DEPARTMENT OF SOIL SCIENCE & AGRICULTURAL CHEMISTRY,
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PARBHANI

PRACTICAL MANUAL

Course No. : BIOCHEM-231

Course Title : Fundamentals of Plant Biochemistry and Biotechnology

Course : B.Sc. (Hons.) Agriculture

Semester : III (New)

Name of the Student :

Registration No. :

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PRACTICAL MANUAL

Course No. : BIOCHEM - 231 (New) Credits : 3 (2+1)
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CERTIFICATE

This is to certify that Shri / Miss ……..…… Reg.No……..... a student of **IIIrd (New)** Semester, **B.Sc. (Hons.) Agriculture** has completed all the exercises successfully for the Course : **Fundamentals of Plant Biochemistry and Biotechnology**, Course No. : **BIOCHEM - 231,** during Monsoon Semester 201 - 201 .

Place :

Date : **COURSE TEACHER**

Remarks :

Exercise No. : 01

Preparation of Solutions, pH and Buffers

Standard solution means a solution which the concentration is known, irrespective of the manner in which its concentration is expressed. The concentration of solutions may be expressed in a number of ways, the general methods are :

1. The per cent solutions
2. Molarity and or molar solutions.

* **Atomic weight** of element is proportional to the **actual weight** of an element, hence it is customary to express in **grams**.

* The **atomic weight** of an element expressed in **gram**, is called **Gram atomic weight** or simply **gram atom**.

* Similarly, **Molecular weight** of any given substance is simply the **Gram molecular weight or gram-mole.**

Gram mole = Mole

Molarity (M) : The conc. of solute in a solution expressed as the **number of moles** of solute **per liter** of solution.

$$\text{Molarity} = \frac{\text{Moles of solute}}{\text{Litre of solution}}$$

Molality (m) : The conc. of solute in a solution expressed as the **number of moles** of solute **per kilogram** of solvent.

$$\text{Molality} = \frac{\text{Moles of solute}}{\text{Kilogram of solvent}}$$

Equivalent weight : Equivalent weight of an element may be **defined** as that quantity of the element in grams which reacts with or displace 1 gram atom of hydrogen or 1 gram atom of oxygen or 1 gram of chlorine.

$$\text{Gm Equivalent weight} = \frac{\text{Molecular weight}}{\text{Replaceable } H^+ \text{ or } OH^- \text{ or } Cl^-}$$

Solution : A solution consists of a **Solute** (one substance) dissolved in **Solvent** (another substance).

Concentration : The concentration of a solution is the amount of the solute dissolved per unit volume or weight of the solvent.

The concentration of solution can be expressed in a number of ways :

(a) Gram of solute per 100 gm of the solvent (w/w) per cent

(b) Gram of solute per 100 ml of the solvent (w/v) per cent

(c) Gram of solute per 1000 ml (litre) of the solvent (w/v) Molar

(d) Gram of solute per 1000 ml (litre) of the solvent (w/w) Molal

(e) Milli litre (ml) per 1000 ml (litre) of the solvent (v/v)

Normal solution (N) : A Normal solution (N) is one which is prepared by dissolving 1 gram equivalent of the solute per 1000 ml (litre) of the solution. The unit symbol N is used to denote "mol/L". Sometimes "Ëq/L" also used..

$$\text{Equivalent weight} = \frac{\text{Molecular weight}}{\text{Valency}}$$

$$\text{Normality} = \frac{\text{Amount of substance gm/L of solution}}{\text{Eq. weight of substance.}}$$

Chemical substance	Equivalent weight	1 N = Eq. wt. x1	2 N = Eq. wt. x 2	0.5 N = Eq. wt.x 0.5(N/2) = Eq. wt /2	1 N = Eq. wt. x 0.1 (N/10) = Eq. wt. /10
HCl	36.45	36.45	72.90	18.23	3.65
H_2SO_4	49.01	49.01	98.02	24.51	4.90
H_3PO_4	32.64	32.64	65.29	16.32	3.26
NaOH	40.00	40.00	80.00	19.99	4.00
$Ca(OH)_2$	37.03	37.03	74.05	18.51	3.70
Na_2CO_3	52.97	52.97	105.94	26.49	5.30

Equivalent weight of acids :

The equivalent weight of an acid is that weight of acid which contains one replaceable H atom. Thus the molecular weight itself is the equivalent weight for monobasic acids, for dibasic acids it is mole weight divided by two, for tri basic molecular weight divided by three --------

Acid	Mol. weight	Valency	Eq. wt.
HCl	36.45	1	36.45
H_2SO_4	98.02	2	49.01
H_3PO_4	97.93	3	32.64

Equivalent weight of bases :

The equivalent weight of an base is that weight of acid which contains one replaceable OH group. 17.008 g OH are equivalent to 1.008 grams of H. Thus, equivalent weight of NaOH, KOH, NH_4OH are equal to its molecular weight, whereas equivalent weight of $Ca(OH)_2$, $Ba(OH)_2$ the molecular weight divided by two.

Base	Mol. weight	Valency	Eq. wt.
NaOH	40.00	1	40.00
$Ca(OH)_2$	74.05	2	37.03
Na_2CO_3	105.94	2	52.97

Equivalent weight of acids :

The equivalent weight of an acid is that weight of it which contains one gram equivalent weight of replaceable hydrogen (all acids contains replaceable H atoms).

Example 1 :

One mole of HCl contains one replaceable hydrogen atom, hence the gram equivalent weight of HCl (1:1) i.e. (1 + 35.5 = 36.5).

$$\text{Equivalent weight of HCl} = \frac{36.5}{1} = 36.5 \text{ gm}$$

Example 2 :

One mole of H_2SO_4 contains two replaceable hydrogen atom, hence the equivalent weight of H_2SO_4 (2:1:4) i.e. (2 + 32.6 + 63.96 = 98.56).

$$\text{Equivalent weight of } H_2SO_4 = \frac{98.565}{2} = 49.28 \text{ gm}$$

Equivalent weight of Bases :

The gram equivalent weight of base is that weight of the base, which contains one gram equivalent weight of the hydroxyl ion (all acids contains replaceable H atoms).

Example 1 :

One mole of NaOH contains one replaceable hydroxyl ion, hence the gram equivalent weight of NaOH (1:1:1) i.e. (23 + 16 + 1 = 40).

$$\frac{40.00}{}$$

$$\text{Equivalent weight of NaOH} = \frac{\text{Molecular weight}}{1} = \frac{40.00}{1} = 40.00 \text{ gm}$$

Example 2 :

One mole of $Ca(OH)_2$ contains two replaceable hydroxyl ion, hence the equivalent weight of $Ca(OH)_2$ (1:2:2) i.e. (40.02 + 31.98 + 2 = 74).

$$\text{Equivalent weight of } Ca(OH)_2 = \frac{74}{2} = 37 \text{ gm}$$

Dilution of acids :

Example 1 : Prepare 500 ml, 1 N solution of Sulphuric acid (H_2SO_4) from concentrated acid (purity 99 %, Specific gravity 1.84 and Molecular weight 98.08).

Eq wt. of H_2SO_4 = 49.04 (98.08 / 2 i.e. replaceable H)

$$\text{Required vol. of conc. acid (Vl) for the solution} = \frac{\text{Eq. wt. of acid} \times V_2 \times \text{Normality} \times 100}{1000 \times \text{Sp. Gravity} \times \text{Purity (\%)}}$$

$$Vl = \frac{49.04 \times 500 \times 1 \times 100}{1000 \times 1.84 \times 99}$$

$$= 13.46 \text{ ml}$$

Answer : 13.46 ml. conc. H_2SO_4 is to be diluted in 500 ml distilled water in a volume flask to get 1 N solution of H_2SO_4 .

Example 2 :

Prepare 750 ml, 0.1 N solution of Orthophosphoric acid (H_3PO_4) from conc. acid (purity 93 %, Sp. Gr. 1.75, and Mol. Wt. 98.00).

Eq wt. of H_3PO_4 = 32.66 (98.00 / 3 i.e. replaceable H)

$$\text{Required vol. of conc. acid (Vl) for the solution} = \frac{\text{Eq. wt. of acid} \times V_2 \times \text{Normality} \times 100}{1000 \times \text{Sp. Gravity} \times \text{Purity (\%)}}$$

$$Vl = \frac{32.66 \times 750 \times 0.1 \times 100}{1000 \times 1.75 \times 98}$$

$$= 1.43 \text{ ml}$$

Answer : 1.43 ml. conc. H_3PO_4 is to be diluted in 750 ml distilled water in a volume flask to get 0.1 N (N/10) solution of H_3PO_4 .

Parts per million (ppm) concept :

ppm solutions are those solutions in which a substance is present in a very small quantity.

It represent Gram of solute per million grams of solution OR Gram of solute per million ml of solution.

Thus, 1 ppm of NaCl solution represents :

1 ml ppm NaCl = 1 mg NaCl / litre of solution
= 1mg NaCl / 1000 ml solution
= 1 μg NaCl / ml of solution.

Therefore , 100 ppm NaCl = 100 mg NaCl in 1000 ml solution
and 1000 ppm NaCl = 1 gm NaCl /litre.

Example :

How would you prepare 100 ppm phosphate solution by using KH_2PO_4 (100 ppm = 100 mg / Lit. or 0.1 gm / Lit PO_4)

Answer : Molecular wt of KH_2PO_4 = 39.1 (2x1) + 30.97 + (4x15.9)
= 136.03
1 gm molecular wt of PO_4 = 30.97

30.97 gm of P content in 136.03 gm of KH_2PO_4**.**

$$\text{Thus, for 100 mg or 0.1 gm p} = \frac{136.03 \times 0.1}{30.97}$$

For 100 ppm P solution = 0.439 gm KH_2PO_4 OR

1000 ppm P solution = 4.39 gm KH_2PO_4.

