B.Sc. BOTANY LAB MANUAL

6th Semester

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C13P: Plant Metabolism

1. Chemical separation of photosynthetic pigments.

Principle: The estimation of chlorophyll 'a' and 'b' is based on their absorption at 645 and 663 nm. Although both the pigments absorb light at both of these wavelengths, molar absorption of chlorophyll 'a' is more pronounced at 663 nm and that of chlorophyll 'b' at 645 nm. No other pigment present in leaves absorb at these wavelengths. Thus estimation of chlorophyll (like any other colored compound) is based on Beer's law, according to which the light absorbed by a solution is directly proportional to the concentration of the compound. Chlorophylls are extracted from the leaves with acetone.

Materials: Sample (leaves of any plant).

ii Acetone (85%) with water.

iii Mortar-pestle, test tubes, measuring cylinder.

iv Centrifuge tubes, centrifuge, spectrophotometer.

Method: 1 . Grind I00 mg green leaves vigorously in mortar pestle with I 0 ml of the acetone.

2. Centrifuge the homogenate at 5000 rpm for I 0 min.

3. Transfer the clear supernatant to a 25 m1 measuring cylinder.

4. Re-extract the residue with 5 ml of the acetone, centrifuge and transfer the supernatant to the measuring cylinder.

5. Make up the volume of supernatant to 20 ml with the acetone.

6. Read O.D. at 645 and 663 nm, taking 85°/o acetone as control.

Observations:

Weight of the sample = 100 mg

ii O.D. at 645 nm = A645

iii O.D. at 663 nm = A663

Calculation:

Chlorophyll'a' (mg/1) = 12.7 A663 - 2.69 A645

Chlorophyll'b' (mg/1) = 22.9 A645 - 4.68 A663

Total chlorophyll {mg/1) = 20.2 A645 + 8.02 A663

From these values chlorophyll (mg/g sample) can be calculated using following expression:

Chlorophlyll (mg/g sample) = Total volume of the extract Chlorophyll (mg/g sample) X Total volume of the extract / 1 000 X Sample wt (g)

Result Chlorophyll contents in the given sample are:

Chlorophyll'a'……. mg/g

Chlorophyll 'b'…… …mg/g

Total chlorophyll = ……..mg/g

2. **To study the effect of light intensity (by changing the distance) on the rate of photosynthesis using aquatic plant**.

Material required: Beaker, funnel, test tubes, watch/clock, Table lamp, scale, blade, clips/rubber ring, sodium bicarbonate and fresh twigs of *Hydrilla/ Vallisneria / Ceratophyllum / Eichhornia / Nymphaea* etc.

Procedure:

- Take a known amount of actively photosynthesizing Hydrilla plants in a beaker full of water.
- Keep a funnel inverted over the plants. Keep plants in such a way that cut end of the twig face upwards.
- Keep a test tube full of water inverted on the funnel.
- Keep the experimental set up in front of table lamp at the distance of 10 cm & switch on the table lamp.
- When bubbles appear, count the number of bubbles for 1 minute.
- Record your observations as shown in table given below. Count the no. of bubbles for 4 more times at same distance by giving 2 minutes resting time between each trial. Record your results in observation table.
- Now repeat this step by keeping the table lamp at the distance of 20 cm & 30 cm & count the number of bubbles produced per minute.
- Plot a graph between numbers of bubbles evolved per minute from Hydrilla plants for different distances..

Observation
The rate of evolution of bubbles varies with distance.
Result: The rate of photosynthesis decreases with increase in the distance from light source.

PRECAUTION:

- Twigs should be placed such that their cut ends are facing upwards in the stem of the inverted funnel.
- Fresh Hydrilla to be used.
- Hydrilla twigs should be obliquely cut.

