KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2017 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT : MEMBRANE BIOLOGY AND BIOENERGETICS PRACTICAL

SEMESTER : I

SUBJECT CODE: 18BCU113 CLASS: I B.Sc., BIOCHEMISTRY

Course objectives

- To introduce and to explain the basic concepts in Membrane biology and Bioenergetics.
- To develop more adequate understanding of cellular and biotechnological processes.
- In practical training students learn the principal research methods in Membrane biology and Bioenergetics, develop skills for problem solving.

Course outcome

- The aim of course is to deepen student's knowledge on structure, function and pathology of biological membranes with particular emphasis on principles of energy transformation (bioenergetics).
- The practical course gives a complete hands-on understanding of the functions of membranes.
- The practical sessions gives practical understanding of basis membrane related experiments.

1. Effect of lipid composition on the permeability of a lipid monolayer.
2. Determination of CMC of detergents.
3. RBC ghost cell preparation and to study the effect of detergents on membranes.
4. Separation of photosynthetic pigments by TLC.
5. Isolation of mitochondria from liver and assay of marker enzyme SDH.
6. Study photosynthetic O_2 evolution in hydrilla plant.
7. Isolation of chloroplast from spinach leaves, estimation of chlorophyll.

REFERENCES

Nelson, D.L. and Cox, M.M., (2013) Lehninger: Principles of Biochemistry 6th ed., W.H.Freeman and Company (New York), ISBN:13: 978-1-4641-0962-1 / ISBN:10:1-4641-0962-1.

Lodish, H., Berk, A., Kaiser, C.A., Krieger, M., Bretscher, A., Ploegh, H., Amon, A. and Scott, M.P., (2013). Molecular Cell Biology 7th ed., W.H. Freeman & Company (New York), ISBN:13: 978-1-4641-0981-2.

Garret, R. H. and Grisham, (2010). Biochemistry 4th ed., C.M., Cengage Learning (Boston), ISBN-13: 978-0-495-11464-2.

Voet, D.J., Voet, J.G. and Pratt, C.W., (2008). Principles of Biochemistry 3rd ed., John Wiley & Sons, Inc. (New York), ISBN:13: 978

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DEPARTMENT OF BIOCHEMISTRY

PRACTICAL PLAN

SUBJECT NAME: MEMBRANE BIOLOGY AND BIOENERGETICS PRACTICAL

SUB.CODE: 18BCU113

SEMESTER: I **CLASS: I B.Sc., BIOCHEMISTRY**

S.No	Topics to be Covered	Supporting material with Page No.
1.	Effect of lipid composition on the permeability of a lipid monolayer	R1: 18-20
2.	Determination of CMC of detergents	R1: 26-29
3.	RBC ghost cell preparation and to study the effect of detergents on membranes	R2: 116-117
4.	Separation of photosynthetic pigments by TLC	R1: 222-224
5.	Isolation of mitochondria from liver and assay of marker enzyme SDH	R1: 250-256
6.	Study photosynthetic O_2 evolution in hydrilla plant	R1: 270-271
7.	Isolation of chloroplast from spinach leaves, estimation of chlorophyll	R2: 496-471

Reference book

R1: Sadasivam S & Manickam A, Biochemical methods 2nd edition, Centre for plant molecular biology, Tamil nadu Agricultural University, Coimbatore, 1996.
R2: Dr. S. Rajan and Mrs. R. Selvi Christy. Experimental procedures in life science. 2011.

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DEPARTMENT OF BIOCHEMISTRY LAB MANUAL

SUBJECT : MEMBRANE BIOLOGY & BIOENERGETICS PRACTICALS
SEMESTER : I
SUBJECT CODE : 18BCU113 CLASS : I B.Sc. Biochemistry

Experiment 1: Effect of lipid composition on the permeability of a lipid monolayer.

