. Using a paint brush or a pasteur pipette, the leaf extract was spotted to the TLC silica gel plate about 1.5 cm away from the edge Of the plate. The area of the spot should be kept to a minimum 1-2mm dia which can be achieved by repeated spotting of the sample volume followed by air drying at the same spot. The loaded TLC plate was carefully placed in the TLC chamber with the sample line toward the bottom.

The plate whose top is leaned against the jar wall should sit on the bottom of the chamber and be in Contact with the developing solvent (solvent surface must be below the extract line). The TLC chamber was covered and allowed to remain undisturbed. The Chromatogram is runned until the solvent front reaches the top edge of the plate. When the solvent front has reached three quarters of the length of the plate, the pate is removed from the developing chamber and the position of the solvent front is immediately marked. The plate was air dried at room temperature and the colored pigments spots were outlined and the Rf values were calculated. For identification of pigments, the R_f values were compared with their standard Rf values

RESULTS

The various pigments of leaves are separated by TLC and its Rf were calculated

OBSERVATION AND TABULATION

S.No	Color	Pigments	R_f value
1	Golden yellow	Carotene	
2	Olive green	Pheophytin	
3	Blue green	Chlorlophyll a	
4	Green yellow	Chlorophyl b	
5	Yellow	Lutein	
6	Yellow	Violxanthin	
7	Yellow	Neaxanthin	

CALCULATION

R_f value = Distance travelled by solute/Distance travelled by solvent

EXP NO: **DATE:**

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

AIM

(i) To get the retention time plot for the benzene, toluene and naphthalene- Acetonitrile mixture and to compare it with the standard plot

(ii) To obtain the parameters measured based on the benzene, toluene and naphthalene peaks

PRINCIPLE

High performance liquid chromatography also known as high pressure liquid chromatography is ideally suited for the separation and identification of amino acids, carbohydrates, lipids, nucleic acids, proteins, steroids, pharmaceuticals and many other biologically active molecules. The instrument used for this consist of the main components namely i. Mobile phase delivery system ii. High pressure pumps iii. Sample injection part iv. Column v. Detector vi. Data processor and vii. Sample collector

MOBILE PHASE DELIVERY SYSTEM

The subcomponent of the mobile phase deliver system consists of solvent reservoir, solvent degassing unit microfilter, microprocessor controlled high pressure pump, PTFE and stainless steel tubing and mixing chamber. Two or three high pressure pumps are used when using gradient mixed solvent mobile phase. The mobile phase should be free from any dust or particulate matter which can damage the pump or the column and hence the liquid is pumped through micro filter. The microprocessor controlled high pressure pump is one of the most important components of HPLC since the performance directly affects the sensitivity and reproducibility of chromatographic separations. The criteria for a good HPLC pump include the capability to operate at pressure upto 500a tm. It should be capable of precise and pulse free delivery of the mobile phase at low flow rate in the range o 0.02 to 10mL/min with minimum drift. Common solvents used include any miscible combination of water or various organics liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in th separation of the anlayte components or compounds such trifluoroacetic acid which acts as on ion pairing agent

SAMPLE INJECTION DEVICE

It is a valve capable of introducing a wide range of sample volumes into the column as a sharp pulsr or plug without adversely affecting the column. Since the system is operating at a high pressure, sample injection is achieved with at typical volume of 10-20µL through a sample loop

COLUMN

It is made of SS tubing 25cm long and 2-4 mm dia for analytical purpose. The pre column is relatively small of about 8-10um long while HPLC columns used for preparative purpose are much wider. The column containing the packed bed of stationary phase particles. Three generally packing materials used are i. superficially porous particles of about 30 mm size. Ii. Very small totally porous particles of 5 mm size and iii. Totally porous particle of 10 mm size for the adsorption and partition mode of HPLC. The guard column is placed between the sample injector and the main column to protect the main column from damage or loss of efficiency

DETECTORS

Two types of detectors are used in HPLC. A bulk or solvent property detector such as refractive index RI or solute property detectors such UV spectral detector. UV diode array detector, UV visible spec, fluorescence detector or electrochemical deter, ELSD – Evaporative light scattering detector. The signal from the detector is amplified and fed to data processor system for visual display and print out of the chromatogram. The fraction collector in most of the sophisticated HPLC systems are microprocessor controlled programmable units and are easily synchronized with detector signals to collect the components of the sample mixture completely

MODE OF OPERATION

The versatility of HPLC is based on the ability of the instrument to be operated in different modes of chromatography simply changing the column. These include adsorption, partition, reversed phase, Ion exchange size exclusion and affinity chromatography. Reversed phase consist of a non polar stationary phase and an aqueous moderately polar mobile phase. One common stationary phase is silica which has been treated with RMe2SiCl, where R is a straight

chain. Alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. The retention time is therefore longer for molecular which are more non polar in nature, allowing polar molecules to elute more readily. Retention Time (R_T) is increased by the addition of polar solvent to the mobile phase and decreased by the addition of more hydrophobic Solvent. The pharmaceutical industry regularly employs RPC to quality drugs before their release.