Example :

How would you prepare 100 ppm potash solution by using KCl (100 ppm = 100 mg / L or 0.1 gm / L K)

Answer : Molecular wt of KCl = 39.1 + 35.45
= 74.55
1 gm molecular wt of KCl = 39.1

39.1 gm of K content in 74.55 gm of KCl**.**

$$\text{Thus, for 100 mg or 0.1 gm K} = \frac{74.55 \times 0.1}{39.1}$$

For 100 ppm K solution = 0.191 gm KCl OR

1000 ppm K solution = 1.91 gm KCl.

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Exercise No. : 2 & 3
Qualitative Tests for Carbohydrates and Amino Acids

(A) Qualitative Tests for Carbohydrates

Carbohydrates are defined as **"aldehyde or ketone derivatives of polyhydroxy alcohols"**. Hence each carbohydrate contains either aldehyde (-CHO) or ketone (C=O) group in its molecular structure. These functional groups may be free or involved in the formation of glycosidic linkages. Most of the chemical properties of the carbohydrates are mainly due to these groups. On the basis of number of sugar units involved in the structure, and their behavior on hydrolysis, carbohydrates, are classified as under:

1. Monosaccharides : Simple sugars that cannot be split further by hydrolysis

a) **Aldoses** : Monosaccharides containing – CHO group e.g. glucose, galactose, mannose.

b) **Ketoses** : Monosaccharides containing C=O group e.g. fructose.

All Monosaccharides are reducing sugars.

2. Oligosaccharides :

Sugars that yield two to ten molecules of Monosaccharides on hydrolysis.

A) **Disaccharides** : Sugars made up of two Monosaccharides, e.g. sucrose, lactose, maltose.

i)**Reducing sugar** : Sugars having free or potentially free – CHO or C=O groups, e.g. lactose, maltose

ii)**Non-reducing sugar** : Sugars having no free or potentially free – CHO or C=O groups e.g. sucrose

B) **Trisaccharides** : Sugars made up of three Monosaccharides e.g. Raffinose

C) **Tetrasaccharides** : Sugars made up to four monosacccharides e.g. stachyose

3. **Polysaccharides** : Carbohydrates made up of more than ten molecules of monosaccharides, e.g. starch, cellulose, glycogen.

The tests for detection of carbohydrates in the sample are usually based on the chemical reaction with functional groups. Following tests are carried out for qualitative detection of carbohydrates in the sample. **The qualitative tests only indicate the presence of compound in the sample**.

TEST - 1 : Anthrone reaction

- **Principle** : The polysaccharids and Oligosaccharides are hydrolyzed to simple sugars by conc. H_2SO_4. All these simple sugars are further dehydrated and cyclized to furfural by conc. H_2SO_4. The furfural so formed reacts with anthrone to produce blue colour complex.
- **Reagents** : **Anthrone solution** (0.2% in conc. H_2SO_4): Dissolve 0.2g anthrone in 100 ml conc. H_2SO_4. Keep the solution in

coloured bottle or in dark. Aalkways prepare fresh anthrone reagents for test.

- **Procedure** : Add 5 drops of sample solution to 2 ml of anthrone reagent, mix, heat in water bath for 5 min. and cool under running tap water. Occurrence of blue colour indicates presence of carbohydrates.

TEST - 2 : Fehling's test

- **Principle** : The reducing sugars under alkaline conditions reduce cupric copper (blue) to cuprous oxide (brick red). **The test is useful only for reducing sugars**.
- **Reagents** :
  1. **Fehling solution A**: Dissolve 35 g of C_uSO_4 $5H_2O$ in water and make up to 500 ml.
  2. **Fehling solution B**: Dissolve 120 g of KOH and 173g Na-K-tartarate (Rochelle salt) in water and make up to 500 ml.
- **Procedure** : Mix equal volumes (2.5 ml each) of Fehling solution A and B. Add 5 ml of the test solution and boil for 5 min. Occurrence of brick red precipitate indicates the presence of reducing sugars.

TEST - 3 : Benedict's test

- **Principle** : Sugars reduce the cupric copper to green, yellow or red precipitate, depending on the concentration of the reducing sugars. Benedict's quantitative reagent (commercially available) contains potassium thiocynate and potassium ferrocyanide. The reduced copper is, therefore, precipitated as white cuprous thiocynate instead of the red cuprous oxide.
- **Reagents** : **Benedict's solution**: Dissolve 173 g of sodium citrate and 100 g of sodium carbonate in about 820 ml water. Filter and make up the volume to 850 ml. Dissolve separately 17.3 g $CuSO_4$. $5H_2O$ in water and make up to 150 ml. Mix both solutions slowly with stirring.
- **Procedure** : Mix 5 ml of Benedict's solution with 5 ml of sample solution and boil in waterbath for 5 minutes. The white precipitate is formed indicates the presence of reducing sugars. .

TEST - 4 : Seliwanoff's Test

- **Principle** : This test is applied for ketose sugars. Ketoses are dehydrated more rapidly than Aldoses to give furfural derivatives, which then condense with resorcinol to form a red colour complex.
- **Reagents** : **Seliwanoff's reagent**: 0.05% resorcinol in 3N HCl.
- **Procedure** : Add 0.5 ml of sample solution to 2 ml of Seliwanoff's reagent and warm in boiling water bath for 1 minute, red colour occurs.

TEST - 5 : Iodine test

- **Principle** : Iodine forms coloured adsorption complexes with polysaccharides. Starch gives a blue colour with iodine, while glycogen and amylopectin form red-brown colour.

- **Reagents** : 1) **Iodine solution** : 0.005 N iodine in 3% potassium iodide.
  2) 1% solutions of cellulose, glycogen, starch.
- **Procedure** : Acidify the test solution with dilute HCl, then add two drops of iodine and compare the colours obtained with that of water and iodine (Blue colour indication).

TEST - 6 : Inversion test of sucrose

- **Principle** : Sucrose is a non-reducing sugar made up of glucose and fructose. The aldehyde group of glucose and ketone group of fructose are involved in the formation of glycosidic linkage. Upon acidification the glycosidic bond is broken and equal amount of glucose and fructose are liberated. The optical rotation is changed from + 66.5^0 (sucrose) to + 52.5^0 (glucose) and – 92^0 (fructose). The result is change in rotation from dextro (+ve) to levo (-ve).
- **Procedure** : Add 5 drops of conc. HCl to 5 ml of sucrose solution. Heat for 5 minutes on a boiling water bath, cool and add carefully solid Na_2CO_3 to give a neutral or slightly alkaline solution. Perform the reduction test (Fehling's test) on the hydrolysed, solution. (Brick red colour is obtained).

Sr. No.	**Test**	**1% solution of**					
		Glucose	Fructose	Lactose	Sucrose	Starch	Hydrolysed starch
1.	Anthrone Test						
2.	Fehling's Test						
3.	Benedicts Test						
4.	Seliwanoff's test						
5.	Iodine Test						

Questions:

1) What is inversion?
2) What is Fehling's solution?
3) Name the common reducing sugars?
4) Name the common non-reducing sugars.
5) Why does starch form blue colour with iodine?
6) What are polysaccharides?
7) What are the storage polysaccharides?
8) What are the structural polysaccharides?

(B) Qualitative Tests for of Amino Acids

Proteins are the high molecular weight organic compounds having complex structures which upon hydrolysis yield either amino acids only or amino acid plus non-protein portion (prosthetic group). Amino acids are organic acids which contain in their molecular structure at last one – COOH group and one – NH_2 groups attached to the carbon chain. There are 20 different amino acids are found in most of the naturally occurring proteins.

TEST - 1 : The Ninhydrin (Triketo hydrindene hydrate) reaction

- **Principle** : Ninhydrin reacts with all ∝- amino acids between pH 4 and 8 to give a purple colourd compound. The amino acids (proline and hydroxyproline) react with Ninhydrin to produce yellow colour.
- **Reagents** :
  1) **Amino acids**: 1% solution of glycine, proline, lysine..
  2) **Ninhydrin**: 0.2% in ethanol/methanol.
- **Procedure** : Plance 1 ml amino acid solution in a test tube and adjust the pH to about neutrality, add 5 drops of Ninhydrin solution and boil for 2 minutes. Note the colour for each amino acids.

TEST - 2 : The Xanthoproteic test

- **Principle** : Amino acids (tryptophan, tyrosine, phenylalanine) which contain an aromatic ring and forms yellow nitro-derivatives on heating with conc. nitric acid.
- **Reagents** :
  1) 1% solutions of above amino acids.
  2) Conc. nitric acid 3) 40% NaOH
- **Procedure** : Mix 1 ml each of nitric acid and amino acid solution, heat, cool and observe the colour. Add sufficient 40% NaOH to make the solution strongly alkaline. A yellow colour in acid solution which turns bright orange with alkali indicates a positive test.

TEST - 3 : The Nitroprusside test

- **Principle** : Thiol groups in amino acids react with sodium nitroprusside in the presence of excess ammonia to give a red colour.
- **Reagents** :
  1) 0.5% solutions of sulphur containing amino acids (methionne, cystine, cysteine).
  2) 2% freshly prepared sodium nitroprusside solution.
  3) Ammonium hydroxide.
- **Procedure** : Mix 0.5 ml of a fresh solution of sodium nitroprusside with 2 ml of the test solution and add 0.5 ml of ammonium hydroxide to form red colour.

TEST 4: The Biuret test

- **Principle** : Alkaline copper sulphate reacts with compounds containing two or more peptide bonds to give a violet blue coloured complex. It is not given by free amino acids.

