

# **Lab Manual on Fundamentals of Plant Biochemistry**

**Course Code: ASBC2101**

**Credits: 3(2+1)**

**By**

**Dr. Raghu Gogada, Associate Professor & HOD**

**Dr. Pradipta Banerjee, Assistant Professor**

**Dr. Udit Nandan Mishra, Assistant Professor**



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*Shaping Lives...  
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**Department of Biochemistry and Plant Physiology**

**M.S. Swaminathan School of Agriculture**

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Alluri Nagar, R. Sitapur, Uppalada, Paralakhemundi, Gajapati, Odisha-761221

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## **Experiment-1: Preparation of Solutions (Percentage solution, Normal Solution and Molar Solution)**

**Aim:** To prepare solutions of given concentration or percentage

### **Principle & Procedure:**

**1. Percent solution** is the solution expressed in the unit %. It may be (a) percentage by weight-w/v or (b) percentage by volume-v/v.

**Percentage solution (w/v):** It is defined as number of grams of solute dissolved in 100 ml of solution.

$$\% \text{ solution} = \frac{\text{wt. of solute (g)}}{\text{vol. of solution (ml)}} \times 100$$

**Example:** If one wished to make a 3% NaCl solution, 3.0 g NaCl would be dissolved in 100 ml of water.

**Percentage solution (v/v):** It is defined as volume of solute present in 100 ml of solution.

$$\% \text{ solution} = \frac{\text{volume of solute (ml)}}{\text{vol. of solution (ml)}} \times 100$$

**Example:** If one wished to make a 70% ethanol solution, 70 ml of 100% ethanol would be dissolved in 30 ml of water.

**2. Normal Solution** or Normality is the number of equivalent weights of a solute dissolved per liter of a solution.

Example: Equivalent weight of  $\text{H}_2\text{SO}_4$  is 49 g. Thus, to prepare 1(N) we need to dissolve  $\text{H}_2\text{SO}_4$  49 g of the acid in 1L of water.

**3. Molar Solution** or Molarity is the number of gram molecular weight of a solute dissolved per liter of a solution.

Example: Equivalent weight of  $\text{H}_2\text{SO}_4$  is 98 g. Thus, to prepare 1(M) we need to dissolve  $\text{H}_2\text{SO}_4$  98 g of the acid in 1L of water.

### **Exercise**

1. Prepare 5% NaOH solution
2. Prepare 300 ml 2 (N) NaOH solution
3. Prepare 500 ml 0.5 (M)  $\text{Na}_2\text{CO}_3$  solution

### **Result**

## **Experiment-2: Measurement of pH of a given solution using digital pH meter**

**Aim:** To measure the pH of a solution/buffer

### **Principle:**

When one metal is brought in contact with another, a voltage difference occurs due to their differences in electron mobility. When a metal is brought in contact with a solution of salts or acids, a similar electric potential is caused, which has led to the invention of batteries. Similarly, an electric potential develops when one liquid is brought in contact with another one, but a membrane is needed to keep such liquids apart.

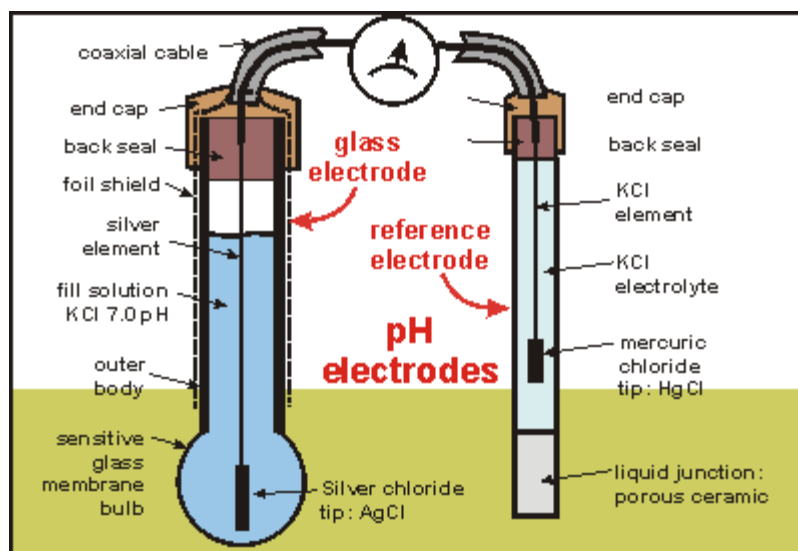
A pH meter measures essentially the electro-chemical potential between a known liquid inside the glass electrode (membrane) and an unknown liquid outside. Because the thin glass bulb allows mainly the agile and small hydrogen ions to interact with the glass, the glass electrode measures the electro-chemical potential of hydrogen ions or the potential of hydrogen. To complete the electrical circuit, also a reference electrode is needed. Note that the instrument does not measure a current but only an electrical voltage, yet a small leakage of ions from the reference electrode is needed, forming a conducting bridge to the glass electrode. A pH meter must thus not be used in moving liquids of low conductivity (thus measuring inside small containers is preferable).