Principle

The lipid composition of a membrane has a considerable effect on its permeability, and in these experiments, a lipid monolayer is used as a membrane model. If butanol is layered on top of water, then two distinct phases are formed, if amphipathic lipids are present, they will move in to the boundary region. The polar part of the molecules will associate with the top aqueous layer and the hydrophobic region with the organic phase. Methylene blue is a highly colored molecule, and its passage across the boundary can be readily followed by the eye. Unlike biological membranes, this model does not contain protein, but useful information can be obtained from this simple experiment as passive diffusion does depend on the lipid composition of the membrane.

Materials

Fatty acids (stearic acid, oleic acid), acylglycerols (triolein, triphalmitin), phospholipid (egg lecithin), sterol (cholesterol), n-butanol, boiling tubes, methylene blue in butanol (0.25 g/L).

Protocol

Set up boiling tubes each containing 5 mL of water. Carefully pipette 5 mL of butanol containing methylene blue and 200 mg of lipid down the side of each tube to form two distinct layers. Leave the tubes to stand at room temperature for 1-2 h and compare the results with the one obtained using a control tube containing water, but no lipid in the butanol. A rough estimate of the effectiveness of the lipids as permeability barriers can be obtained by measuring the extinction of the methylene blue in the aqueous phase.

Experiment 2: Determination of CMC of detergents.

Purpose:

To determine the critical micelle concentration (CMC) of non-ionic detergent using the surface tension method.

Glassware:

1. 25 µL disposable pipette
2. 6 x 50 nm culture tubes

Reagent:

Prepared freshly prior to the experiment

1. Detergent solution:

   A solution of the detergent should be prepared in distilled water at a concentration that is 10=15 times expected CMC.

Procedure:

1. Height of water in micropipette:

   In to a disposable culture tube pipette 500 µL of distilled water, then insert a 25 µL pipette. Measure the height of the water in the micropipette when the water start rising in the pipette. Record the height of the water in cm and repeat this measurement two more times. It is important to use the same pipette each time and to use the same pipette and to dry it out between measurements. Simply blowing air into the micropipette will dry it sufficiently.

Addition of detergent solution:

Add aliquots of detergent solution to the culture tube containing 500 µL distilled water such that atleast 4 are below and 4 are above the expected CMC for each concentration of detergent the height of the solution in the micropipette is measured 3 times. Micropipette must be dried between measurements to ensure consistency and reproducible result. Blowing the micropipette out several times is sufficient to dry the micropipette.

Determination of CMS

Plot the mean peak heights (cm) versus the millimolar concentration of the detergent. The CMC

is read off from the intersection of the two straight line. One is descending past of the curve and other to the pleateau.

Experiment 3: RBC ghost cell preparation and to study the effect of detergents on membranes.
1

Principle:

Several detergents like molecules disrupts membranes by "dissolving" the phospholipid components. In the case of erythrocytes, this effect can be readily followed in a colorimeter by measuring the absorbance of hemoglobin released from the disrupted cells.

Materials:

Fresh rat blood or time-expired human blood used for transfusion, isotonic saline 8.9 g/L; detergents (1%): Triton X-100 (neutral), cetyltrimethylammonium bromide (cationic), sodium dodecyl sulfate (anionic).

Lysophosphatidyl choline (lysolecithin, 10 mmol/L), progresterone (100 mmol/L in ethanol), hydrocortisone (saturated solution in ethanol, approximately 5 mmol/L), centrifuge tubes (10 mL), incubator at 37°C, colorimeter or spectrophotometer.

Protocol:

Use human blood from a transfusion bottle or collect the blood from a freshly killed rat into a tube containing 0.5 mL of an anticoagulant (40 g/L) trisodium citrate). Centrifuge the cells, wash them twice with isotonic saline, and resuspend them in the same volume as the original blood. Dilute the erythrocyte suspension with saline so that when 0.5 mL is added to 4.5 mL of Triton X-100, an extinction of about 0.8-0.9 is obtained at 540 nm after centrifugation. This represents 100% lysis and all subsequent extinction values should be expressed as percent lysis. Pipette in duplicate 0.5 mL samples of the diluted erythrocytes into 10 mL centrifuge tubes containing 4.5 mL of saline. Mix thoroughly and add 50 µL of the test compound, mix by gentle swirling, and place in an incubator at 37°C for 20 min. Separate any unbroken cells by centrifugation on a bench centrifuge and measure the absorbance of the supernatant solution at 540 nm.