- **Reagents** : 1) 1% copper sulphate. 2) 40% sodium hydroxide
  2) 0.5% solution of bovine serum albumin, egg albumin, casein.
- **Procedure** : Add 5 drops of copper sulphate solution to 2 ml of the test solution followed by 2 ml of NaOH, mix thoroughly and note the colour produced (violet).

TEST 5: Acid precipitation of proteins

- **Principle** : The positive charges on the protein are neutralized by acid to form an insoluble salt of protein.
- **Reagents** : 1) 1% solution of protein (casein, albumin)
  2) Acid reagent (20% w/v Trichloroacetic acid)
- **Procedure** : Mix 5 ml of protein solution with 2 ml of Trichloroacetic acid and stir. Observe the precipitate.

TEST 6: Salting out test

- **Principle** : Salts at high concentration compete with proteins for solvent which results in rendering proteins insoluble and precipitate (white).
- **Reagents** : 1) 2% solution o albumin
  2) Ammonium sulphate
- **Procedure** : To 10 ml of protein solution add increasing amounts of ammonium sulphate with stirring, till the proteins are precipitated.

Questions:

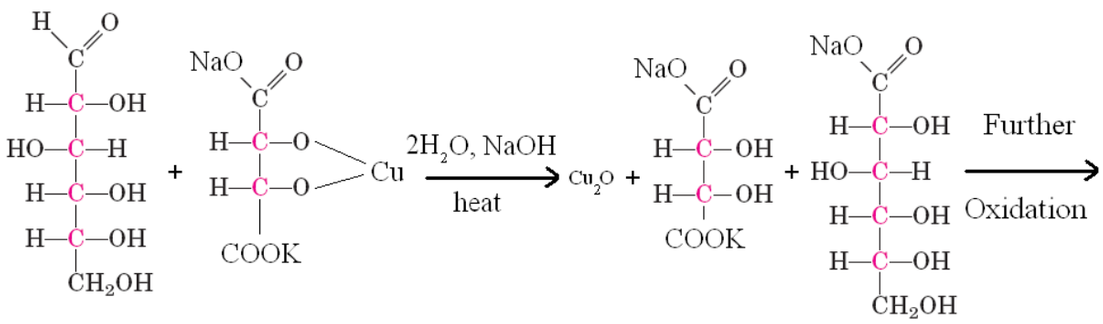
1) Define proteins.
2) Write the chemical reaction of biuret test.
3) What is denaturation of proteins?
4) Why do proteins get precipitated on addition of acid.

Exercise No. : 4

Estimation of reducing sugar by Nelson- Somogyi method

Principle

The reducing sugars when heated with alkaline copper tartrate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 620 nm.



Glucose | Sodium potassium tartarate-copper complex | Cuprous Oxide (Red) | Sodium Potasium tartarate | Sodium D-glucose

Reagents

1. *Alkaline copper tartrate*:

   (a) Dissolve 2.5 g anhydrous sodium carbonate, 2g sodium bicarbonate, 2.5g potassium sodium tartrate and 20g anhydrous sodium sulphate in 80 ml water and make up to 100 ml.

   (b) Dissolve 15g copper sulphate in a small volume of distilled water. Add one drop of sulphuric acid and make up to 100 ml. Mix 4ml of (b) and 96ml of solution (a) before use.

2. *Arsenombolybdate reagent*: Dissolve 2.5g ammonium molybdate in 45 ml water. And 2.5ml sulphuric acid and mix well, then add 0.3g disodium hydrogen arsenate dissolved in 25ml water. Mix well and incubate at 37°C for 24 to 48h.

3. *Standard stock glucose solution*: 100 mg in 100ml distilled water.

   Working standard: Dilute 10ml of stock solution to 100ml with distilled water (100mg/ml)

Method

1. Weigh 100 mg of the sample and extract the sugars with hot 80% alcohol twice (5ml each time).
2. Collect the supernatant and evaporate on water bath.
3. Add 10 ml of water and dissolve the sugars.
4. Pipette out aliquots of 0.1 or 0.2 ml of alcohol-free extract to separate

test tubes.

5. Pipette out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard solution into a series of test tubes.
6. Make up the volume in both sample and standard tubes to 2ml with distilled water.
7. Pipette out 2ml distilled water into a separate tube to serve as a blank.
8. Add 1ml of alkaline copper tartrate reagent to each tube.
9. Place the tubes in a boiling water for 10 min.
10. Cool the tubes and add 1ml of arscnomoybdic acid reagent to all the tubes.
11. Make up the volume in each to 10 ml with water.
12. Read the absorbance of blue colour at 620 nm after 10 min.
13. From the graph drawn, calculate the amount of reducing sugars present in the sample.

Calculation

$$\text{Reducing sugars In sample (\%)} = \frac{\text{Sugar value from graph (µg)}}{\text{aliquot sample Used (0.1 or 0.2 ml)}} \times \frac{\text{Total volume of alcohol - free extract (10ml)}}{\text{weight of sample (100 mg)}} \times \frac{1}{100}$$

Note: Although the colour is stable, determine the absorbance at a fixed lime after the addition of arsenomolybdate reagent.

DNSA Method

Several reagents have been employed which assay sugars by their reducing properties. One such compound is 3, 5-dinitrosalicylic acid (DNS) which in alkaline solution is reduced to 3-amino-5- nitrosalicyclic acid.

Reducing sugar (Glucose) + DNS (yellow) = 3-amino-5-nitrosalicyclic acid (orange- red)

Reagents

1. *Dinitrosalicylic acid (DNS) reagent*: Dissolve simultaneously Ig of dinitrosalicylic acid, 200mg of crystalline phenol and 50mg of sodium sulphite in 100ml of 1% NaOH solution by stirring. Store the reagent in a stoppered bottle at 4°C. The reagent deteriorates, during storage due to atmospheric oxidation of the sulphite present. If required to be stored, prepare the reagent without sulphite and add it just before use.
2. 40% Rochelle salt solution (sodium-potassium tartrate solution).
3. Standard sugar solution: (see under Nelson-Somogyi method)

Method

1. Follow the steps 1 lo 3 as in Nelson-Somogyi method to extract the reducing sugars from the sample.
2. Pipette out 0.5 to 3ml of alcohol-free extract into test tubes and make up the volume to 3ml with water in all the tubes.
3. Add 3ml of DNS reagent and mix.
4. Heat, for 5 min in a boiling water bath.
5. After the colour has developed, add 1ml of 40% Rochellc salt solution (when the contents are still warm) and mix.
6. Cool the tubes under running tap and measure the absorbance at 510 nm using reagent blank adjusted to zero absorbance.
7. Calculate the amount of reducing sugar in the sample using a standard graph prepared from working ‘standard glucose solution (0 to 500 µg) in the same manner.

Calculation

$$\text{Amount of carbohydrate In sample (\% mg)} = \frac{\text{Sugar value from graph (mg)}}{\text{aliquot sample}} \times \frac{\text{Total volume of alcohol - free extract (ml)}}{\text{weight of sample (mg)}} \times \frac{1}{100}$$

Used (0.1 or 0.2 ml)

Note:

1. It is a convenient and sensitive method for the estimation of reducing sugars, particularly when large number of samples are to be analyzed.
2. This method is not suitable for determination of a complex mixture of reducing sugars as the standard curves do not always pass through the origin and different sugars give different colour yields.

Estimation of non-reducing sugars by Nelson-Somogyi Method

Principle

Non-reducing sugars present in the plant extracts are first hydrolyzed with either sulphuric acid or formic acid to reducing sugars. Then, the total reducing sugars are estimated either by Nelson-Somogyi or DNS method.

Reagents

1. 1N H_2SO_4
2. 1N NaOH
3. Methyl red indicator

Method

1. Follow the steps 1 to 3 as given in Nelson-Somogyi method for sample preparation.
2. Pipette out 1ml of extract and add 1ml of IN H_2SO_4.
3. Hydrolyze the mixture by heating at 49°C for 30 min (the acid hydrolysis is effective in splitting the sucrose-type linkages).
4. Cool the tubes and add 1 or 2 drops of methyl red indicator.
5. Neutralize the contents by adding IN NaOH drop-wise from a pipette. Maintain appropriate reagent blanks.
6. Estimate the total reducing sugars by either Nelson-Somogyi's or DNS method as described earlier.

Calculation

$$\text{Non – Reducing sugar In sample (\% mg)} = \frac{\text{Sugar value from graph (µg)}}{\text{aliquot sample Used (1 ml)}} \times \frac{\text{Total volume of extract - (10 ml)}}{\text{weight of sample (mg)}} \times \frac{1}{100}$$

Note:- The content of non-reducing sugars can also be calculated by subtract-ing the reducing sugars from total carbohydrate content. Reading

Exercise No. : 5

Estimation of starch by Anthrone reagent

Starch is an important polysaccharide. It is the storage form of carbohydrate in plants abundantly found in root, tubers, stems, fruits and cereals. Starch which is composed of several glucose molecules, is a mixture of two types of components, namely amylose and amylopectin. Starch is a hydrolyzed into simple sugars by dilute acids and the quantity of simple sugars is measured colorimetrically.

Principle

The sample is treated with 80% alcohol to remove sugars and then starch is extracted with perchloric acid. In hot acidic medium starch is hydrolyzed to glucose and dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone.

Reagents

1. *Anthrone*: Dissolve 200mg anthrone in 100ml of ice-cold 95% sulphuric acid.
2. *80% Ethanol*
3. *52% Perchloric acid*
4. *Standard glucose*: Stock-100 mg in 100ml water.
   Working standard -10 ml of stock diluted to 100ml with water (100/µg/ml).