IMPORTANT PARAMETERS DETERMINED BY HPLC

RETETNION TIME

A fundamental retention parameter in column chromatography is the retention time. The retention time is defined as the time taken by the solute to reach the detector from the moment of its injection into the column and determined by the measuring the distance between the sample injection point ot the apex of the peak from an online chromatogram. The time at which a specific analyte elutes is called retention time and is considered a reasonably unique identifying characteristics of a given analyte

RETENTION VOLUME

Retention volume may defined as the volume of the mobile phase required to eluate a solute from the point of its injection into the column and its passage through the column to the detector

It is obtained by $V_R = t_R \times F$

F = flow rate of mobile phase mL/min

RESOLUTION

Resolution is a measure of their separation or amount of separation between two adjacent peaks and is defined as the distance between peaks maxima compared with the average base widths of the two peaks

$Rs = 2 [t_{R2}-t_{R1}]/w_{b1}+w_{b2}]$

t_{b1} = Retention time of component 1

t_{b2} = Retention time of component 2

w_{b1} = Peak width of component 1

w_{b2} = Peak width of component 2

MATERIALS REQUIRED

- Toluene
- Benzene
- Napthalene
- Micro injector
- HPLC set up
- Micropipettes
- HPLC grade acetonitrile
- Water
- Beakers
- Conical flasks

PROCEDURE

Preparation of sample

0.2 mL of Benzene, Toluene and 0.1mg naphthalene is mixed with 100 mL of HPLC grade water

Preparation of Mobile phase

70 mL of HPLC grade acetonitrile mixed with 30 mL of water

Optimizing the HPLC set up:

(i) Switch on the pumps A and B, detector in their respective order
(ii) Create new method in the prescribed software for HPLC
(iii) Adjust the pressure in the pumps A and B optimized to 45kgf/cm^2
(iv) The flow is set as binary gradient
(v) The lamp in the UV visible detector is set to ON position. The wavelength in the UV detector is set as 254mm and the retention time is set to 12 min
(vi) The optimization parameters are applied and are downloaded to the instrument
(vii) The tubing of the pumps is purged for any air bubble to tbe remove by allowing a small quantity of the solvent to pass through 10uL of the sample is injected by means of injection method
(viii) The baseline origin for the used defined type of plot between volts and time is set up
(ix) As the runtime proceeds, peaks are observed between 6 min
(x) The retention time plot and parameters are obtained and compared with the standard graph

RESULT

The retention time plot and parameters measured based on the sample peaks is obtained and compared with the standard graph