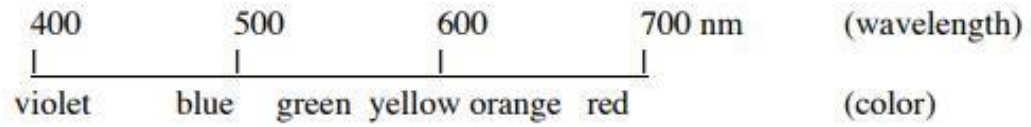
Experiment 4: Separation of photosynthetic pigments by TLC

Plant leaves contain a number of important pigments including chlorophylls, carotenes, and xanthophylls. During the summer when leaves contain large amounts of chlorophyll (and are thus green), the presence of the other pigments is not obvious to the eye. During the fall, however, after most of the chlorophyll has been degraded, these other pigments can be more readily observed, and the

leaves of many plants take on the variety of beautiful colors that are typical of fall foliage. During this lab you will have a chance to collect leaves of various colors and analyze the different pigments that are present.

General information about leaf pigments.

VISIBLE LIGHT SPECTRUM



CHLOROPHYLL
- absorbs red and blue light
- reflects green
- membrane-bound

CAROTENES
- absorb violet, blue, and green light
- reflect yellow, orange, and red
- membrane-bound

XANTHOPHYLLS
- absorb violet, blue, and green light
- reflect yellow, orange, and red
- membrane-bound

ANTHOCYANINS
- absorption variable (green, yellow, orange, red)
- reflected colors variable (violet, blue, red)
- water-soluble

Collection of leaf samples.

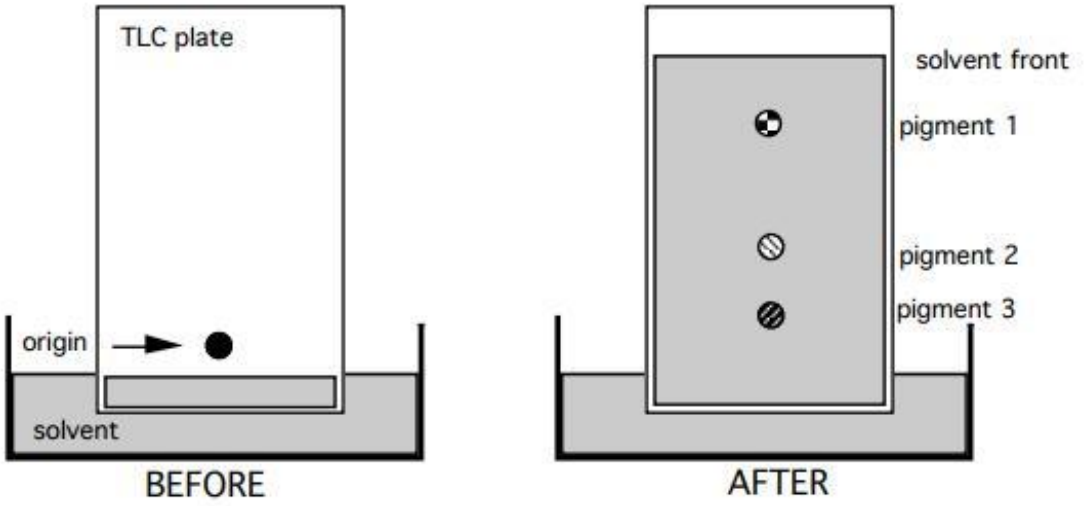
Collect three good sized leaves - one that is fully green and two others that more brightly colored (e.g. red or yellow). Record where you collected these samples and the scientific name of the species from which they were taken.

Separation of pigments by TLC.

Principles of TLC.