Method

1. Homogenize 0.1 to 0.5g of the sample in hot 80% ethanol to remove sugars. Centrifuge and retain the residue. Wash the residue repeatedly with hot 80% ethanol till the washings do not give colour with anthrone reagent. Dry the residue well over a water bath.
2. To the residue add 5.0 ml of water and 6.5 ml of 52% perchloric acid.
3. Extract at 0*C for 20 min. Centrifuge and save the supernatant.
4. Repeat the extraction using fresh perchloric acid. Centrifuge and pool the supernatants and make up to 100ml.
5. Pipette out 0.1 or 0.2 ml of the supernatant and make up to the volume to 1ml with water.
6. Prepare the standards by taking 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard and make up the volume to 1ml in each tube with water.
7. Add 4ml of anthrone reagent to each tube.
8. Heat for 8 min in a boiling water bath.
9. Cool rapidly and read the intensity of green to dark green colour at 630 nm.

Calculation

Find out the glucose content in the sample using the standard graph. Multiply the value by a factor 0.9 to arrive at the starch content.

Exercise No. : 6

Determination of Soluble Protein by Folin-Lowry Method

Principle:

Protein reacts with the folin-Ciocalteau reagent to give a coloured complex. The colour so formed is due to the reaction of the alkaline copper with the protein as in the biuret test and the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

Materials:

1. Alkaline sodium carbonate solution.
   (2% Na_2Co_3 in 0.1 N NaOH)
2. Copper sulphate – sodium potassium tartarate solution.
   (0.5% $CuSO_4$ in 1% Na & tartarate)
3. 'Alkaline solution' – prepare on day of use by mixing 50 ml of 1st solution and 1 ml of 2nd solution.
4. Folin – Ciocalteau reagent – Dilute the commercial reagent with an equal volume of water on the day of use. This is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid.
5. Standard protein – Albumin solution 0.2 mg/ml.

Method:

Add 5 ml of the 'alkaline solution' to 1 ml of the test solution. Mix thoroughly and allow to stand at room temperature for 10 minutes or longer. Add 0.5 ml of diluted Folin – Ciocalteau reagent rapidly with immediate mixing. After 30 minutes read the extinction against the appropriate blank at 750 nm.

Estimate the protein concentration of an unknown solution after preparing a standard curve.

Exercise No. : 07

Estimation of free Amino acids by Ninhydrin method

The amino acids are the basic building blocks of all proteins. Those amino acids which exist in free form in tissues and not bound to proteins are **called as free amino acids.** Normally, during diseased conditions in plants, there will be a change in the total free amino acid composition. Hence, estimation of total free amino acids gives an indication about the physiological and health condition of the plants. Moor and Stein (1948) introduced the **Ninhydrin method** and it is widely used for estimation of total free amino acids.

Principle :

Ninhydrin (triketohydrindene hydrate) a powerful oxidizing agent reacts with α- amino acids between pH 4 and 8 and decarboxylates to give an intensly bluish-purple coloured compound which is measured colorimetrically at 570 nm. The amino acids proline and hydroxyproline give a yellow colour.

Reagents :

1. 80% Ethanol.
2. 0.2 M Citrate buffer, pH 5.0
3. **Ninhydrin reagent :** Dissolve 0.8 g of stannous chloride ($SnCl_2.2H_2O$) in 500 ml of 0.2 m citrate buffer, pH 5.0. Add this solution to 20 g nynhydrin in 500 ml of methyl cellosolve (2-methoxyethanol). Prepare fresh and store in a brown bottle (care : Carcinogenic).
4. **Diluent solvent :** Mix equal volumes of water and n-poropanol.
5. **Working standard leucine solution :** Dilute 10 ml of stock leucine solution to 100 ml with water.

Sample extraction :

1. Grind a known weight of sample (500 mg) in a pestle and mortar with a small quantity of acid-washed sand.
2. Add 5 - 10 ml of 80% ethanol (if the tissue is tough, use boiling 80% ethanol). Filter or centrifuge.
3. Repeat the extraction twice and pool all the supernatants.
4. Reduce the volume if required by evaporation and use the extract for estimation of total free amino acids.

Estimation method :

1. To 0.1 ml of extract, add 1 ml of ninhydrin reagent and mix.
2. Make up the volume to 2 ml with water.
3. Heat in a boiling water bath for 20 minutes.
4. Add 5 ml of the diluent while still on the water bath and mix.
5. After 15 minutes of boiling, cool the tubes under running tap and read the absorbance of the purple colour against a reagent blank (prepare by taking 0.1 ml of 80% ethanol instead of extract) at 570 nm (green filter).
6. Calculate the amount of total free amino acids using standard curve prepared from leucine by pipetting out 0.1 - 1.0 ml (10-100 μg range) of working standard solution. Express the results as percentage equivalent of

leucine.

Notes :

1. Use glass distilled water and reagent grade chemicals, otherwise the blank will also give a colour reaction.
2. Commercial ninhydrin should be recrystallized. Ninhydrin is carcinogenic. Wear gloves while handling it.
3. The method described estimates only the α -aminoacids, non-protein amino acids are not accounted.
4. Standard graph is different for different amino acids. So it is advisable to prepare a composite mixture of alanine, aspartic acid, tryptophan, proline and lysine (in equal weights) and use it for standardization. Such standard kits are available commercially.
5. The colour intensity in the presence of methyl cellosolve is stable for a longer time. If the ninhydrin reagent is prepared in acetone or water, the colour is not stable.

Readings :

1. Moore, S. and W.H. Stein (1948). In : *Methods Enzymol* (eds. Colowick, S.O, and Kaplan N.D.), Academic press, New York, 3, 468.
2. Misra, P.S., E.T. Mertz, and D.V. Glover (1975). *Cereal Chem*. 52, 844.

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Exercise NO. 8

Determination of total Crude Fat / Oil by Soxhlet's method

Principle: Lipids in sample is dissolved in organic non polar solvents like petroleum spirit, benzene, hexane etc. Lipids/ Fat dissolved in solvent can be extracted by heating and cooling simultaneously in a condenser.

Material:

1. **Soxhlet apparatus** : It consists of three parts fitted into one another. These three parts are extraction flask, extraction thimble and water condensor.
2. Petroleum spirit B.P. 40-60° C.
3. Whatman No. 1 filter paper sheet..

Procedure:

1) Weigh 2 to 5 gm of 60 mesh oil seed sample (previously ground and dried at 105 ^{o}C for 24 hours for removing moisture) or 10 gm of other sample.
2) Prepare a small packet of sample with Whatman No.1 filter paper.
3) Take weight of empty dry extraction flask.
4) Plug the bottom of thimble by putting cotton or glass wool to avoid the possibility of passing out the sample particles in extraction flask.
5) Connect the rubber tube, water tap to condenser. See that water supply to the condenser is constantly flowing.
6) Put the packet of sample in thimble and pour organic solvent to 2/3 capacity of thimble. Take extraction flask containing 2/3 organic solvent.
7) Connect these extraction flask and thimble to the condenser unit with heating coil.
8) Put the apparatus on heating mantle and start water supply to the condenser. Regulate the rate of heating to allow continuous volatilization of solvent, its simultaneous condensation.
9) Continue heating slowly till 6-8 siphoning collected in extraction flask. And stop heating.
10) Take out extraction flask from the extraction unit. Which contains crude fat with little ether. .
11) Evaporate excess ether on water bath **OR** in open air.
12) Keep the flask in the oven at 105 ^{o}C for 1 hour and evaporate remaining spirit.
13) Cool to the room temperature and weigh it accurately to know the quantity of crude fat / oil extracted.

Observations:

i) Wt. of sample taken = 5 gm (X)
ii) Wt. of empty flask = ….. g (W_1)
iii) Wt. of flask + oil = ……..g (W_2)

Calculation:

$$\% \text{ Crude fat / oil} = \frac{(W_2 - W_1)}{X} \times 100$$

Results: Given sample contains……% of oil.

Questions :

1. What is meant by crude fat ?
2. State the different parts of Soxhlet apparatus.
3. Explain the principle of extraction of crude fat by Soxhlet method.
4. Name the solvent for extraction of fat.

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Exercise No. : 9

Qualitative Tests for Oil

Lipids are characterized by their limited solubility in water considerable solubility in organic solvents (benzene, acetone, ether). It is a heterogeneous class of compounds and traditionally classified as simple lipids (oils, fats and waxes) and compound lipids (phospholipids, sphingolipids, sulfolipids, glycolipids, sterols, carotenoids and fat soluble vitamins).

Test 1 – Lipid's solubility

Principle: "Like dissolves like" A major portion of lipids being nonpolar, they are soluble only in nonpolar solvents. These solvents are also called **"organic"** or **"fat solvents"**.

Reagents: (1) Vegetable oil samples

(2) Organic solvents : Acetone, Ether and Benzene..

Procedure: Mix 1 ml of oil in water and other solvents. Observe the solubility. Place 1 drop of solution from each group on a filter paper and leave it to dry. Observe the formation of a clear greasy spot in solutions containing oil after drying of filter paper..

Test 2 - Unsaturation test

Principle : The vegetable oils contain relatively higher proportion of unsaturated fatty acids. The unsaturated fatty acids contain one or more double bonds. Halogens such as iodine, chlorine or bromine can be easily added at these double bonds. This process is called "**Halogenation**". Halogenation results in the decolourization of a solution of bromine or iodine by a lipid which indicates the presence of double bond.

Reagents : 1. Vegetable oil

2. Bromine water.

Procedure: Slowly add bromine water to the test solution drop by drop, shaking after each addition, until the bromine just fails to be decolorized. Compare the ability of test compounds to decolorize the solutions of iodine and bromine.

QUESTIONS

1. What are lipids?
2. What is the difference between fats, oils and waxes?
3. What is halogenation?
4. Give examples of monoethenoid acids, diethenoid acid, triethenoid acid and tetraethenoid acids.
5. Enlist different fat solvents.