Observation Table:

Distance from source of light (cm)	Number of air bubbles evolved per minute					Average
	Trials					
	1	2	3	4	5	
10						
20						
30						

3. Effect of carbon dioxide on the rate of photosynthesis.

Material required:

Beaker, funnel, test tubes, sodium bicarbonate solution (0.05%,0.1%,0.15%,0.2% and 0.25%) and fresh twigs of *Hydrilla,Vallisneria, Ceratophyllum, Eichhornia, Nymphaea, Elodea, Cabomba* etc.

Procedure:

1. Take a known amount of actively photosynthesizing Hydrilla plants in a beaker containing 0.05% of sodium bicarbonate solution.
2. The amount of Hydrilla plants taken must be kept constant throughout the experiment. Keep a funnel inverted over the plants. Keep plants in such a way that all the cut ends of the twigs face upwords.
3. Now keep a test tube full of water inverted on the funnel.
4. Keep the experimental set up in light and count the number of air bubbles evolved in each minute for 10 minutes.
5. Record your results in observation table
6. Repeat the above procedure with 0.1%, 015%, 0.20% and 0.25% solutions of sodium bicarbonate in the beaker.
7. Count the air bubbles evolved for 00.5%, 0.1%, 0.15%, 0.20% and 0.25% solutions. The volumes of the solutions taken should be same for all the strengths of the solutions.
8. Plot a graph between numbers of O2 bubbles evolved per minute from Hydrilla plants for different concentrations of CO2.

Observation:

The rate of evolution of bubbles varies with concentration.

Table: The number of air bubbles evolved per minute from Hydrilla plants in different concentration of carbon dioxide.

Concentration of $NaHCO_3$ solution (%)	**Number of air bubbles evolved per minute**										**Average**
	Trials										
	1	2	3	4	5	6	7	8	9	10	
0.05											
0.10											
0.15											
0.20											
0.25											

Result: As concentration of CO2 increases rate of photosynthesis (no. of bubbles evolved) also increases.

C14P: Plant Biotechnology

Preparation of MS medium

MAJOR COMPONENTS OF MS MEDIA

The media involves the following four major components:

1. Inorganic nutrient: It includes mineral salts that are important for the growth and development of the plants. It is categorized into two groups: Macronutrients (Calcium, magnesium, nitrogen) and micronutrients (copper, iron, and zinc).
2. Organic nutrient: It mainly includes vitamins and amino acids, required for the growth and differentiation of the cultures.
3. Growth hormones: It includes auxins, cytokinins, and gibberellins. It is essential for the growth and development of tissues and organs.
4. Gelling agents: It includes agar and gelatin. It provides support to the cultures for their establishment.

You can refer to the article “Major Components of Tissue Culture Media” to read more about the components of the media.

MEDIA PREPARATION

Preparation of stock

1. Micronutrient Stock (100X)

- Take 400 ml double-distilled water in a 1L beaker, then weigh the salts given in the table below and dissolve it in the water.
- Transfer the solution to the 1L volumetric flasks, and make up the volume to 1L. Pipette out 10 ml of the solution to make 1L MS media.

Salts	Concentration - 100X (mg/l)
MnSO4.4H2O	2230
ZnSO4.4H2O	860
H3BO3	620
KI	83
Na2MoO4.2H2O	25
CuSO4.5H2O	2.5
COCl2.6H2O	2.5

2. Iron Stock (20x)

- Take 80 ml double-distilled water in a 100 ml beaker, weigh the components given in the table and dissolve it completely (in the same order as given in the table).
- Transfer the solution to a volumetric flask of 100 ml and makeup to the final volume.
- Pipette 5 ml of the stock solution for 1L of MS media.

Components	Concentration-20X (mg/100 ml)
Na2EDTA	672
FeSO4.7H2O	556

3. Vitamin Stock (100x)

- Take a 100 ml beaker and add 50 ml double-distilled water in it. Weigh the vitamins given in the table below and dissolve it completely in the water.
- Transfer the solution to a volumetric flask of 100 ml and makeup to the final volume.
- Pipette out 1 ml of the vitamin stock solution for 100ml of MS media.
- Add vitamins after the media is autoclaved to protect it from heat degradation. Vitamins can be sterilized by ultrafiltration technique.