Thin-layer chromatography (TLC) is a convenient technique for separating and analyzing the different pigments present in a leaf. A leaf extract containing a mixture of many compounds is spotted onto a TLC plate and an organic solvent is allowed to move up the plate, potentially carrying with it the

various compounds in the leaf extract. The different components of a leaf extract are separated based on their affinities for the stationary phase (the silica on the TLC plate) and for the mobile phase (the solvent that is moving up the plate). Compounds with more affinity for the silica (i.e. hydrophilic compounds) will not move very far, while compounds with a high affinity for the organic solvent (i.e. hydrophobilic compounds) will move much farther.



In this example, a leaf extract containing three pigments was spotted onto a TLC plate at the "origin". The TLC plate was then placed into a container of solvent. As the solvent front moved up the plate, the three pigments moved at different rates. Pigment 3 is apparently quite hydrophilic and therefore moved much more slowly that the solvent front while pigment 1 is apparently more hydrophobic since it moved almost as fast as the solvent front. The mobility of a compound in a particular TLC system is the Rf value.

$$Rf = \frac{\text{distance traveled by the compound}}{\text{distance traveled by the solvent front}}$$

Pigment extraction.

1. Label four 1.5 ml tubes, one for each of your three leaf samples collected from the woods and one for a sample of spinach leaf. Place 250 mg of leaf tissue into each tube and add 400 µl of 90% acetone. 2. Grind each sample thoroughly with a small plastic pestle. Add an additional 600 µl of 90% acetone to each tube and mix them thoroughly using a vortex mixer.

3. Centrifuge your tubes for 3 minutes in a microcentrifuge. Carefully transfer the supernatants to fresh tubes and dispose of the pellets.

4. Add 100 µl of hexane to each tube. Add 200 µl of water to each tube. Mix thoroughly using a vortex mixer so that the leaf pigments will partition into the hexane phase.

5. Centrifuge for 1 minute in a microcentrifuge. *Observe what colors have partitioned into the aqueous and hexane phases. What does this mean?* Carefully transfer the hexane fractions into new tubes. Dispose of the aqueous phases.

6. Add 200 µl of water to each of your pigment extracts. Mix them thoroughly using a vortex mixer.

7. Centrifuge for 1 minute in a microcentrifuge. Carefully transfer the hexane fractions into new tubes. These will be the final pigment extracts that you will use. Each one should contain a concentrated mixture of whatever pigments were present in the leaf from which it was derived.

TLC plates.

1. Draw a faint pencil line (without damaging the silica surface) 1.5 cm from the bottom edge of a TLC plate. Mark four spots along this line (one for each of your pigment extracts), leaving 2 cm of space between each spot.

2. Measure out 10 µl aliquots of each pigment extract and place them into fresh tubes. Place a yellow pipet tip into each tube. Using the pipet tip, apply a small amount of each pigment extract to the appropriate spot. Allow the hexane to evaporate. Apply a little bit more of each extract to the TLC plate and allow the hexane to dry. Keep repeating this process until the entire 10 µl of each pigment extract has been applied to the plate. Allow the TLC plate to dry completely before proceeding to the next step.

3. Place the TLC plate into a chromatography chamber containing petroleum ether acetone-chloroform (3:1:1). Since these organic solvents are quite volatile, smell bad, and are not good for your health if you breathe them, do this step completely inside a fume hood.

4. Observe as the solvent front moves up the TLC plate. You should also be able to see the pigments separating as the TLC plate "develops". Allow the solvent front to travel about 6 cm past the origin. Once the development is complete (about 15 minutes) remove the plate from the chromatography chamber and quickly mark the location of the solvent front with a pencil.

Allow the plate to dry completely before removing it from the fume hood. Photograph your TLC plate with a digital camera as soon as it dries (the colors will fade within a few hours) and then print out a copy (on a color printer) for your notebook.