Exercise No. : 10

Determination of Alpha amylase activity from germinating seed

Amylases :

Starch is the principal storage polysaccharide in plant cells. It is made up of about 10-20 % of amylose and about 80-90% of amylopectin. Amylose is a linear homopolymer of D-glucose units linked by α-1, 4-glycosidic bonds without any branches. While amylopectin is a branched polymer of D-glucose units linked by both α-1, 4 and at branching with α-1, 6-glycosidic bonds.

α- and β- Amyases are the hydrolytic enzymes which hydrolyze starch, α- Amylases hydrolye α-1, 4 linkage of starch in a random manner producing a mixture of D-glucose and dextrins (short chains of glucose with 6-12 units). β-Amylases hydrolyz alternate α-1, 4 glycosidic bonds from the non-reducing end of starch yielding successively the disaccharide, maltose. Hence, amylases play an important role in starch metabolism in plants.

Principle :

The reducing sugars produced by the action of α- and/or β-Amylases react with dinitrosalicylic acid and reduce it to a brown colouring product nitroaminosalicylic acid.

Materials and Reagents :

1. 0.1 M Sodium acetate buffer, pH 4.7.
2. **1% Starch solution :** Prepare a fresh solution by dissolving 1g starch in 100 ml acetate buffer. Slightly warm, if necessary.
3. Dinitrosalicyclic acid reagent.
4. 40% Rochelle salt solution (potassium sodium tartrate).
5. **Maltose solution :** Dissolve 50 mg maltose in 50 ml distilled water in a volumetric flask and store it in a refrigerator.

Method :

Extraction of amylases :

Extract 1 g of sample material with 5-10 volumes of ice-cold 10 m M calcium chloride solution overnight at 4^0C or for 3 h at room temperature. Centrifuge the extract at 54,000 g at 4^0C for 20 minutes. The supernatant is used as enzyme source.

Extraction of β-Amylases (free and bound) :

The free β-Amylases is extracted from acetone defatted sample material in 66mM phosphate buffer (pH 7.0) containing 0.5 M NaCl. The extract is centrifuged at 20,000 rpm for 15 minutes. The supernatant is used as a source of free β-Amylase.

The pellet is then extracted with phosphate buffer containing 0.5 % 2-mer-captoethanol. The clear extract is used as source of bound β-Amylase. All operations are carried out at 4^0C.

Enzyme Assay :

1. Pipette out 1 ml of starch solution and 1 ml of properly diluted enzyme in a test tube.
2. Incubate it at 27^0C for 15 minutes.
3. Stop the reaction by the addition of 2 ml dinitrosalicylic aid reagent.
4. Heat the solution in a boiling water bath for 5 minutes.
5. While the tubes are warm, add 1 ml potassium sodium tartrate solution.
6. Then cool it in running tap water.
7. Make up the volume to 10 ml by addition of 6 ml water.
8. Read the absorbance at 560 nm.
9. Terminate the reaction at zero time in the control tubes.
10. Prepare a standard graph with 0-100 μg maltose.

Calculation:

A unit of α or β-Amylase is expressed as mg of maltose produced during 5 minutes incubation with 1% starch.

Notes :

1. Alternative amylase assay methods are also available. For example, starch is made to react with iodine to give a blue colour. However, this reaction is not stoichiometric i.e. the intensity of blue colour is not linearly proportional) to starch concentration. It is only of qualitative value. The maltose assay is stoichiometric.
2. The method of extraction of amylases described is preferred for extraction of amylases from other sources.

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Exercise No. : 11

Determination of invivo Nitrate reductase activity from leaf tissue

IN VIVO ASSAY

Nitrate reductase (NR) activity can be directly assayed using cut leaf segments. In a solution containing nitrate, phosphate buffer and propanol, nitrate can readily enter leaf cells. In this case, the leaf cell supplies NR and the reductant NADH. The propanol in the assay medium increases cell permeability and makes the leaf segments essentially anaerobic. Under anaerobic conditions, the reduction of nitrite to ammonia is inhibited, and the conversion of nitrate to nitrite is enhanced Because of the sensitivity of the reaction to molecular oxygen all operations should be carried out in dim room light to reduce photosynthetic activity. With this approach the NR activity associated with all four inducing treatments can be assayed by one group.

1. Weigh out four 0.3g leaf samples for each of the inducing treatments: dark minus NO_3^-, dark plus NO_3^-, light minus NO_3^-, light plus NO_3^-. Cut the leaves into segments 1.0-1.5 cm squares.
2. Prepare duplicate 25 x 100 screw cap test tubes for each inducing condition. You will need one set for time=0 (t_o) and another set for time=30 min. (t_{30}). You should have a total of 16 tubes (4 conditions x 4 tubes
3. Place the leaf samples in the appropriate tubes.
4. Add 10 ml of In vivo assay solution to each tube and cap the tube.

In vivo assay solution: 100 mM phosphate buffer, pH 7.5

30 mM KNO_3
5%(v/v) propanol

5. Place the t_o tubes in a boiling water bath for 5 minutes and then allow to cool to room temperature.
6. Place all tubes, t_o and $t_{30,}$ in a shaking water bath at 30^{o}C and incubate for 30 min.
7. At the end of the 30-min. incubation period, place the t_{30} tubes in a boiling water bath for 5 min. and then cool to room temperature.

Preparation of a Standard Curve: For the **in vivo assay,** you will also need a standard curve to calibrate the color reactions. You will be provided with a stock solution of nitrite.

Nitrite stock: 25 :M (nmol/ml) KNO_2 in water

8. Follow the table below to prepare the standard curve.

Tube #	nmol nitrite	ml nitrite	ml water
1	0	0	10
2	25	1	9
3	50	2	8
4	100	4	6
5	150	6	4
6	200	8	2
7	250	10	0

9. To detect nitrite in the assay tubes, add 10 ml of color development reagent.

Color Development Reagent: 1% sulfanilamide in 3N HCl

0.02% N-(1-naphthyl)-ethylenediaminehydrochloride

10. Mix thoroughly and place tubes in the dark at room temperature for 15 min.
11. Determine OD of each standard tube at 540 nm.
12. Add 10 ml of color reagent to each assay tube and determine the OD at 540 nm. Compare with standard curve.

Exercise No. : 12

Paper Chromatography / TLC demonstration for separation of amino acids

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Principle:

The separation of compounds in TLC is based on differential adsorption as well as partitioning of the analytes between the liquid stationary phase and mobile solvent phase. This technique is rapid as compared to paper chromatography. Molecules get separated between the hydrated stationary phase and non-polar mobile phase. Hydrophilic analytes have more affinity to the polar stationary matrix, while less hydrophilic molecules tend to have more affinity towards mobile phase, resulting in its faster movement and separation. The separated analytes are identified by comparing their R_f values to that of reference standards. The R_f value of an analytes depends upon the (i) solvent system (ii) degree of saturation of the mobile phase in the chromatographic chamber (iii) particle size of the adsorbent (iv) type of adsorbent (v) temperature and humidity. Thus, the R_f value for an analytes is constant for a given set of experimental conditions.

$$R_f = \frac{\text{Distance traveled by the analyte}}{\text{Distance traveled by the mobile phase (solvent)}}$$

Commonly used stationary matrix for TLC include, silica gel – G, silica gel H, micro-porous cellulose, alumina, florisil, polyamide and octadecylsilance. Normally, glass, aluminium or polyester supports (size 20 x 20 cm) are used for coating stationary matrixes.

Material: (i) Glass plates (20 x 20 cm, thickness 3 mm) (ii) Chromatographic glass jar with vacuum greased lid (iii) TLC applicator with plastic plate support and TL\c rack (iv) TLC-spotting guide (v) Calibrated capillary tubes (SPL/10 uL capacity0 or micro-syringe (10 uL). Silica gel-G, TLC grade.

Sample: *Spirulina* powder (commercially available in drug stores/medical shops as capsules).

Solvent system: Petroleum ether: Acetone (7:3 v/v).

Procedure:

i) Arrange 5 clean glass plates on the plastic support unit along with silica gel applicator. Weight 40 g of silica gel-G into a 250 ml iodine flask. Add 100 ml of distilled water and thoroughly shake the contents. Immediately, pour the slurry into the applicator and coat the gel along the glass plate, by rapidly moving the applicator. The coating of the adsorbent should be of uniform thickness (25-75 um). Air dry the plates (placed in TLC rack) at room temperature for 5 hours. Activate the coated TLC plates in a hot air oven at 110^0C for 1 hour. Later, the plates are removed and allowed to cool at room temperature in a desiccated chamber.

ii) Prepare on acetone extract of the Spirulina powder (500 mg), by transferring the contents of one capsule into a 20 ml glass test tube

containing 5 ml of acetone. Vortex and allow the contents to stand at room temperature for 30 minutes. Collect the acetone extract by filtering through glass wool. The clear filtrate is concentrated by evaporation to a small volume (500 uL volume).

iii) Spot equidistantly 5, 10, 15 and 20 uL aliquots of the acetone extract onto the activated silica gel plate, about 3 cm away from the edge of the plate by using a TLC guide and capillary tube. The area of the spot should be kept to minimum (1-2 mm dia), which can be achieved by repeated spotting of the sample volume followed by air drying at the same spot. The chromatogram is developed by placing the TLC plate vertically in a TLC chamber, saturated with the mobile phase, in such a manner that the spotted edge dips into the solvent system. Run the chromatogram until the solvent front reaches the top edge of the plate. Remove and mark the solvent front as soon as the plate is removed from the chamber. Air dry the plate at room temperature. Outline the coloured pigment spots using poker and calculate the R_f values of the pigments.

Caution: Do not touch or damage the coated area and the edges of the plate.
For the purpose of identification, following are the reference R_f values (Table 8.1) of the pigments in the petroleum ether: acetone solvent system.

