

1. ESTIMATION OF PROTEIN BY LOWRY'S METHOD

Aim:

To estimate the amount of protein in the given sample by Lowry's method.

PRINCIPLE:

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids [Dunn, 13]. The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5.

The Lowry method is sensitive to low concentrations of protein. Dunn [1992] suggests concentrations ranging from 0.10 - 2 mg of protein per ml while Price [1996] suggests concentrations of 0.005 - 0.10 mg of protein per ml. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we will be using very small volumes of sample, which will have little or no effect on pH of the reaction mixture.

A variety of compounds will interfere with the Lowry procedure. These include some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids and sulphhydryl reagents [Dunn, 1992]. Price [1996] notes that ammonium ions, zwitter ionic buffers, nonionic buffers and thiol compounds may also interfere with the Lowry reaction. These substances should be removed or diluted before running Lowry assays.

Reagents

A. 2% Na₂CO₃ in 0.1 N NaOH

B. 1% NaK Tartrate in H₂O

C. 0.5% CuSO₄.5 H₂O in H₂O

D. Reagent I: 48 ml of A, 1 ml of B, 1 ml C

E. Reagent II- 1 part Folin-Phenol [2 N]: 1 part water

BSA Standard - 1 mg/ ml**Procedure:**

- 0.2 ml of BSA working standard in 5 test tubes and make up to 1ml using distilled water.
- The test tube with 1 ml distilled water serve as blank.
- Add 4.5 ml of Reagent I and incubate for 10 minutes.
- After incubation add 0.5 ml of reagent II and incubate for 30 minutes
- Measure the absorbance at 660 nm and plot the standard graph .
- Estimate the amount of protein present in the given sample from the standard graph.

Tabulation:

S.No	Vol. of BSA (ml)	Conc of BSA (mg/ml)	Vol. of Distilled water (ml)	Vol. of reagent I (ml)	Incubation for 10 min	Vol. of Reagent II (ml)	Incubation for 30 min	OD At 660nm	

Calculations:**Result:**

The amount of protein present in the given sample was found to be

2. ESTIMATION OF PROTEINS BY BRADFORD METHOD

Aim:

To estimate the amount of protein in the given sample by Bradford Assay.

Principle

The protein in solution can be measured quantitatively by different methods. The methods described by Bradford uses a different concept-the protein's capacity to bind to a dye, quantitatively. The assay is based on the ability of proteins to bind to coomassie brilliant blue and form a complex whose extinction coefficient is much greater than that of free dye.

List of Reagents and Instruments

A. Equipment

- Test tubes
- Graduated cylinder
- Weight Balance
- UV spectrophotometer

B. Reagents

- Dissolve 100mg of Coomassie-Brilliant blue G250 in 50 ml of 95% Ethanol.
- Add 100 ml of 85% phosphoric acid and make up to 600 ml with distilled water.
- Filter the solution and add 100 ml of glycerol, then make upto 1000ml.
- The solution can be used after 24 hrs.
- BSA

Procedure

- Prepare various concentration of standard protein solutions from the stock solution (say 0.2, 0.4, 0.6, 0.8 and 1.0 ml) into series of test tubes and make up the volume to 1 ml .

- Pipette out 0.2ml of the sample in two other test tubes and make up the volume to 1ml.
- A tube with 1 ml of water serves as blank
- Add 5.0 ml of coomassie brilliant blue to each tube and mix by vortex or inversion.
- Wait for 10-30minutes and read each of the standards and each of the samples at 595nm.
- Plot the absorbance of the standards verses their concentration.
- Plot graph of optical density versus concentration. From graph find amount of protein in unknown sample.

Tabulation:

S.No	Vol. of BSA (ml)	Conc of BSA (mg/ml)	Vol. of Distilled water (ml)	Vol. of Bradford reagent (ml)	Incubation for 10 min	OD at 595 nm

Calculations:**Result:**

The amount of protein present in the given sample was found to be

3. CELL DISRUPTION BY ULTRA SONICATION

AIM

To disrupt the microbial cells by sonication and to determine the amount of protein released and rate constant for cell disruption.

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.

MATERIALS REQUIRED:

Overnight grown bacterial culture, sonicator, eppendorf tubes, ice cubes, Spectrophotometer, Alkaline copper sulphate solution , Folin's reagent.