Components	Concentration-100X (mg/100ml)
Glycine	20
Nicotinic acid	5
pyridoxine.HCl	5
Thiamine.HCl	1

NOTE: Discard the vitamin solution after 30 days.

3. Cytokinin Stock (100X)

- Weigh “10mg kinetin” and dissolve it into a few drops of 1N HCl.
- Add a few ml of double-distilled water to the above solution and transfer it to the 100 ml volumetric flask and makeup to the volume.
- Store the stock in the refrigerator.
- Use 1 ml of the stock for 1L of the MS media.

Steps of the Preparation

Take 400 ml double-distilled water in a 1L beaker. Weigh the macronutrients given in the table below, and dissolve them completely into the water.

Macronutrients	Concentration-100X (mg/L)
(NH4)NO3	1650
KNO3	1900
CaCl2.2H2O	440
MgSO4.7H2O	370
KH2PO4	170

1. From the prepared stock solutions, pipette out 5ml iron, 10 ml micronutrient, and 1ml kinetin to the 1L beaker of the media.
2. Weigh 100 mg Myo-inositol and dissolve it in the previous mixture.
3. Weigh 10 mg IAA and dissolve it into a few drops of 1N NaOH. Then, transfer the solution to the previous mixture.

NOTE: The stock of IAA is not prepared because of its oxidative degradation.

1. Add 800 ml of double-distilled water in the beaker and adjust the pH of the media to 5.7.
2. Transfer the prepared solution to a 1L volumetric flask and make up the final volume to 1L.
3. Keep the solution in the refrigerator.

Final steps

1. Sterilize all the equipment and glass used for the tissue culture process.
2. Weigh 0.8g of supreme grade agar and 3.0g reagent-grade sucrose and transfer them to 250 ml Erlenmeyer flask.
3. Add 100 ml of the stored MS media, in the flask and seal the cap with aluminium foil.
4. Sterilize the flask with the media.
5. After sterilizing the media for 15-20 minutes, add 1 ml vitamin solution.
6. Swirl the flask for the dissolution of the vitamin, agar, and sucrose into the media, before pouring it into the culture bottles.
7. Pour the media into culture jars and store them in the refrigerator for 1 hour, before the culturing process.

Now, your culture bottles are ready for the tissue-culture processes.

Isolation of Protoplasts:

Protoplasts are isolated by two techniques

1. Mechanical method

2. Enzymatic method

Mechanical Method:

Protoplas isolation by mechanical method is a crude and tedious procedure. This results in the isolation of a very small number of protoplasts.

The technique involves the following stages (Fig. 44.1):

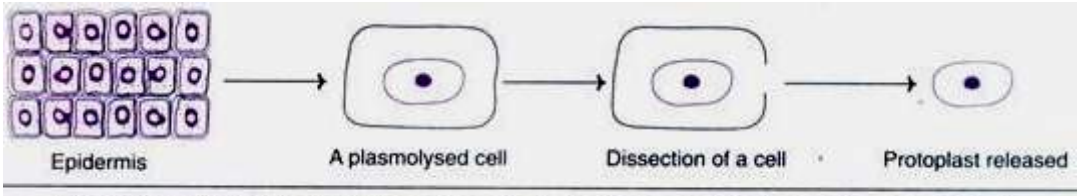

Fig. 44.1 : A diagrammatic representation of mechanical method for the isolation of protoplasts.

1. A small piece of epidermis from a plant is selected.

2. The cells are subjected to plasmolysis. This causes protoplasts to shrink away from the cell walls.

3. The tissue is dissected to release the protoplasts.

Mechanical method for protoplast isolation is no more in use because of the following limitations:

i. Yield of protoplasts and their viability is low.

ii. It is restricted to certain tissues with vacuolated cells.

iii. The method is laborious and tedious.