The pH meter measures the electrical potential (follow the drawing clock-wise from the meter) between the mercuric chloride of the reference electrode and its potassium chloride liquid, the unknown liquid, the solution inside the glass electrode, and the potential between that solution and the silver electrode. But only the potential between the unknown liquid and the solution inside the glass electrode change from sample to sample. So all other potentials can be calibrated out of the equation. The calomel reference electrode consists of a glass tube with a potassium chloride (KCl) electrolyte which is in intimate contact with a mercuric chloride element at the end of a KCL element. It is a fragile construction, joined by a liquid junction tip made of porous ceramic or similar material. This kind of electrode is not easily 'poisoned' by heavy metals and sodium.

The glass electrode consists of a sturdy glass tube with a thin glass bulb welded to it. Inside is a known solution of potassium chloride (KCl) buffered at a pH of 7.0. A silver electrode with a silver chloride tip makes contact with the inside solution. To minimise

electronic interference, the probe is shielded by a foil shield, often found inside the glass electrode.

Most modern pH meters also have a thermistor temperature probe which allows for automatic temperature correction, since pH varies somewhat with temperature.



### Procedure

1. Turn on your pH meter. Before you begin to calibrate and use your pH meter you will first need to turn it on and allow adequate time for the meter to warm up. This should generally take around 30 minutes, but check your pH meter's operating manual for exact times.

2. Clean your electrode. Take the electrode out of its storage solution and rinse it with distilled water under an empty waste beaker. Once rinsed, blot dry with Kimwipes.

- Be sure to rinse your electrode in a waste beaker that is different from the beaker you will be calibrating in.
- Avoid rubbing the electrode as it has a sensitive membrane around it.
- If you find the electrode to be particularly dirty consult your operating manual for recommended cleaning solutions.

3. Prepare your buffers. You will generally need more than one buffer for calibrating a pH meter. The first will be a “neutral” buffer with a pH of 7, and the second should be near the expected sample pH, either a pH of 4 or 9.21. Buffers with a higher pH (9.21) are best for measuring bases, whereas buffers with a low pH (4) are best for measuring acidic samples. Once you have chosen your buffers allow them to reach the same

temperature as the pH meter because pH readings are temperature dependent. Pour your buffers into individual beakers for calibration.

- Check with your pH meter manufacturer, or current educational or professional institution, about acquiring pH buffer solutions.
- Buffers should be kept in a beaker for no longer than two hours.
- Discard the buffer when you are finished. Do not return it to its original container.

## Experiment-3: Preparation of Buffers

**Aim:** To prepare 1x PBS buffer of pH 7.4

### Principle:

A buffer is an aqueous solution containing a weak acid and its conjugate base or a weak base and its conjugate acid. A buffer's pH changes very little when a small amount of strong acid or base is added to it. It is used to prevent any change in the pH of a solution, regardless of solute. Buffer solutions are used as a means of keeping pH at a nearly constant value in a wide variety of chemical applications. For example, blood in the human body is a buffer solution. Buffer solutions are resistant to pH change because of the presence of an equilibrium between the acid (HA) and its conjugate base (A<sup>-</sup>). The balanced equation for this reaction is:  $HA \rightleftharpoons H^+ + A^-$ . When some strong acid (more H<sup>+</sup>) is added to an equilibrium mixture of the weak acid and its conjugate base, the equilibrium is shifted to the left, in accordance with Le Chatelier's principle. This causes the hydrogen ion (H<sup>+</sup>) concentration to increase by less than the amount expected for the quantity of strong acid added. Similarly, if a strong base is added to the mixture, the hydrogen ion concentration decreases by less than the amount expected for the quantity of base added. This is because the reaction shifts to the right to accommodate for the loss of H<sup>+</sup> in the reaction with the base. Buffer solutions are necessary in a wide range of applications. In biology, they are necessary for keeping the correct pH for proteins to work; if the pH moves outside of a narrow range, the proteins stop working and can fall apart. A buffer of carbonic acid (H<sub>2</sub>CO<sub>3</sub>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>) is needed in blood plasma to maintain a pH between 7.35 and 7.45. Industrially, buffer solutions are used in fermentation processes and in setting the correct conditions for dyes used in coloring fabrics.