PROCEDURE:

- Take 2 ml of bacterial culture in 5 eppendorf tubes and centrifuge at 10000 rpm for 5 minutes.
- Discard the supernatant and dissolve the pellet in 1ml of distilled water.
- Keep the eppendorf tubes in ultrasonic wave generator tip for different time intervals (30-150 sec).
- Set the controller power 50% and frequency of 25kHz.

- After sonication, centrifuge the eppendorf tubes at 10000 rpm for 5 minutes.
- Take 0.2 ml of supernatant and determine the amount of protein in the sample by Lowry's method.

Tabulation:

S.No	Sonication Time (sec)	OD at 660 nm	Conc of released protein (mg/ml)

Calculations:

Result:

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

The rate constant (θ) for the cell disruption by Ultrasonication =.....

4. CELL DISRUPTION BY HIGH PRESSURE HOMOGENIZER

AIM:

To disrupt the given bacterial cell/ culture by high pressure homogenizer and to find the rate constant for cell disruption.

PRINCIPLE:

A high pressure homogenizer consists of a high pressure positive displacement pump coupled to an adjustable discharge valve with a restricted orifice. The cell suspension is pumped through the homogenizing valve at 200-1000 atm atmospheric pressure depending on the type of microorganism and concentration of the cell suspension. The disrupted cell suspension is cooled as it exits the valve to minimize thermal denaturation of sensitive products. Cell disruption occurs due to various stresses developed in the fluid. The primary mechanism seems to be the stress developed due to the impingement of high velocity jet suspended cells on the stationary surface. Other stresses are generated by the homogenizer including normal and shear stresses. The normal stress is generated as the fluid passes through the narrow channel of the orifice and the shear stress as the pressure rapidly reduces to almost atmospheric pressure as the cell suspension passes out of the orifice. Different parameters influence the degree of cell disruption and rate of release of the product. These include the nature of microorganism, its size, cell wall composition and thickness, and concentration of microbial cells, product location within the cell, type of homogenizer, operating pressure, temperature, number of passes of the cell suspension through the homogenizer.

$$\ln(C_{\max}/C_{\max}-C)=-kN$$

where

N=number of passes through the valve

K=first order rate constant depending on operating pressure

MATERIALS REQUIRED:

Overnight grown bacterial culture, Alkaline CuSO₄ solution, Folin's reagent, High pressure homogenizer, spectrophotometer, test tubes.

PROCEDURE

1. A culture flask containing given bacterial culture is taken and subjected to high pressure homogenization.
2. Homogenization was carried out at different atmospheric pressure of 100-500 bar.
3. Two passes are done in the homogenizer.
4. The homogenized culture is centrifuged at 10,000 rpm for 5 minutes.
5. 0.2 ml of the supernatant is taken and the protein concentration is estimated using Lowry's Method.
6. The graph was plotted between concentration and pressure applied.

Tabulation:

S.No	Pressure (bar)	Conc of released protein (r)		r/r_{max}	r/r_{max}	ln (1-r)	
		Single Pass	Double Pass	Single Pass	Double Pass	Single Pass	Double Pass
01	100						
02	200						
03	300						
04	400						
05	500						

Calculations:**Result:**

Maximum amount of protein released (C_{max}) = at bar

The rate constant (k) for the cell disruption by high pressure homogenization in

Single pass =

Double pass =

5 .CELL DISRUPTION BY ENZYMATIC DIGESTION

AIM:

To disrupt the given bacterial cell/ culture by enzymatic method.

PRINCIPLE:

Digestion of cell wall is achieved by the addition of lytic enzymes to a cell suspension. Enzymes are highly selective, gentle and most effective. Lysozyme is widely used to lyse bacterial cells. The enzyme hydrolyses α -1,4 glycosidic bond in the mucopeptide moiety of bacterial cell wall of gram positive bacteria. The final rupture of the cell often depends on the osmotic pressure of the suspending medium. In case of gram negative bacteria like Escherichia coli pretreatment with detergent such as Triton X-100 or addition of EDTA is necessary. EDTA is used to destabilize the outer membrane thereby making the peptidoglycan layer accessible to lysozyme. Yeast cell lysis requires a mixture of different enzymes such as glucanase, protease, mannanase or chitinase. Sequential disruption of microbial cells for selective product release involves the use of lytic enzymes based on the type of microbial cells and process conditions.

MATERIALS REQUIRED:

Overnight grown bacterial culture, Alkaline CuSO₄ solution, Folin's reagent, Lysis buffer containing Lysozyme.