However, some workers prefer mechanical methods if the cell wall degrading enzymes (of enzymatic method) cause deleterious effects to protoplasts.

Enzymatic Method:

Enzymatic method is a very widely used technique for the isolation of protoplasts. The advantages of enzymatic method include good yield of viable cells, and minimal or no damage to the protoplasts.

Sources of protoplasts:

Protoplasts can be isolated from a wide variety of tissues and organs that include leaves, roots, shoot apices, fruits, embryos and microspores. Among these, the mesophyll tissue of fully expanded leaves of young plants or new shoots are most frequently used. In addition, callus and suspension cultures also serve as good sources for protoplast isolation.

Enzymes for protoplast isolation:

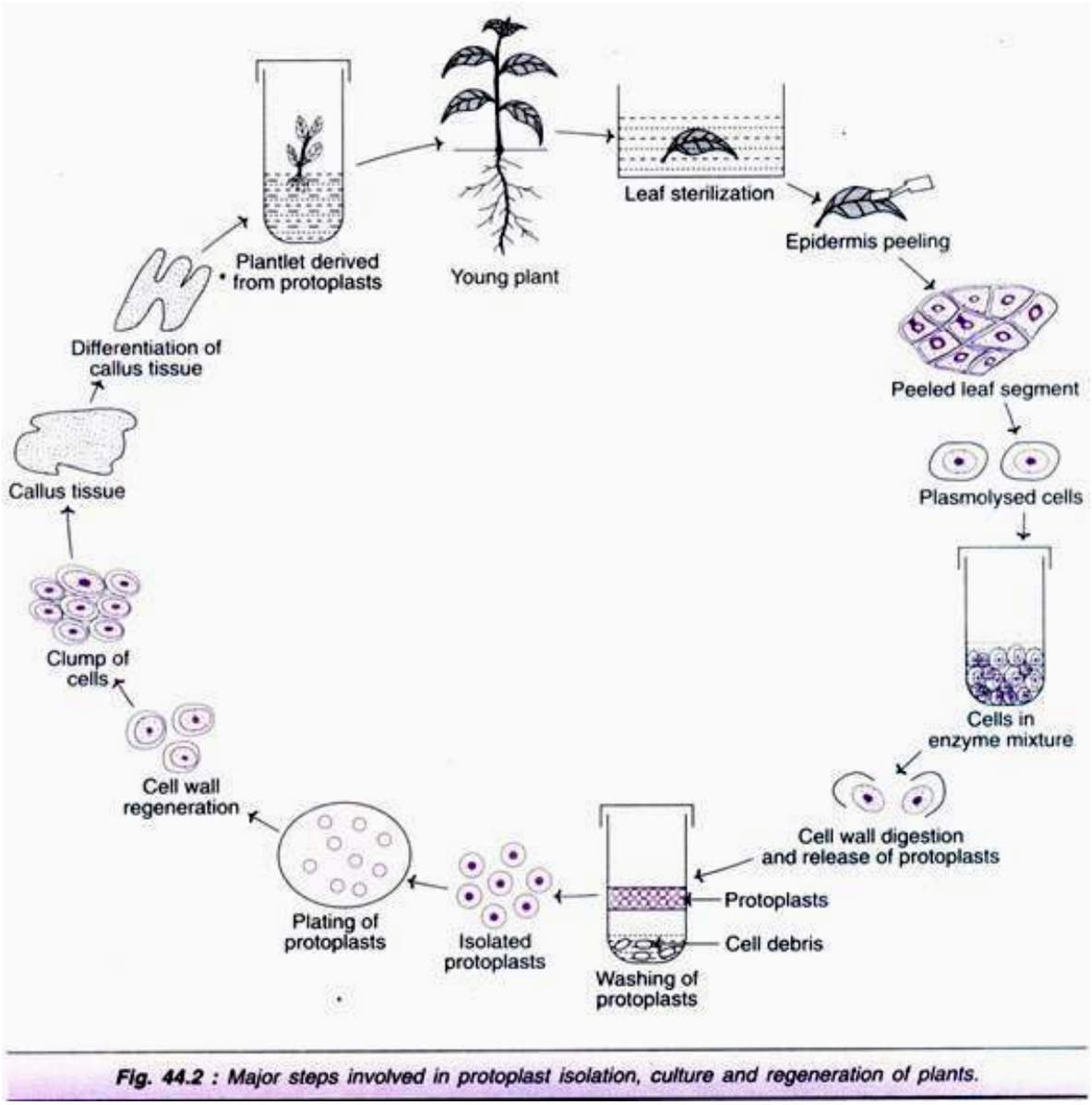

Fig. 44.2 : Major steps involved in protoplast isolation, culture and regeneration of plants.

The enzymes that can digest the cell walls are required for protoplast isolation. Chemically, the plant cell wall is mainly composed of cellulose, hemicellulose and pectin which can be respectively degraded by the enzymes cellulose, hemicellulose and pectinase. The different enzymes for protoplast isolation and the corresponding sources are given in Table 44.1.

In fact, the various enzymes for protoplast isolation are commercially available. The enzymes are usually used at a pH 4.5 to 6.0, temperature 25-30°C with a wide variation in incubation period that may range from half an hour to 20 hours.

The enzymatic isolation of protoplasts can be carried out by two approaches:

1. Two step or sequential method:

The tissue is first treated with pectinase (macerozyme) to separate cells by degrading middle lamella. These free cells are then exposed to cellulose to release protoplasts. Pectinase breaks up the cell aggregates into individual cells while cellulose removes the cell wall proper.

2. One step or simultaneous method:

This is the preferred method for protoplast isolation. It involves the simultaneous use of both the enzymes — macerozyme and cellulose.

Isolation of protoplasts from leaves:

Leaves are most commonly used, for protoplast isolation, since it is possible to isolate uniform cells in large numbers.