### Procedure

1. Start with 800 ml of distilled water
2. Add 8 g of NaCl + 0.2 g of KCl + 1.44 g of Na<sub>2</sub>HPO<sub>4</sub> + 0.24 g of KH<sub>2</sub>PO<sub>4</sub>.
3. Adjust the pH to 7.4 with HCl.
4. Add distilled water to a total volume of 1 liter.
5. Dispense the solution into aliquots and sterilize by autoclaving (20 min, 121°C, liquid cycle). Store at room temperature.

### Result

## Experiment-4: Qualitative tests of Carbohydrates

**Aim:** To determine the presence of carbohydrates in the given sample qualitatively

### Principle:

Carbohydrates are widely prevalent in the plant kingdom, comprising the mono-, di-, oligo-, and polysaccharides. The common monosaccharides are the glucose, fructose, galactose, ribose etc. the disaccharides, i.e., the combination of two monosaccharides include sucrose, lactose and maltose. Starch and cellulose are polysaccharides consisting of many monosaccharide residues. Cellulose is most abundant organic compound on this planet since it forms part of cell wall in plants.

Aldehydes (-CHO) and ketones (=CO) are active groups in carbohydrates. Carbohydrates contain many hydroxyl groups as well. The number of hydroxyl groups varies with the number of carbon atoms. Monosaccharides contain the free aldehyde group (maltose) and some do not have the free ones (sucrose). The polysaccharides, starch and cellulose, are polymers of monosaccharides linked through the active groups.

The chemical properties of the saccharides vary depending upon the number of hydroxyl groups and the presence or absence of -CHO/ =CO groups. These variations are the basis in the development of color reactions to identify the saccharides.

### Chemicals and Reagents:

#### Reagents

- a) **Iodine Solution:** Add a few crystals of iodine to 2% potassium iodide solution till the color becomes deep yellow.
- b) **Fehling's Reagent A:** Dissolve 34.65 g copper sulphate in distilled water and make up to 500 mL.
- c) **Fehling's Reagent B:** Dissolve 125 g potassium hydroxide and 173 g Rochelle salt (potassium sodium tartrate) in distilled water and make up to 500 mL.
- d) **Benedict's Qualitative Reagent:** Dissolve 173 g sodium citrate and 100 g sodium carbonate in about 800 mL water. Heat to dissolve the salts and filter, if necessary. Dissolve 17.3 g copper sulphate in about 100 mL water and add it to the above solution with stirring and make up to volume to 1L with water.
- e) **Barfoed's Reagent:** Dissolve 24 g copper acetate in 450 mL boiling water. Immediately add 25 mL of 8.5% lactic acid to the hot solution. Mix well. Cool and dilute to 500 mL.
- f) **Seliwanoff's Reagent:** Dissolve 0.05g resorcinol in 100 mL dilutes hydrochloric acid (1:2).

- g) **Bial's Reagent:** Dissolve 1.5 g orcinol in 500 mL of concentrated HCl and add 20 to 30 drops of 10% ferric chloride.

### Procedure:

Substantiated by the observations the presence or absence of different carbohydrates needs to be determined.