Table 8.1 R_f values of plant pigments

Pigment	R_f value
Chlorophyll a	0.68
Chlorophyll b	0.54
Chlorophyll c	0.03
β - carotene	0.94
Fucoxanthin	0.51
Lutein	0.43
Violaxanthin	0.22

Workout: Analyse the pigments present in Spinach leaves, by TLC and compare the profile with Spirulina.
Note: The separated pigment spots on the TLC plate should be scored immediately, as the pigment undergoes destruction/bleaching in the presence of light.

Exercise No. : 13

TLC for separation of sugars

Separation of sugars by silica gel thin layer chromatography

Principle

Separation of compounds on thin layer is similar in many ways to paper chromatography, but has the added advantage that a variety of supporting media can be used so that separation can be by adsorption, ion exchange, partition chromatography or gel filtration depending on the nature of the medium employed. The method is very rapid and many separations can be completed in an hour. Compounds can be detected at a lower concentration than on paper as the spots are compact.

Reagent

1. Slurry of silica gel G: mix 25g of silica gel G powder with 50 ml of water or with 0.2 M sodium borate buffer, PH 8.0 or 0.02M sodium acetate or boric acid.
2. Solvent system (see table)
3. Spray reagents (see table)
4. Standard: 0.01 M sugar in water

Procedure

1. Using an applicator (spreader) uniformly spread slurry of silica gel G (25Qu thick) on glass plates (5x 20cm and 20x20cm and 0.4cm thickness).
2. Air dry the plates for l-2 hr to allow the binder to set.
3. Place the plates in a rack and activate them at 110-120°C for hr. If not used immediately, store the<dried plates in a desiccator and activate just before use.
3. Spot 5µ1 of samples, dry with cold hair dryer and repeat spotting once more.
4. Develop the plates in a solvent (see Table), when the solvent has reached the top, dry the plates in a stream of warm air and spray with an appropriate developing reagent (take care to spray gently as other-wise the coating material may peel off).
5. Heat the plate, measure the R_f values of sugars from unknown mixture and identify using standards.

Notes

1. Some of the solvent systems, adsorbents and mode of identification of sugars are given in Table.

Detection of sugars by thin-layer chromatography.

Compounds	Adsorbent	Solvent system	Mode of identification	Colour
Mono-and disaccharides	Kieselghur G (sodium acetate)	Ethyl acetate/propanol (65/35)	(a) spray with 2% solution of diphenylamine in water saturated with n-butanol or in butanol and methanol (1:1) containing 5% trichloroacetic acid. Heat at 100 ^{o}C for 10-15 min.	Aldohexoses give brown spots. Aldopentoses give purple spots.
	Kieselghur G (sodium phosphate)	Butanol / acetone / phosphate buffer, PH 5.0 (40/50/10)	(b) Spray with 0.5% solution of 3,5 dinitrosalicylic acid in 4% NaOH. Heat at 100 ^{o}C for 5 min.	Reducing sugars give brown spots on a yellow background
	Silica gel G impregnated with 0.2M sodium borate buffer, PH 8.0 to 0.02 M sodium acetate or boric acid	Benzene/acetic acid/ methanol (20/20/60)	(c) srapy with 0.2% naphthoresorcinol in ethanol and 2% aq. TCA (1:1) (mix just before use). Heat at 100 ^{o}C for 10 Min.	Fructose, sucrose, sorbose and raffinose give red colour. Pentoses and uronic acid give blue colour on standing.

Exercise No. : 14 &15

Isolation of genomic DNA from plant. Purification, Quantification and quality determination

ISOLATION OF GENOMIC DNA

PRINCIPLE

The extraction of genomic DNA from plant material requires cell lysis, inactivation of cellular nucleases and separation of the desired genomic DNA from cellular debris. Ideal lysis procedure is rigorous enough to disrupt the complex starting material (plant tissue), yet gentle enough to preserve the target nucleic acid. The cetyltrimethylammonium bromide (CTAB) protocol (developed by Murray and Thompson in 1980) is appropriate for the extraction and purification of DNA from plants and plant derived foodstuff and is particularly suitable for the elimination of polysaccharides and polyphenolic compounds otherwise affecting the DNA purity and therefore quality. Plant cells can be lysed with the ionic detergent CTAB, which forms an insoluble complex with nucleic acids in a low-salt environment. Under these conditions, polysaccharides, phenolic compounds and other contaminants remain in the supernatant and can be washed away. The DNA complex is solubilised by raising the salt concentration and precipitated with ethanol or isopropanol.

Leaf Sample Collection

1. Harvest leaves from green house or field grown plants. It is preferable to use young leaves without necrotic areas or lesions, although older leaves, which are not senescent, may be used.
2. If the midrib is thick and tough, remove it. Cut or fold the leaves into 10-15 cm sections and place in a plastic bag along with the tag identifying the sample. Place the bags on ice chest or other container with ice to keep samples cool.
3. The leaf samples may be stored at – 80^0C until it is ground.

Grinding

1. Cut the leaf samples into 1-2 cm sections and grind it with liquid N_2 in a mortor and pestle. Ensure that a fine leaf powder is obtained.
2. The Sample may be used immediately for DNA extraction or can be stored at -80^0C.

DNA Extraction

Extraction of high molecular weight DNA free from protein and RNA is essential for all molecular biology investigations. The cell walls must be broken or digested away in order to release cellular constituents, which is usually done by grinding tissue in dry ice or liquid N_2. Cell membrane must be disrupted which is done by a detergent usually SDS (Sodium Dodecy1 Sulphate) or CTAB (Cetryl Trimethy1 Ammonium Bromide). DNA must be protected from the endogenous nucleases. The detergent used for this purpose is EDTA (Ethylene Diamine Tetra Acetic Acid). EDTA is a chelating agent, which binds magnesium ions (Mg^{++}), generally considered as a co-factor for most nucleases. The tissue mixture is emulsified with either chloroform or phenol to denature protein from

DNA. Shearing of DNA should be minimized of quickly drawing through a small orifice. Time between thawing of frozen pulverized tissue and exposure to extraction buffer should be minimized to avoid nucleolytic degradation of DNA. The crop species where excess polysaccharides are present must be eliminated by $CsCl_2$ density gradient or by using CTAB method. The resulting DNA would be about 50-400μg. The DNA extraction protocols described here are reproducible and are applicable for diverse crop species.

Preparation of Reagents for Plant Genomic DNA Isolation

Materials

1. 2% CTAB buffer (100 ml)
   2.0 g CTAB (Hexadecyl trimethyl-ammonium bromide)
   10.0 ml 1M Tris pH 8.0
   4.0 ml 0.5M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di-sodium salt)
   28.0 ml 5M NaCl
   40.0 ml H2O
   1 g PVP 40 (polyvinyl pyrrolidone (vinylpyrrolidine homopolymer) Mw 40,000)
   Adjust all to pH 5.0 with HCl and make up to 100 ml with H_2O.
2. Microcentrifuge tubes
3. Mortar and Pestle
4. Liquid Nitrogen
5. Micro centrifuge
6. Absolute Ethanol (ice cold)
7. 70 % Ethanol (ice cold)
8. 7.5 M Ammonium Acetate
9. Water bath
10. Chloroform : Iso Amyl Alcohol (24:1)
11. Water (sterile)
12. Agarose
13. 6x Loading Buffer
14. 1x TBE solution
15. Agarose gel electrophoresis system
16. Ethidium Bromide solution

1 M Tris pH 8.0

Dissolve 121.1 g of Tris base in 800 ml of H_2O. Adjust pH to 8.0 by adding 42 ml of concentrated HCl. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 1 L with H_2O. Sterilize using an autoclave.

5x TBE buffer

54 g Tris base
27.5 g boric acid
20 ml of 0.5M EDTA (pH 8.0)
Make up to 1L with water.

To make a 0.5 X working solution, do a 1:10 dilution of the concentrated stock.

1% Agarose gel

1g Agarose dissolved in 100 ml TBE

Procedure

1. Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB buffer.
2. Transfer CTAB: plant extract mixture to a microcentrifuge tube.
3. Incubate the CTAB: plant extract mixture for about 15 min at 55° C in a water bath.
4. After incubation, spin the CTAB: plant extract mixture at 12000 rpm for 5 min to spin down cell debris. Transfer the supernatant to clean microcentrifuge tubes.
5. To each tube add 250 µl of Chloroform: Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 12000 rpm for 1 min.
6. Transfer the upper aqueous phase only (contains the DNA) to a clean microcentrifuge tube.
7. To each tube add 50 µl of 7.5 M Ammonium Acetate followed by 500 µl of ice cold absolute ethanol.
8. Invert the tubes slowly for several times to precipitate the DNA. Generally the DNA can be seen as a white precipitate. Alternatively the tubes can be placed at 20°C for 1 hr after the addition of ethanol to precipitate the DNA.
9. Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. (Alternatively the precipitate can be isolated by spinning the tube at 12000 rpm for a minute to form a pellet and discard the supernatant)To wash the DNA, transfer the precipitate into a microcentrifuge tube containing 500 µl of ice cold 70 % ethanol and slowly invert the tube. Repeat the step 9.
10. After the wash, spin the DNA into a pellet by centrifuging at 12000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry for approximately 10-15 min. Do not allow the DNA to over dry since it will be hard to redissolve the DNA.
11. Resuspend the DNA in sterile DNase free water (approximately 20-50 µl H_2O; the amount of water needed to dissolve the DNA can vary, depending on how much quantity is isolated). RNaseA (10 µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µl RNaseA in 10ml H_2O).
12. After re-suspension, the DNA is incubated at 65°C for 20 min to destroy any DNaes store at 4°C.
13. Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

Protocol for Rapid Isolation of DNA

Isolation of DNA may be a limitation if the breeder wants to use PCR/RAPD analysis for MAS programme since the population size in breeding programme is quite large. A simple protocol is needed for DNA marker-assisted rice breeding programme. The protocols for DNA isolation given to you will produce reproducible results, however the step by step procedure is time consuming and labour intensive and hence are not suitable for large scale DNA analysis needed for a MAS programme. The following protocol gives you a simplified protocol, which needs a small amount of leaf sample and is

comparatively faster than other protocols. Moreover, the DNA quality is suitable for PCR analysis and comparable to other protocols.