Record how many pigments were present in each of your samples. Identify what each of these pigments is based on your knowledge of the structure and light absorption properties of plant pigments. Calculate the Rf values for each of the pigments you observed. Previously obtained Rf values for several spinach pigments in a similar TLC plate/solvent system to the one you used are listed below.

carotene	0.98
chlorophyll a	0.59
chlorophyll b	0.42
xanthophyll 1	0.28
xanthophyll 2	0.15

(https://www.colby.edu/academics_cs/courses/BI214/upload/lab3-pigments.pdf)

Experiment 5: Isolation of mitochondria from liver and assay of marker enzyme SDH. ***Principle***: To isolate mitochondria from mouse liver. ***Materials & Reagents:***

1. Centrifuge tubes
2. Mice
3. Potassium salts
4. Sodium salts
5. Sucrose
6. Bovine serum albumin (BSA)
7. Disodium ethylenediaminetetraacetate dihydrate (EDTA)
8. Ethylene glycol-bis(2-aminoethylether)-N, N, N’, N’-tetraacetic acid (EGTA)
9. Dithiothreitol (DTT)
10. HEPES
11. Protease inhibitors (100x)
12. D-Mannitol
13. Magnesium chloride hexahydrate ($MgCl_2$)
14. Potassium hydroxide (KOH)
15. Sodium hydroxide (NaOH)
16. Extraction buffer

Equipment

1. Dounce homogenizer and pestles (A and B)
2. Small scissors
3. Tweezers

Procedure

A. Preparation

1. Starve animals overnight (8-16 h during the dark cycle)

Note: Starvation is optional but leads to more reproducible results, especially in the liver.

2. Prepare a container with ice and place:
   a. Dounce homogenizer and pestles
   b. Three centrifuge tubes per sample (20-50 ml)
   c. One beaker containing extraction buffer per sample
3. Cool centrifuge and rotor to 4 °C. Cool on ice Dounce and pestles (Figure 1).
4. Animal dissection material (Figure 1)
   a. Small scissors
   b. Tweezers

B. **Procedure**

1. Sacrifice the mouse by cervical dislocation, immediately remove the liver and place it in the ice-cold beaker with extraction buffer.
2. Rinse the liver by adding and removing cold fresh extraction buffer until most of the blood is removed (5-6 washes).
3. Mince the liver in the beaker in ice extensively using small scissors until homogeneus (Figure 3)
4. Transfer the minced liver into a Dounce homogenizer and add approximately 3 ml of cold extraction buffer.
5. With the homogenizer placed in the ice container, gently grind the tissue ten times with the A pestle (looser) and another ten with the B pestle (tighter). Avoiding the formation of bubbles is critical to obtain high quality mitochondria (Figure 4).
6. Transfer the homogenate into a centrifuge tube (Figure 5) and complete to 30-40 ml with fresh cold extraction buffer. Follow the differential centrifugation steps (Figure 2)

7. Centrifuge 10 min at 700 *x g* and 4 °C. Pour supernatant to a new ice-cold tube and discard the pellet containing nuclei and intact cells (Figure 6).
8. Repeat the operation centrifuging again at 700 *x g* for 10 min at 4 °C and subsequently pouring the supernatant to a new ice-cold tube.
9. Centrifuge at 10,000 *x g* for 15 min at 4 °C. Discard the supernatant and re-suspend the pellet (Figure 7) in ice-cold extraction buffer.
10. Centrifuge at 10,000 *x g* for 15 min at 4 °C, discard the supernatant and re-suspend the final pellet in the minimal possible volume (around 0.3 ml) of extraction buffer or the specific experimental buffer (Figure 8).

Notes:

1. After isolation, protein concentration is determined by standard methods. Typically, around 30-40 mg of mitochondrial protein are obtained from one liver.

2. The quality of the isolated mitochondria can be determined their respiratory control ratio (RCR) using an oxygen electrode and measuring their oxygen consumption rate in the presence and in the absence of ADP. RCR should range 4-6 with pyruvate plus malate and 1.5-3 with succinate plus rotenone.