CALCULATION

From the graph

Retention time

$V_R = t_R \times F$

F = flow rate of mobile phase mL/min

Resolution

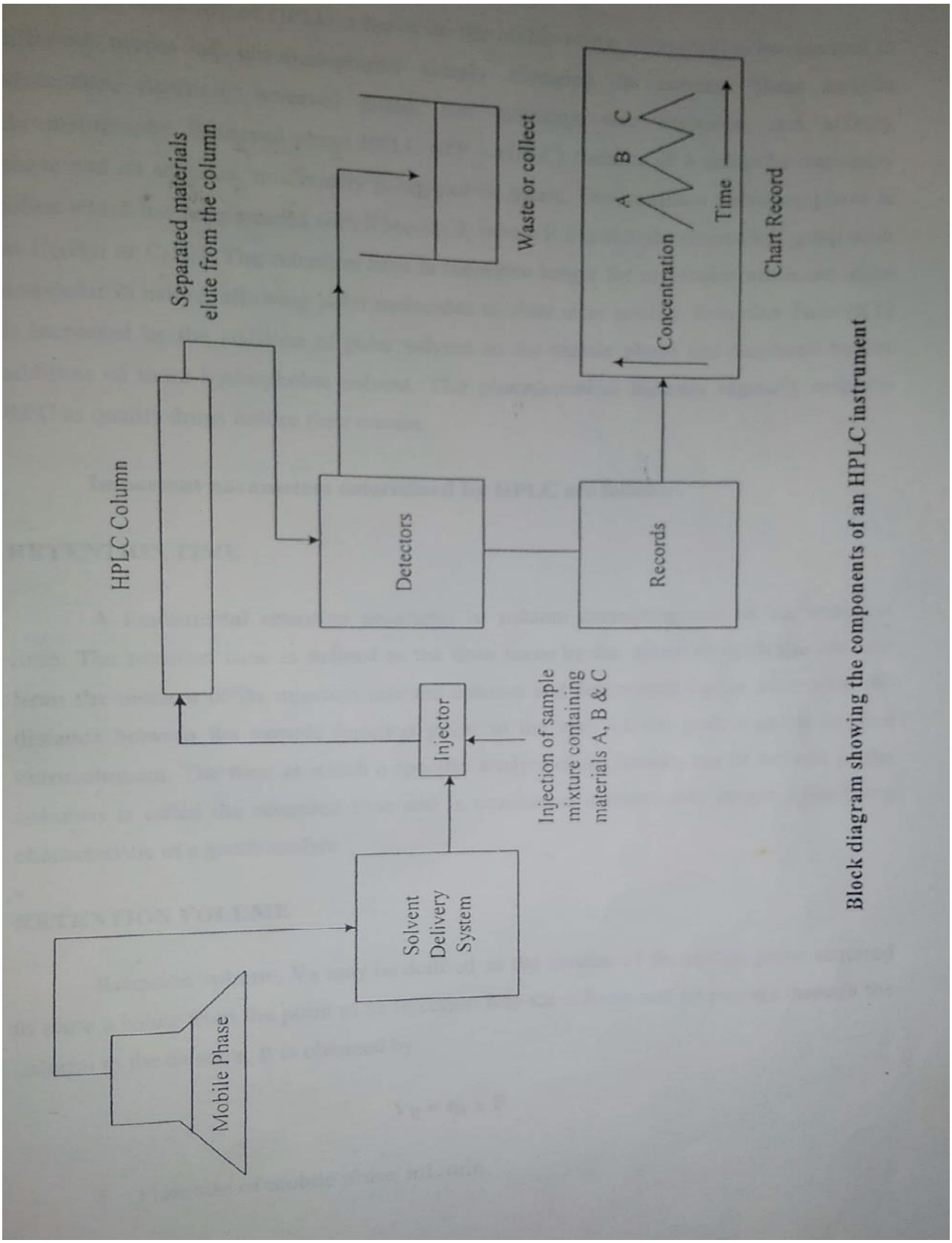
$Rs = 2 [t_{R2}-t_{R1}]/w_{b1}+w_{b2}]$

t_{b1} = Retention time of component 1

t_{b2} = Retention time of component 2

w_{b1} = Peak width of component 1

w_{b2} = peak width of component 2



Block diagram showing the components of an HPLC instrument

EXP NO: **DATE:**

CYSTALLIZATION OF A PRODUCT

I.ISOLATION OF BENZOIC ACID AND CRYSTALLIZATION OF BENZOIC ACID FROM SOFT DRINK

AIM: To isolate benzoic acid from soft drink and produce crystals from impure sample

MATERIALS REQUIRED

Crude sample of benzoic acid, 250 ml measuring flask, funnel,a glass rod, and a trough, conical flask. HCL, Methylene chloride, UV spectrophotometer, distilled water, charcoal

PROCEDURE

Synthesis of benzoic acid

1.150ml of soft drink (coca cola) was poured into a conical flask and acidified with 2 drops of dilute hydrochloric acid. 50ml of methylene chloride was added and the flask swirled gently for at least 5 minutes.

2.The mixture was transferred into 250ml separating funnel and allowed to settle for about 5 – 10 minutes.

3. The organic layer was drained into a beaker and allowed to evaporate on a water bath, leaving a residue of benzoic acid.

4. The residue was diluted with methylene chloride and sent to the UV for the absorbance to be taken at 272 nm

CRYSTALLIZATION OF BENZOIC ACID

1. Take about 2-3ml of the crude sample of benzoic acid in a 250ml beaker, in another take about 150ml of water and keep it for boiling.

2. Add slowly with stirring least amount of boiling water to the beaker containing crude sample of benzoic acid so that it gets dissolved easily. Add 0.5g of animal charcoal to the solution and boil for a minute.

3. Filter the solution while hot using a fluted filter paper place d in a stemlessfunnel. Collect the clear filtrate in a beaker.

4. Allow the filtered solution to cool at room temp. Now cool it by placing it on a beaker filled with cold water.

5. Separate the crystals by suction using Buchner funnel. Wash the crystals with water, dry the crystals.

6. Record the weight of the crystals.

RESULT

Concentration of benzoic acid in soft drink.....
The crystals of benzoic acid are shining white and yield.....gm

Standard Con'(ppm)	UV Absorbance
25	0.030
20	0.027
15	0.011
10	0.006

II.CRYSTALLIZATION OF NAPHTHALENE

AIM: To prepare crystals of pure naphthalene from an impure sample using ethyl alcohol as solvent

MATERIALS REQUIRED: Crude sample of naphthalene, 250 ml beakers, stemless funnel, glass rod

PROCEDURE

1. Take about 2-3ml of the crude sample of naphthalene in a 250ml beaker, in another beaker take about 50ml of ethanol and heat it on a water bath

2.add slowly with stirring least amount of hot ethyl alcohol to the beaker containing crude sample of naphthalene so that it gets dissolved easily.

3. Filter the solution while hot using a fluted filter paper placed in a stemless funnel and collect the clear filtrate in a beaker.

4. Allow the filtered solution to cool at room temp. and now cool it by placing it on a beaker filled with cold water.

5. Separate the crystals by suction using Buchner funnel. Wash the crystals with water, dry the crystals.

6. Record the weight of the crystals.

RESULT The crystals of naphthalene are shining white. Yield of crystals =…..gm

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