Procedure

1. Collect a healthy leaf sample (2 cm long) into a 1.5ml eppendorf tube and label the tube with the plant number. Cap the tube and place on ice.
2. In the laboratory, cut the leaf tissue into small pieces and put the tissue on spot plate or in a small mortar.
3. Add 400μl of DNA extraction buffer and grind the tissue with a thick glass rod or with a pestle and grind smoothly.
4. Grind the tissues until the buffer turns dark green, which indicates cell breakage and release of cholorophyll.
5. Add 400μl more of DNA extraction buffer. Mix and transfer 400μl into 1.5ml eppendorf tube and write the plant number. Do not place tubes back on ice, as SDS will precipitate.
6. Add 400μl Chloroform. Mix well and spin for 30 seconds. Transfer the top (aqueous phase) into another 1.5ml tube and put label. Do not disturb interface above the chloroform layer. Avoid the particulate matter while pipetting.
7. Add proteinase K to a final concentration of 50μg/ml and incubate at room temperature for 5 minutes.
8. Add 800μl 100% Ethanol and mix well. Spin for 5 minutes in a microfuge at 10,000 rpm. Decant the supernatant.
9. Wash the pellet with 70% ethanol and air-dry or vacuum dry the DNA. Do not overdry the pellets as this reduces their solubility.
10. Resuspend the DNA in 50μl of TE. Store DNA at -20^0C. Avoid vortexing samples at any step in this protocol. Flick the tubes repeatedly with your finger.
11. Use 1μl of DNA for every PCR/RAPD analysis. Rice DNA should be stable for 6-8 weeks. Do not store longer for use.

Estimation of DNA

The isolated DNA needs to be estimated for quality and quantity before starting of molecular biology experiments. There are **two methods of DNA estimations**.

1. Quantitative Estimation of DNA 2. Qualitative Estimation of DNA

1. Quantitative Estimation of DNA

Nucleic acid (DNA and RNA) has maximum absorbance at about 260nm. An OD of 1.0 corresponds to approximately 50μg/ml for dsDNA, 40 μg/ml for ssDNA and RNA. The ratio between the readings at 260 nm and 280 nm (OD 260/OD 280) provides as estimate for the purity of nucleic acid. Pure preparation of DNA and RNA has a ratio of approximately 1.8 and 2.0, respectively. If there is contamination with protein or phenol the ratio will be significantly less than this value (< 1.8). A ratio greater than 2.0 indicates a high proportion of RNA per DNA in the sample. To measure the concentration of your DNA, you need to make a dilution. Add 10 μl of sample to 2390 μl of TE, vortex and measure absorbance at 260nm and 280nm in spectrophotometer. This corresponds to 20-fold dilution and the OD reading can be directly converted into μg/ml for the original sample by multiplying by 1000. For example, if the OD of the dilution = 0.450, then the original sample must have a concentration of 20 x 50 x 450 = 450μg/ml. Write the name of the sample and

concentration on each tube with a black marker. If 40 fold dilutions are measured the concentration must be multiplied by two again.

2. Qualitative Estimation of DNA

It is critical that plant DNA must be of high molecular weight for molecular analysis (> 30 kb). To check this you will need to run an aliquot (2μl) of each sample on a 1% agarose gel. You will need to run molecular weight standards of uncut Lambda Phage, Lambda phage cut with Hind III OR ∅X174 phage cut with Hae III. Stain the gel in a tray containing 10μl of ethidium bromide solution (10μl in 500ml of distilled water)
for 30 minutes. Destaine for 30 minutes in distilled water and view the gel under UV transilluminator. High molecular weight DNA will reveal single band near high molecular weight size band of molecular weight standard. If a second band is revealed at the lower protein of the gel, it indicates contamination of RNA and a smear in the gel shows protein contamination. A further purification is needed according to DNA quality observation if necessary. High molecular weight and high quality DNA can be used for all types of molecular studies.

3. Agarose gel electrophoresis

Electrophoresis through agarose is the standard method used to separate, identify and purify DNA fragments. Agarose gels can be poured in a variety of shapes, sizes and porosities and can be run in number of different configuration. The choices within these parameters depend primarily on the sizes of the fragments being separated. Agarose is a polysaccharide extracted from seaweed. It is typically used at concentrations of 0.5 to 2%. The higher the agarose concentration the "stiffer" will be the gel. Agarose gels are extremely easy to prepare: you simply mix agarose powder with buffer solution, melt it by heating, and pour the gel. It is also non-toxic.

Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50,000 bp can be separated using standard electrophoretic techniques. Agarose gels have lower resolving power than polyacrylamide gels but have a greater range of separation. DNA from 200 bp to approximately 50 kb in length can be separated on agarose gels of various concentrations.

Exercise No. : 16

Amplification of genomic DNA using different primers and resolution of PCR products on agarose gel

A) DNA Amplification by PCR :

Polymerase chain reaction (PCR) is an ingenious new tool for molecular biology that has had an effect on research similar to that of restriction enzymes and southern blot. PCR is very sensitive method that a single DNA molecule has been amplified and single copy genes are routinely extracted out of complex mixtures of genomic sequences and visualized as distinct bands on agarose gels.

Principle :

Polymerase is an enzyme that polymerizes any DNA material *in vitro* if it is supplemented with other minimal requirements such as dNTPs (nucleotide triphosphates) and primer. The cocktail is incubated at different temperatures i.e. 94^0C, 35^0C and 72^0C which are responsible for the denaturation of the DNA, annealing of primer and DNA and then polymerization of nucleotides on template DNA strand respectively. The PCR protocol differs with different situations.

Materials :

1. Sample DNA
2. 25mM dNTPs
3. $MgCl_2$ (25mM)
4. 10x PCR buffer
5. Primer
6. Sterile distilled water
7. Taq DNA polymerase

Procedure :

1. Label the PCR tubes properly and arrange them open in a rack.

2. Pipette out 1 µl of DNA preparation in the appropriate eppndorf (or quantity containing 0.05 µg of DNA).

3. Prepare a master mix in one eppendorf as follows :

   For one sample :

1.	PCR buffer (10X)	2.5 µl
2.	dNTPs stock	2.5 µl
3.	$MgCl_2$	2.5 µl
4.	Primer	2.5 µl
5.	Sterile distilled water	8.0 µl
6.	Taq polymerase	1.0 µl
	Total	**19 µl**

4. Mix the master mixture properly and distribute 19 µl to each PCR tube containing DNA sample and mix it.

5. Keep the tubes in PCR machine.

6. Set the temperature to 94 ^{0}C for 5 minutes (denature the ds DNA)

7. Set the program given as follows :

Step 1	Temperature 94 ^{0}C, Time - 1 minute
Step 2	Temperature 35 ^{0}C, Time - 30 seconds
Step 3	Temperature 72 ^{0}C, Time - 45 seconds
Step 4	45 cycles
Step 5	Temperature 72 ^{0}C, Time - 5 minutes
Step 6	Temperature 4 ^{0}C

8. After completion of all the cycles take out the tubes and preserve at 4 ^{0}C.

9. Run the amplified sample on agarose gel (1.5 %) and observe the banding pattern.

10. Document the results by gel documentation system.

B) Checking of Amplified DNA by Gel Electrophoresis :

Many different techniques are available for the fractionation and characterization of nucleic acids including the recombinant DNA. Electrophoresis of DNA or RNA in gels is rapid and relatively inexpensive method and can be easily set up in any laboratory. DNA can be checked for size, intactness, homogeneity and purity by this technique. Importantly this technique is central to gene manipulation and DNA amplification fingerprinting (DAF) experiments.

Separation of DNA can be achieved in polyacrylamide or most preferably in agarose gels. Agarose vertical gels give reproducible results and can be completed more rapidly than the horizontal systems. Horizontal agarose gel electrophoresis has the advantage that much lower concentrations of agarose can be utilized than in a vertical gel system allowing the separation of large DNA fragments, and also handling of the gel is easier. An effective compromise between the standard vertical and horizontal slab gel is the 'submarine minigel'. These small (10x6x0.5 cm) agarose slab gels are run horizontally under approximately 2 to 5 mm buffer and have the advantages of being easier to prepare and faster running than conventional gels.

Principle :

Agarose form a gel by hydrogen bonding and the gel pore size depends on the agarose concentration. The DNA molecules are separated by electrophoresis on the basis of their size, shape and the magnitude of the net charge on the molecules. The dye ethidium bromide intercalates between the bases of RNA and DNA and fluorescence orange when irradiation with UV light. Low concentration agarose gels with large pore permit fractionation of high MW molecules and vice versa.

Materials :

1. Tris Borate (EDTA (TBE) buffer (10X ; pH 8.2)

0.9 M Tris HCl	:	113.0 g
0.025 M EDTA Na_2	:	9.3 g
0.9 M Boric acid	:	55.0 g
Double distilled water to	:	1000 ml

2. Agarose : 1.5 % (w/v) in single strength TBE buffer. Boil to dissolve the agarose; then maintain at 50 ^{0}C in a flask until used.