**Table 1.1: Qualitative reactions of Carbohydrates:**

	<b>Experiment</b>	<b>Observation</b>	<b>Remarks</b>
<b>1.</b>	<p><b>Molisch's Test</b> Add two drops of Molisch's Reagent (5% 1-naphthol in alcohol) to about 2mL of test solution and mix well.</p> <p>Incline the tube and add about 1 mL of concentrated sulphuric acid along the sides of the tube.</p> <p>Observe the color at the junction of the two liquids.</p>	A red-cum-violet ring appears at the junction of the two liquids	The color formed is due to the reaction of alpha-naphthol with furfural and/or its derivative formed by the dehydration of sugars by concentrated sulphuric acid. All carbohydrates react positively with this reagent.
<b>2.</b>	<p><b>Iodine Test</b> Add a few drops of iodine solution to about 1 mL of the test solution</p>	Appearance of deep blue color	<p>This indicates the presence of starch in the solution</p> <p>The blue color is due to formation of starch-iodine complex.</p>
<b>3.</b>	<p><b>Fehling's Test</b> To 1 mL of Fehling's solution 'A', add 1 mL of Fehling's solution 'B' and a few drops of the test solution. Boil for a few minutes.</p>	Formation of yellow or brownish-red precipitate	The blue alkaline cupric hydroxide present in solution, when heated in the presence of reducing sugars, gets reduced to yellow or red cuprous oxide and it gets precipitated. Hence, formation of the colored precipitate indicates the presence of reducing sugars in the test solution.
<b>4.</b>	<p><b>Benedict's Test</b> To 2 mL of Benedict's reagent add five drops of the test solution. Boil for five minutes in a water bath. Cool the solution.</p>	Formation of red, yellow or green color/precipitate.	As in Fehling's test, the reducing sugar because of having potentially free aldehyde or keto group reduce cupric hydroxide in alkaline solution to red colored cuprous oxide. Depending on the sugar concentration yellow to

			green color is developed.
5.	<b>Barfoed's Test</b> To 1 mL of the test solution add about 2 mL of Barfoed's reagent. Boil it for one minute and allow to stand for a few minutes.	Formation of brick-red precipitate.	Only monosaccharides answer this test. Since Barfoed's reagent is weakly acidic, it is reduced only by monosaccharides.
6.	<b>Seliwanoff's Test</b> To 2 mL of Seliwanoff's reagent add two drops of test solution and heat the mixture to just boiling.	Appearance of deep red color	In the concentrated HCl, ketones undergo dehydration to yield furfural derivatives more rapidly than do aldoses. These derivatives form complexes with resorcinol to yield deep red color. It is a timed color reaction specific for ketones.
7.	<b>Bial's Test</b> To 5 mL of Bial's reagent add 2-3mL of solution and warm gently. When bubbles rise to the surface cool under the tap	Appearance of green color or precipitate	It is specific for pentoses, they get converted to furfural. In the presence of ferric ion orcinol and furfural condense to yield a coloured product.
8.	<b>Test for non-reducing sugars such as sucrose:</b>		
a)	Do Benedict's test with the test solution	No characteristic color formation	Indicates the absence of reducing sugars in the given solution
b)	Add 5 drops of concentrated HCl to 5mL of test solution in another test tube. Heat for five minutes on a boiling water bath. Add 10% sodium hydroxide solution to give a slightly alkaline solution (test with red litmus paper). Now perform Benedict's test with this hydrolyzed solution	Appearance of red or yellow color	Indicated the formation of reducing sugars from non-reducing sugars after hydrolysis with acid.
9.	<b>Mucic Acid Test</b> Add a few drops of conc. HNO <sub>3</sub> to the concentrated test solution or substance directly and evaporate it over a boiling water bath till the acid fumes are expelled. Add a few drops of water and leave it overnight	Formation of crystals	The both end carbon groups are oxidized to carboxylic groups. The resultant saccharic acid of galactose is called mucic acid which is insoluble in water.
10	<b>Osazone Test</b> To 0.5g of phenyl hydrazine hydrochloride add 0.1g of sodium acetate and 10 drops of glacial acid. To this mixture add 5mL of test solution and heat on a boiling water	Glucose, fructose and mannose produce needle-shaped yellow osazone crystals. Whereas	The ketoses and aldoses react with phenyl-hydrazine to produce a phenylhydrazone which in turn reacts with another two molecules of

	bath for about half an hour. Allow the tube to cool slowly and examine the crystals under a microscope.	lactosazone in mushroom-shape d. Different osazones show crystals of different shapes. Maltose produces flower-shaped crystals.	phenylhydrazine to form the osazone.
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**Notes:**

1. For osazone test, the reaction mixture should be between pH 5 and 6. Fructose takes 2min to form the osazone whereas for glucose it is 5min. the disaccharides take a longer time to form osazone. Disaccharides form crystals only on cooling.
2. When a mixture of carbohydrates is present in the test sample, chromatographic methods should be employed to identify the individual sugars.