PROCEDURE:

- Take 1.5 ml of overnight grown bacterial culture in eppendorf tubes and centrifuge at 10000 rpm for 5 minutes.
- Discard the supernatant and suspend the biomass/ pellet in 1 ml of PBS (phosphate buffered saline) buffer.
- Add 200 μ l of lysis buffer to all the tubes.
- Incubate at different time intervals 10,20, 30, 40, 50 mins.
- Centrifuge all the eppendorf tubes at 10000 rpm for 5 minutes.
- Take 0.2 ml of supernatant and estimate the amount of protein present in each sample by Lowry's method.

- Plot the graph between time vs protein released.

Tabulation:

S.No	Incubation Time (min)	OD at 660 nm	Conc of released protein (mg/ml)

Calculations:

CMR

Result:

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

The optimum time for maximum protein release =

6. FLOCCULATION

AIM:

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

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 $0.1\ \mu\text{m}$ 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 aluminium, 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 , $\text{Al}_2(\text{SO}_4)_3$,

Acrylamide, Test tubes, cell debris solution, spectrophotometer.

PROCEDURE:

- Take 5 ml of cell debris solution in 3 test tubes and measure the absorbance at 610 nm in colorimeter.
- Add 2 ml of flocculants to the corresponding test tubes and wait for 30 minutes of incubation.
- Measure the absorbance at 610nm in spectrophotometer and Calculate Debye radius for each flocculent

Tabulation:

S.No	Flocculants	OD at 600 nm
1	Control	
2	KCl	
3	NaCl	
4	CaCl ₂	
5	Al ₂ (SO ₄) ₃	
6	Acrylamide	

Calculations:**Result:****Debye radius of**

KCl =

NaCl =

CaCl₂ =Al₂SO₄ =

Acrylamide =

Inference:

7.BATCH FILTRATION

AIM

To filter the given slurry and to determine specific cake resistance α and medium resistance R_m .

PRINCIPLE

Filtration is the conventional unit operation aimed at the separation of particulate matter. Filtration is defined as the separation of solid in a slurry consisting of the solid and fluid by passing the slurry through a septum called the filter medium. The filter medium allows the fluid to pass through and retains the solids. The separated solid called the filter cake forms a bed of particles on the filter medium. The thickness of the cake increases from an initial value of zero to a final thickness at the end of filtration. During filtration the filtrate passes first through the cake and then the filter medium. The rate of filtration is defined as the volume of filtrate collected per unit time per unit area of the filter medium. The resistance of the medium is constant and is independent of the cake.

MATERIALS REQUIRED:

CaCO₃ solution, filter paper, funnels, measuring cylinder:

PROCEDURE:

- Take 50 ml of 10% CaCO₃ and filter it through filter paper.
- Note down the volume of filtrate collected in measuring cylinder every minute until the end of filtration.
- Calculate area of funnel.
- Plot the graph between At/V vs V/A and find slope and intercept.
- From the values of slope and intercept calculate α and R_m .

Tabulation:

Time (sec)	Volume of filtrate collected (ml)	At/V (s/cm)	V/A (cm)

Calculations:

CMR

Result:

The specific cake resistance (α) =

The medium resistance (R_m) =

8. BATCH SEDIMENTATION

AIM

To study the settling velocity of solid particles under batch sedimentation and to find the area of the continuous thickener.

PRINCIPLE:

There are several stages in the settling of flocculated suspension and different zones are formed as sedimentation proceeds. Usually, concentration of the solids is high enough, that sedimentation of individual particles or flocs are hindered by other solids to such an extent that all solids at a given level settle at a common velocity. At first the solid is uniformly distributed in the liquid, the total depth of the suspension is Z_0 . After a short time the solids settle to give a zone of clear liquid.

Formula:

$$(i) \text{Area of the thickener} = FC_0 / (V_L / (1/C_L - 1/C_u)) \text{ m}^2$$

$$(ii) V_L = (Z_i - Z_L) / \theta_L$$

$$(iii) C_i = C_0 Z_0 / Z_i$$

Where,

$$F = \text{Flow rate (m}^3\text{)}$$

$$C_0 = \text{Initial concentration of the slurry (kg/m}^3\text{) at time 0}$$

$$C_u = \text{Underflow concentration (kg/m}^3\text{)}$$

$$V_L = \text{Velocity of settling (m/s)}$$

Materials Required:

Measuring cylinder 1000ml, stop clock, CaCO₃ and scale.