The procedure broadly involves the following steps (Fig. 44.2):

1. Sterilization of leaves.

2. Removal of epidermal cell layer.

3. Treatment with enzymes.

4. Isolation of protoplasts.

Besides leaves, callus cultures and cell suspension cultures can also be used for the isolation of protoplasts. For this purpose, young and actively growing cells are preferred.

Purification of protoplasts:

The enzyme digested plant cells, besides protoplasts contain undigested cells, broken protoplasts and undigested tissues. The cell clumps and undigested tissues can be removed by filtration. This is followed by centrifugation and washings of the protoplasts. After centrifugation, the protoplasts are recovered above Percoll.

Viability of protoplasts:

It is essential to ensure that the isolated protoplasts are healthy and viable so that they are capable of undergoing sustained cell divisions and regeneration.

There are several methods to assess the protoplast viability:

1. Fluorescein diacetate (FDA) staining method—The dye accumulates inside viable protoplasts which can be detected by fluorescence microscopy.

2. Phenosafranine stain is selectively taken up by dead protoplasts (turn red) while the viable cells remain unstained.

3. Exclusion of Evans blue dye by intact membranes.

4. Measurement of cell wall formation—Calcofluor white (CFW) stain binds to the newly formed cell walls which emit fluorescence.

5. Oxygen uptake by protoplasts can be measured by oxygen electrode.

6. Photosynthetic activity of protoplasts.

7. The ability of protoplasts to undergo continuous mitotic divisions (this is a direct measure).

Isolation of plasmid DNA. Introduction

The isolation of plasmid DNA from *E. coli* using an alkaline lysis is a well-established method. *E. coli* with plasmid is cultured in media with antibiotics to a high cell density, harvested, and then lysed with a SDS/NaOH solution. Rapid acidification using concentrated potassium acetate causes the precipitation of protein and chromosomal DNA. Plasmid DNA, which is supercoiled, remains in solution and can be captured on a silica spin column. The plasmid DNA is washed with an ethanol solution and then eluted in water or TE buffer.