**Result:**

## Experiment-5: Qualitative determination of Amino acids

**Aim:** To determine the presence of Proteins in the given sample qualitatively

**Principle:** Following tests are used to determine the amino acids qualitatively

**a) Solubility test**

Polar amino acids are more soluble in water than non-polar, due to presence of amino and carboxyl group which enables amino acids to accept and donate protons to aqueous solution.

**b) Ninhydrin test for alpha L amino acids**

Ninhydrin (tri ketohydrindene hydrate) degrades amino acids into aldehydes, ammonia and CO<sub>2</sub> (on pH range 4-8) through a series of reactions. The net result is ninhydrin in a partially reduced form from hydrindantin. Ninhydrin then condenses with ammonia and hydrindantin to produce an intensely blue or purple pigment, sometimes called Ruhemann's purple. The color varies slightly from acid to acid. Proline and hydroxy-proline (amino acids) give yellow color. Many substances other than amino acids, such as amines will yield a blue color with ninhydrin, particularly if reaction is carried out on filter paper.

**c) Xanthoproteic test for Aromatic amino acids**

Conc. HNO<sub>3</sub> reacts with the aromatic rings that are derivatives of benzene giving the characteristic nitration reaction. Amino acids tyrosine and tryptophan contain activated benzene rings which are easily nitrated to yellow colored compounds. The aromatic ring of phenyl alanine does not react with nitric acid despite it contains a benzene ring, but it is not activated, therefore it will not react.

**d) Millon's test for amino acids containing hydroxy phenyl group**

The phenol group of tyrosine is first nitrated by nitric acid in the test solution. Then the nitrated tyrosine complexes mercury ion in the solution to form a brick-red solution or precipitate of nitrated tyrosine, in all cases, appearance of red color is positive test.

**e) Sakaguchi Test**

In alkaline solution, arginine reacts with alpha-naphthol and sodium hypobromite /chlorite as an oxidizing agent, to form red complexes as a positive result.

### Materials and Reagents

Hydrochloric acid

Sodium hydroxide

Chloroform

Ninhydrin

Nitric acid

Phenol solution

10% NaOH

α-naphthol in 10% ethanol

5% sodium hypobromate (or sodium hypochlorite)

Test tubes

Water bath

### Procedure

### Solubility Test

Place 0.5 mL of amino acid sample in 4 test tubes clean, dry containing 4 mL of different solvents (chloroform and hot water), Shake the tubes thoroughly, and then leave the solution for about one minute, Record your result.

### Ninhydrin Test

- i. Place 1 mL of each of the solutions in a test tube and add 2 drops of Ninhydrin solution.
- ii. Boil the mixture over a water bath for 2 min.
- iii. Allow to cool and observe the blue color formed
- iv. Complete the below table

### Xanthoproteic Test

- i. Label five tubes (1 - 5), then add 0.5 mL of each amino acid solution and phenol solutions to those test tubes each alone.
- ii. Add a few drops of concentrated HNO<sub>3</sub>.
- iii. Compare the color with that given by blank using water instead.  
Now COOL THOROUGHLY under the tap and CAUTIOUSLY add 5 drops of 10M NaOH to make the solution strongly alkaline.

### Millons Test

- i. Label 4 test tubes (1 - 5).
- ii. Add 1 mL of test solutions in separate tubes and the phenol solution in one tube.
- iii. Add to each tube 2 mL Millon's reagent and shake it well.
- iv. Place the test tubes in the boiling bath with care, for 10 min.
- v. Write your observation in the following table.

### Sakuguchi Test

- i. Label 3 test tube and put in each one 1 mL of the aminoacid solution.
- ii. Add to each tube 2 mL of NaOH solution. Mix well.
- iii. Add to each tube 2 mL of  $\alpha$ -naphthol solution. Mix well.
- iv. Add to each tube 3 drops of sodium hypobromite solution, and record your result.