3. Gel loading solution :

Sucrose	:	30 %
Bromo phenol blue (BPB)	:	0.25 %
Xylene cyanole FF	:	0.25 %

All w/v in single strength TBE buffer

4. DNA preparations (plant DNA, plasmid DNA, amplified DNA etc.)
5. Marker DNA for size determination (Lambda DNA digested with restriction endonucleases EcoR I and Hind III).
6. Gel casting plate
7. Gel tank
8. Power pack
9. UV transilluminator with photography system.
10. Safety glasses

Procedure :

1. Form a wall around clean dry, gel casting glass plate (10 X 60.6 cm) using zinc oxide tape. This should give a leak proof wall about 1 cm high all around the plate. Alternatively, the plate is placed in a suitable gel casting tray purchased from a commercial supplier. Place the set up perfectly horizontal over a leveled plate.

2. Pour 30 ml of agarose solution maintained at 50 ^{0}C onto the casting plate. Immediately place a suitable well forming comb about 1 cm from one end of the plate. The teeth of the comb should not touch the wall of the glass plate. Allow the gel to set for 1 hour.

3. Remove the comb from the gel, carefully. Transfer the gel along with the gel plate to the electrophoresis tank such that the wells are near the cathode (+ve electrode). Pour 1x TBE buffer into the tank until gel is submerged completely.

4. Connect the electrodes to the power pack properly.

5. Prepare DNA samples in the gel loading buffer (10 µl DNA sample in 4 µl gel loading buffer) and load approximately 5 to 20 µl of sample using micropipettes.

6. Turn on the power supply and run at 50 V. Monitor the progress of fast running dye (BPB) and tracking dye (Xylene cyanole) during electrophoresis. Terminate the run when tracking dye is about to leave the gel.

7. After disconnecting the power supply, transfer the gel to a staining tray containing 250 µg ethidium bromide (50 µl of 5 mg/ml solution) in 250 ml of used TBE buffer. Stain the gel about 30 minutes.

8. Transfer the gel onto a thick plastic sheet, place on an UV transilluminator and view the gel under ultra violet light (300 nm). Nucleic acids (DNA) on the gel will appear orange owing to the fluorescence of bound ethidium bromide. Photograph as soon as the gel has been checked for the presence of bands. Use the photograph for further interpretation of band patterns.

9. Measures (from photograph) the distance moved by each band from the loading well. Plot the distances against log molecular weight of standards (ƛ DNA fragments) to give a calibration curve. Deduce the size of DNA and / or restricted fragment of samples using the curve.

Note :

1. Ethidium bromide can be directly added to the agarose solution in a concentration of 20 µg / 100 ml).

2. Use gloves for handling ethidium bromide as it is mutagenic agent.

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(i)
Syllabus

Course :	**BIOCHEM 231**	**Credit:**	3(2+1)	**Semester-III**
Course title:	Fundamentals of Plant Biochemistry and Biotechnology			

Theory

Importance of Biochemistry. Plant cell structure and function of cell orgenells. Properties of Water and significance of weak interactions and biomolecules. Bioenergetics and significance of redox reactions. Carbohydrate: Structure, classification, properties and functions. Lipid: Structures and properties of fatty acids. Structure, classification, properties and functions. Amino acids and Proteins: Structure, classification, properties and functions. Structural organization of proteins. Enzymes: General properties; Classification; Mechanism of action and enzyme kinetics and inhibition. Enzyme regulation. Nucleic acids: Importance and classification; Structure of Nucleotides, DNA structure and forms of RNA and function. Mitochondrial electron transport and oxidative phosphorylation. Metabolism of carbohydrates, lipids and nucleic acids. Biochemistry of nitrate assimilation and photosynthesis. Introduction to recombinant DNA technology: PCR techniques and its applications.

Concepts and applications of plant biotechnology: Scope, organ culture, embryo culture, cell suspension culture, callus culture, anther culture, pollen culture and ovule culture and their applications; Micro-propagation methods; organogenesis and embryogenesis, Synthetic seeds and their significance; Embryo rescue and its significance; somatic hybridization and cybrids; Somaclonal variation and its use in crop improvement; cryo-preservation; Introduction to recombinant DNA methods: physical (Gene gun method), chemical (PEG mediated) and Agrobacterium mediated gene transfer methods; Transgenics and its importance in crop improvement; PCR techniques and its applications; RFLP, RAPD, SSR; Marker Assisted Breeding in crop improvement; Biotechnology regulations.

Practical

Preparation of solution, pH & buffers, Qualitative tests of carbohydrates and amino acids. Quantitative estimation of soluble sugars and amino acids. Estimation of starch, total carbohydrate and soluble proteins . Determination of crude fat and qualitative tests of fats and oils. Enzyme assay: Alpha amylase , Nitrate reductase, lipase and protease. Paper chromatography/ TLC demonstration for separation of amino acids/ Monosaccharides. Demonstration on isolation of DNA. Demonstration of gel electrophoresis techniques and DNA finger printing.

(ii)

Teaching Schedule

a) Theory

Lecture	Topic	Weightage (%)
1	Importance of Biochemistry	2
2-3	Properties of Water, pH and Buffer	5
4	Biomolecules - Definition, types, structure, properties and its applications	5
5-7	Carbohydrate: Importance and classification. Structures of Monosaccharides, Reducing and oxidizing properties of Monosaccharides, Mutarotation; Structure of Disaccharides and Polysaccharides.	8
8-9	Lipid: Importance and classification; Structures and properties of fatty acids; storage lipids and membrane lipids.	6
10-12	Proteins: Importance of proteins and classification; Structures, titration and zwitterions nature of amino acids; Structural organization of proteins.	8
13-14	Enzymes: General properties; Classification; Mechanism of action; Michaelis & Menten and Line Weaver Burk equation & plots; Introduction to allosteric enzymes.	8
15	Nucleic acids: Importance and classification; Structure of Nucleotides, A, B & Z DNA; RNA: Types and Secondary & Tertiary structure.	5
16-18	Metabolism of carbohydrates: Glycolysis, TCA cycle, Glyoxylate cycle, Electron transport chain.	8
19-20	Metabolism of lipids: Beta oxidation, Biosynthesis of fatty acids.	5
21	Biochemistry of nitrate assimilation	5
22	Photosynthesis	5
23-24	Introduction to recombinant DNA technology: PCR techniques and its applications.	5
25-26	Organ culture, embryo culture, cell suspension culture, callus culture, anther culture, pollen culture and ovule culture and their applications;	5
28-29	Micro-propagation methods; organogenesis and embryogenesis, Synthetic seeds and their significance; Embryo rescue and its significance; somatic hybridization and cybrids	8
30	Somaclonal variation and its use in crop improvement	4
31-32	Physical (Gene gun method), chemical (PEG mediated) and Agrobacterium mediated gene transfer methods	4
33	Marker Assisted Breeding in crop improvement	4
	Total	**100**

b) Practical

Experiment	Topic
1	Preparation of solution, pH & buffers
2-3	Qualitative tests for carbohydrates and amino acids
4	Estimation of reducing sugars by Nelson-Somogyi method
5	Estimation of starch by Anthrone method
6	Determination of soluble protein by folin-lowry method
7	Estimation of free amino acids by Ninhydrin method
8	Determination of total crude fat/oil by Soxhlet method
9	Qualitative tests for oil
10	Determination of alpha amylase activity from germinating seed
11	Determination of invivo nitrate reductase activity from leaf tissue
12	Paper chromatography/ TLC demonstration for separation of amino acids
13	TLC for separation of sugars
14-15	Isolation of genomic DNA from plant. Purification, Quantification and quality determination
16	Amplification of genomic DNA using different primers and resolution of PCR products on agarose gel

Suggested Readings:

1) Bhatia S. C., 1984, Biochemistry in Agricultural Sciences, Shree Publication House, New Delhi.
2) Purohit S.S. 2009, Biochemistry - Fundamentals and Applications, Agrobios, Jodhpur
3) Singh M. 2011, A Textbook of Biochemistry, Dominant Publishers & Distributors, New Delhi
4) Veerkumari L. 2007, Biochemistry, MIP Publishers, Chennai
5) Jain J. L. etal 2005, Fundamentals of Biochemistry, S. Chand & Company Ltd. , New Delhi
6) *Rastogi S. C.*. 2003 - *Biochemistry* Tata McGraw-Hill Education, New Delhi.
7) *Rama Rao A. V. S. S.*, 2002 A *Textbook of Biochemistry*. Edition, 9, illustrated. Publisher, Sangam Books Limited, New Delhi.
8) Com EE &Stumpf PK. 2010.Outlines of Biochemistry.5th Ed. John Wiley Publications.
9) Donald Voet and Judith G. Voet. 2011. Biochemisry, 4th Ed. John Wiley and Sons, Inc., NY, USA.
10) Goodwin, TW & Mercer EI. 1983. Introduction to Plant Biochemistry. 2nd Ed. Oxford, New York.Pergaman Press.
11) David L. Nelson and Michael M. Cox.2012. Lehninger Principles of Biochemisry, 6th Ed .Macmillan Learning, NY, USA
12) Jeremy M. Berg, John L. Tymoczko, LubertStryer and Gregory J. Gatto, 2002.Biochemisry, 7th Ed. W.H. Freeman and Company, NY, USA
13) Jayaram. T. 1981. Laboratory manual in biochemistry, Wiley Estern Ltd. New Delhi:
14) Plummer D. 1988. An Introduction to Practical Biochemistry. 3rd ed. Tata McGraw Hill, New Delhi.
15) Practical biochemistry: R. L. Nath. A treatise on Analysis of Food, Fats and Oils: A. R. Sen, N.K. Pramanik and S.K. Roy
16) *Sadasivam* S, *Manickam* A (1996) *Biochemical methods*. 2nd edition, New Age International (p) Ltd. Publisher, New Delhi..