Material

- Microfuge tubes
- Resuspension buffer (50 mM Tris HCl, pH 8, 10 mM EDTA, 100 µg/ml RNase A)
- Lysis buffer (0.2 N NaOH, 1% SDS)
- Neutralization buffer (3/5 M Potassium acetate, pH 6)
- Spin columns (Product Number SSC 100-01)
- Isopropanol
- Wash buffer (70% Ethanol)
- Elution buffer (water or TE buffer- 10 mM Tris, pH 8, 1 mM EDTA)

Equipment

- Vortexer
- Centrifuge

Protocol

1. Culture *E. coli* with plasmid in LB media with antibiotic selective pressure, overnight on a shaker at 37°C.
2. Pellet 1.5 ml of bacterial culture in a microfuge tube by centrifuging for 2 minutes at 10,000 rpm.
3. Decant the supernatant and add 200 µl of the resuspension buffer. In order to resuspend the pellet you may have to vortex.
4. Add 250 µl of the lysis buffer, invert the tube 10 times to mix thoroughly. The solution should become clear and viscous.
5. Add 350 µl of the neutralization buffer, invert the tube 10 times or until a precipitate forms. The precipitate is a mixture of protein and chromosomal DNA.
6. Centrifuge the tube for 10 minutes at 10,000 rpm. Transfer the supernatant to a microfuge tube and add 0.7 isopropanol. Incubate at -20°C for 15 minutes.
7. Transfer the solution to a spin column.
8. Centrifuge the spin column for 1 minute at 7,000 rpm. Discard the flow through.
9. Add 400 µl of the wash buffer and centrifuge for 1 minute at 7,000 rpm. Discard the flow through. Repeat this step.
10. Centrifuge for an additional 2 minutes at 10,000 rpm to remove residual wash buffer.
11. Transfer the column to a clean microfuge tube. Add 50 µl of elution buffer and centrifuge for 1 minute at 10,000 rpm.

Results

Table 1: Eluted plasmid was read on a DeNovix spectrophotometer, to determine concentration. The machine was blanked with DI water.

Sample Name	Concentration (ng/µl)	260/280	260/230
E. coli DH5α with pSVβ	1143.41	2.06	2.38

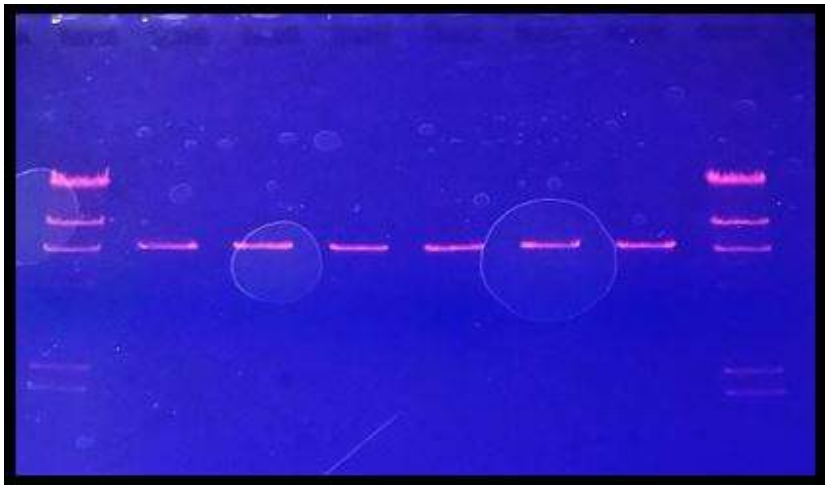

Figure 1: The isolated plasmid under went an enzymatic digest with HindIII (sourced from NEB) and was run on an Agarose Gel with Ethidium Bromide, along side two lanes of digested Lambda ladders (