PROCEDURE:

- 50 gms of Calcium carbonate was measured and placed in a 1 litre of measuring cylinder.
- One litre of water is poured into the cylinder and the slurry is stirred well.
- The level of interface between the clear liquid and the slurry of original cone was noted at various time interval.
- The height and time was tabulated.
- A graph between height and time was plotted.

Observation

S. no	Height of interface $\times 10^{-2}$ m	Time (sec)

Calculation Table

Z_i (m)	Z_L (m)	θ_L (s)	$C_L = C_0 Z_0 / Z_i$ (kg/m ³)	$V_L = Z_i - Z_L / \theta_L$ (m/s)	$V_L / (1/C_L - 1/C_U)$ (kg/m ² s)	Area m ²

Result:

The area of the thickener required = _____

9. AQUEOUS TWO PHASE EXTRACTION

AIM

To isolate the given protein by aqueous two phase extraction and to find the partition co-efficient.

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 purification techniques. 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, eco-friendly simple separation techniques. Thus, for separation of biomolecules, aqueous two phase systems (ATPS) offer an attractive alternative that meets the above-mentioned requirements as well as the criteria 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, polyvinyl alcohol, 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 molecules¹⁴ 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_{top}) and bottom (C_{bottom}) phase, respectively.

$$K_{\text{part}} = C_{\text{top}}/C_{\text{bottom}}$$

MATERIALS REQUIRED:

PEG 4000, Sodium carbonate, Sodium phosphate, Sodium sulphate, Distilled water, Centrifuge tube (15 ml), Alkaline copper sulphate solution, Folin's reagent.

PROCEDURE:

- Prepare 40% (w/w) of PEG 6000 and 25% (w/w) of Sodium salt solutions.
- Take 5 ml of salt solution in three different centrifuge tube and add preweighed 30 mg of BSA and mix well.
- Add 5 ml of PEG solution to all the tubes.
- Centrifuge the tube at 5000 rpm for 10 mins.
- Carefully transfer the top phase solution to the test tube.
- Estimate the amount of protein present in the top and bottom phase by Lowry's method.
- Calculate the partition coefficient.

Calculations:

Result:

Partition coefficients of BSA in following systems are

PEG 4000 – Carbonate system =

PEG 4000 – Phosphate system =

PEG 4000 – Sulphate system =

10. PROTEIN PRECIPITATION. (Ammonium Sulfate Precipitation)

AIM:

To precipitate the given protein sample by using Ammonium sulphate and to determine the protein recovery.

PRINCIPLE

Proteins are usually soluble in water solutions because they have hydrophobic amino acids on their surfaces that attract water molecules and interact with them. This solubility is a function of the ionic strength and pH of the solution. Proteins have iso electric points at which the charges of their amino acid side groups balance each other. If the ionic strength of a solution is either very high or very low proteins will tend to precipitate at their iso electric point. The solubility is also a function of ionic strength and as you increase the ionic strength by adding salt, proteins will precipitate. Ammonium sulfate is most common salt used for this purpose because it is unusually soluble in cold buffers (we have to keep proteins cold!) and is economically viable. Ammonium sulfate fractionation is commonly used in research laboratories as the first step in protein purification because it provides some crude purification of proteins away from non- proteins and also separates some proteins. Ammonium sulfate also yields the precipitated proteins slurry that is usually very stable, so the purification can be stopped for a few hours.

MATERIALS REQUIRED

Ammonium sulphate, BSA, Alkaline copper sulphate solution, Folin's reagent, Magnetic stirrer, Centrifuge tube, Centrifuge, Test tube, Spectrophotometer.

PROCEDURE

- Dissolve 5 mg of BSA in 10 ml of distilled water in 50 ml beaker.
- Add the corresponding saturation amount (50-100 %) of ammonium sulphate to the beaker with stirring.
- After adding ammonium sulphate completely to the beaker , leave it undisturbed for 30 mins.

- Transfer the solution the 15 ml centrifuge tube abd centrifuge at 10000 rpm for 5 mins.
- Discard the supernatant and dissolve the pellet in distilled water and centrifuge again.
- Discard the supernatant and dissolve the pellet in 1 ml of distilled wate
- Take 200µl of sample and estimate the amount of protein present by Lowry's method.
- Calculate the percentage of recovery by using the formula

Recovery percentage =(Final protein concentration / Initial protein concentration) *100

Tabulation:

Percentage of Ammonium Sulphate	Protein concentration	Percentage of recovery
50		
60		
70		
80		
90		
100		

RESULT

Maximum percentage recovery of BSA is----- at -----% of Ammonium sulphate saturation.