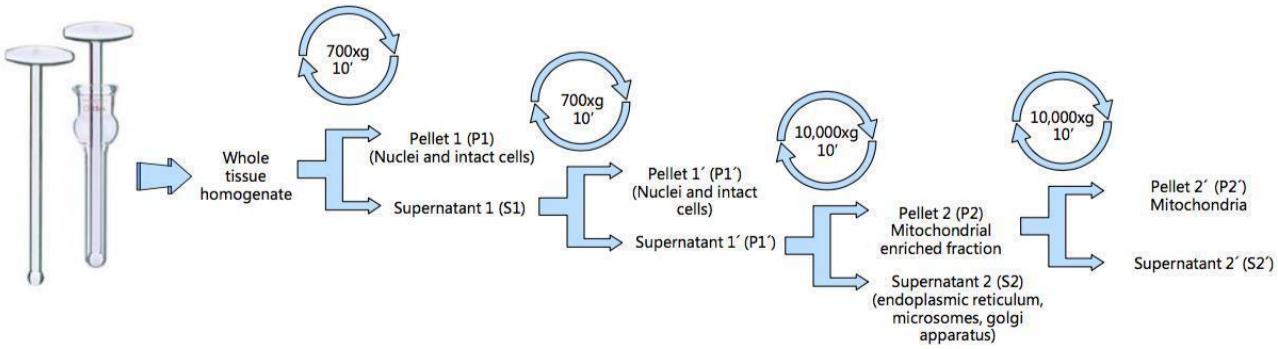


Figure: Mitochondrial isolation by differential centrifugation

Extraction buffer (freshly prepared)

250 mM sucrose
250 mM mannitol
25 mM HEPES
10 mM KCl
0.25 mM EDTA
10 mM EGTA
1.5 mM $MgCl_2$
1 mM DTT
0.1% BSA
1x protease inhibitors
pH 7.4 with KOH or NaOH

Note: The type of salt used can interfere with some functional assays. KOH is recommended for calcium handling experiments, as it prevents the efflux of calcium from the mitochondria through the Na_+/Ca_{2+} exchanger. For membrane potential experiments using safranin O, NaOH is recommended to allow calibration with KOH and valinomycin.

Determination of succinate dehydrogenase activity (SDH)

SDH activity was estimated by the method of Nulton-Persson and Szweda (2001).

Principle:

The activity was determined from the rate of decrease in absorbance at 600 nm after treating the mitochondria with the reaction mixture containing sodium succinate in the presence of the electron accepter DCPIP which is converted to its reduced form

Procedure:

The reaction mixture contained 10 millimol/l sodium succinate, 0.5 mg BSA, 0.9 millimol/l KCN, mitochondrial protein (approximately 20 µg), 80 µmol/l DCPIP and 100 millimol/l phosphate buffer (pH 7.4) in a final volume of 1 ml. The reaction was monitored after the addition of DCPIP at 600 nm for 2 min at 30 sec interval. The activity was calculated using the extinction coefficient of DCPIP (19.1 mM-1 cm -1) and expressed as µmoles of DCPIP reduced/min/mg protein.

Experiment 6: Study photosynthetic O2 evolution in hydrilla plant.

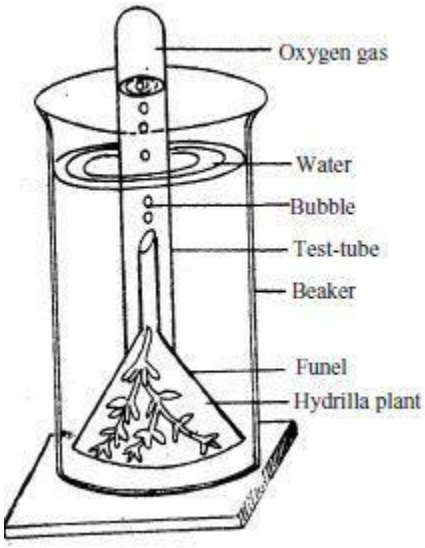
***Requirements*:** A large Beaker, Some Hydrilla Plant, One Funnel, One Test tube, water and a glowing splinter of wood.