**Table 2.1: Qualitative reactions of Amino acids**

S. No	Name of the Test	Observation

**Result:** The amino acids in the given sample are qualitatively determined and results were tabulated in Table 2.1

## **Experiment-6: Determination of Proteins using Biuret Protein Assay**

**Aim:** To determine the presence of Proteins in the given sample using Biuret test

### **Principle:**

Under alkaline conditions substances containing two or more peptide bonds form a purple complex with copper salts in the reagent.

### **Reagent:**

A formula for biuret reagent is (per liter final volume): 9 gm Sodium potassium tartrate, 3 gm Copper sulfate, 5 gm Potassium iodide, dissolved in order in 400 mL 0.2 M NaOH before bringing to final volume.

### **Procedure:**

1. Volumes sample, reagent can be scaled up/down and/or volume ratios varied, as with any assay.
2. Warm up the spectrophotometer 15 min. before use.
3. Prepare standards from bovine serum albumin, preferably calibrated using absorbance at 280 nm and the extinction coefficient. Using 5 mL color reagent to 1 mL sample a recommended range is 0.5 to 20 mg protein.
4. Prepare a reference tube with 1 mL buffer.
5. If possible, dilute unknowns to an estimated 1 to 10 mg/mL with buffer; a range of dilutions should be used if the actual concentration cannot be estimated. Tabulate the values in Table 3.1.
6. Use 1 mL sample per assay tube
7. Add 9 mL Biuret reagent to each tube, vortex immediately, and let stand 20 min.
8. Read at 550 nm.

### **Analysis**

Prepare a standard curve of absorbance versus micrograms protein (or vice versa), and determine amounts from the curve. Determine concentrations of original samples from the amount protein, volume/sample, and dilution factor, if any.

**Table 3.1: Table for BSA Standard and Test Samples**

S. No	Vol of BSA (mL)	Conc. of BSA (mg/mL)	Vol of distilled water (mL)	Vol of Biuret reagent	Duration of Incubation	OD at 550nm
					20min	
Samples						

**Result:**

The amount protein present in the given sample was.....

## Experiment-7: Titration Methods for Estimation of Amino acids

**Aim:** Estimation of Amino acids by Titration methods

### Principle:

Amino acids react with formalin to form methylene amino acids. But it was found that the reaction was more complex and the main product is monomethylol and di methylol amino acids. Amino acids exist in zwitter ionic form and cannot be titrated directly with alkali. Thus amino groups of amino acids are blocked by reaction with formaldehyde. But formaldehyde does not react with the charged amino groups ( $-\text{NH}_3^+$ ), thus first the amino acid reacts with sodium hydroxide solution to give glycine which condenses with formaldehyde to give a stable anion.

Further formaldehyde solution contains formic acid and also amino acids are not exactly neutral species, thus it is necessary that both the formaldehyde and amino acid solution should have the same pH before mixing and for this purpose each solution is first made just alkaline to phenolphthalein by means of dilute sodium hydroxide solution.

### Reagents:

**a. Neutral formalin solution**

It is prepared by neutralization of formalin solution with alkali to phenolphthalein end point.

**b. Standard Glycine solution**

An accurately weighed 0.8 grams of Glycine is dissolved in distilled water in a measuring flask 100 mL.

**c. Unknown Glycine solution**

**d. Sodium hydroxide solution 0.1 N**

**e. Phenolphthalein indicator**

### Procedure:

1. A standard glycine solution (20 mL) is pipetted out into a conical flask and phenolphthalein (2-3 drops) added.
2. It is titrated with sodium hydroxide solution until a faint pink color appears.
3. Then the neutralized formaldehyde solution (10 mL) is added.
4. The pink color of the solution in the titration flask disappears immediately and the solution becomes acidic.
5. Then the contents of the flasks are titrated with sodium hydroxide solution till the pink color is obtained.
6. Same experiment repeated with unknown glycine solution.