***Procedure*:** The Hydrilla plant should be kept in the beaker and covered with the funnel. Hydrilla plant be placed in such a way that its cutting ends remain towards the stem of the funnel. Water should be poured in the beaker in such a way that the stem of the funnel kept beneath the water level. A test tube completely filled with water should be inverted over the stem of the funnel. At this position the

experimental set should be placed in Sunlight (or under electric light in the laboratory).

Observation: After sometimes it will be seen that bubbles are coming out from the Hydrilla plant and are accumulating in the test tube. After accumulation of some gas the test tube should be taken out carefully and a glowing splinter of wood be thrusted into the test tube, it will burst into flame.

Conclusion: As the gas helps the glowing splinter to flame so the gas is Oxygen, because Oxygen does not flame itself but helps other to flame.



Experiment 7: Isolation of chloroplast from spinach leaves, estimation of chlorophyll

Materials Required

1.Spinach leaves 30 grams.
2.Knife and scissors.
3.Cutting board.
4.Kitchen blender.
5.Muslin cloth.
6.Glass beaker.
7.50 ml centrifuge tubes.
8.1.5 ml centrifuge tubes.
9.Micropipette.

10.Glass pipette.

11.Cooling centrifuges.

12.Spectrophotometer.

Reagents and buffers

1. 1x Chloroplast isolation buffer without BSA:- 0.33M sorbitol, 0.1M tris-Cl ph 7.8, 5mM MgCl2, 10mM NaCl, 2mM EDTA.
2. 1x Chloroplast isolation buffer with BSA (0.1%w/v):
3. 40% percoll: 4ml percoll and 6 ml 1x CIB buffer with BSA to make 10 ml of 40% percoll. (Use 10 ml of 40% percoll for 6ml of chloroplast suspension)
4. 80% acetone.

Procedure

1. Wash 30 gms of spinach leaves thoroughly first with tap water and then with distill water.
2. Remove the midrib veins of the leaves and cut into small pieces.
3. Add 120 ml of 1xCIB buffer with BSA to the cut leaves in a blender. Blend with 2-3 strokes.
4. Filter the blended leaves through 6 layers of muslin cloth.
5. The filtrate is then evenly divided into four 50 ml centrifuge tubes.
6. Centrifuge the tubes for 3 minutes at 200xg. A white pellet will be obtained.
7. Transfer the supernatant into chilled 50 ml centrifuge tubes and centrifuge at 1000xg for 7 minutes. A green pellet will be obtained.
8. Discard the supernatant and break the green pellet gently by finger tapping.
9. Resuspend the pellet in 2ml of 1x CIB buffer with BSA and mix gently by pipetting up and down.
10. Pool the suspended pellet into one centrifuge tube.
11. Preparation of 40% percoll layer: Mix 4 ml percoll with 6 ml of 1x CIB buffer with BSA.
12. Gently overlay 6ml of the chloroplast suspension over this 40% percoll layer.
13. Centrifuge at 1700 xg for 6 minutes. The intact chloroplast will sediment to the bottom of the tube as a green pellet and the broken chloroplast will form the upper layer.
14. Carefully remove the upper layer of the chloroplast suspension leaving only the pellet containing the intact chloroplast.

15.Mix the pellet with 500 ul of 1x CIB buffer without BSA.

Estimation of chlorophyll concentration

16.Add 10 ul of chloroplast suspension to 990ul of 80% acetone solution and mix gently.

17.Centrifuge at 3000xg for 2 minutes.

18.Take 100ul of the supernatant and transfer into a cuvette and measure the absorbance at 650 nm. Use 100 ul of 80% acetone as blank.

19.Take duplicate OD 650 values.

20.Take the average of the two values and estimate the mg/ml chlorophyll concentration using the following formula:

A 650 x 100/36 = mg/ml chlorophyll.

Where A 650 is the absorbance at 650 nm, 100 is the dilution factor and 36 is the extinction coefficient of chlorophyll.

Difference Encountered in a Real Laboratory

In an actual laboratory setting, there are certain important steps that are not necessarily applicable in a virtual lab:

1. Before starting the experiment sterile the laminar air flow chamber using spirit.
2. Always disinfect your work area when you are finished.