### Observations:

Weight of Glycine dissolved in 100 mL = w g

Volume of sodium hydroxide solution used with standard glycine solution =  $v_1$  mL

Volume of sodium hydroxide solution used with unknown glycine solution =  $v_2$  mL

Volume of glycine solution taken = 20 mL

### Calculations

V<sub>1</sub> mL of NaOH in 20mL standard glycine solution = (w/5) g of glycine

V<sub>2</sub> mL of Na OH in 20mL of unknown glycine solution = (w/5) x (v<sub>2</sub>/v<sub>1</sub>) g of glycine

Thus strength = (w/5) (v<sub>2</sub>/v<sub>1</sub>) (1000/20) g/L

$$= (w \times v_2 \times 10) / v_1 \text{ g/L}$$

### Result:

## Experiment-8: Effect of pH, temperature and substrate concentration on enzyme action

**Aim:** To determine the effect of pH temperature and substrate concentration on enzyme action

### Principle:

Enzyme reaction rates are influenced by several factors such as pH, temperature and the concentration of reactants/substrate concentration. For all enzymes there is an optimum temperature and pH for the formation of enzyme-substrate complexes.

As the temperature increases there is more movement of molecules and therefore more collisions between enzymes and substrates – so the enzyme activity increases. However, there is a limit to which enzyme activity can increase because at a certain temperature an enzyme will denature.

When the pH is too acidic or too basic for an enzyme, its hydrogen bonds begin to break, causing its active site to change its shape. An altered active site can't bind with its substrate so enzyme activity decreases. If the pH is too unfavorable then covalent bonds can break, causing the enzyme to denature.

It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum.

### Materials:

pH meter, Water baths-2, ice, 500 mL beaker, test tubes, droppers, white spot plates, starch, iodine.

### Procedure:

#### Effect of pH:

- Switch on the water bath and keep it at 40°C.
- Take 3 test tubes (16x125mm) and name them 1 2 3.
- Prepare solutions as per the table 5.1.
- Place test tubes in water bath at 40°C for 30 min.
- Then place 3 drops from each test tube separately on white spot plate and add 2 drops of iodine solution to each. Observe the change in the colour which corresponds to the extent of hydrolysis by colour of test.

**Table 5.1: Amylase enzyme activity with different pH buffers**

Test tube Number	1% starch Solution (mL)	2mL buffer solution of pH	Amylose or saliva
1	2	2	2
2	2	7	2
3	2	12	2

### Effect of Temperature:

1. Switch on the water bath and keep it at 40°C.
2. Take 3 test tubes (13x100mm) and name them 1 2 3. Take 6 mL of your own saliva (which will have salivary amylase) in a 10 mL graduated cylinder.
3. Take 2 mL of saliva in each tube and add 2 mL of 2% starch solution to each tube.
4. Place tube 1 in ice bath (10°C), 2 in water bath with 40°C, and 3 in water bath with 100°C for 30 minutes.
5. Then place 3 drops from each test tube separately on white spot plate and add 2 drops of iodine solution to each. Observe the change in the colour which corresponds to the extent of hydrolysis by colour of test.

**Table 5.2: Enzyme activity at different Temperatures**

Test tube Number	1% starch Solution (mL)	Amylose or saliva	Temperature
1	2	2	10 °C
2	2	2	40 °C
3	2	2	100 °C

### Effect of Substrate:

1. Switch on the water bath and keep it at 40°C.
2. Take 3 test tubes (13x100mm) and name them 1 2 3
3. Prepare solutions as per the table 5.3.
4. Add 1 mL of 3 M HCl to each test tube. Place test tubes in water bath at 40°C. Observe precipitate formation in the test tube. Note the time in seconds and observe which test tube forms the precipitate first. Note down the time for remaining two test tubes also.

**Table 5.3: Enzyme activity with different substrates**

Test tube Number	2 % Renin Solution (mL)	Distilled Water (mL)	Milk (mL)
1	4	0	3
2	2	2	3
3	1	3	3

### Result:

## **Experiment-9: Paper Chromatography for separation of Amino acids**

**Aim:** To separate Amino acids by paper chromatography.

### **Principle:**

Amino acids are the building blocks of peptides and proteins. They possess two functional groups: the carboxylic acid group gives the acidic character, and the amino group provides the basic character.

The R-represents the side chain that is different for each of the amino acids that are commonly found in proteins. However, all 20 amino acids have a free carboxylic acid group and a free amino (primary amine) group except proline, which has a cyclic side chain and a secondary amino group.

We use the properties provided by these groups to characterize the amino acids. The common carboxylic acid and amino groups provide the acid–base nature of the amino acids. The different side chains, and the solubilities provided by these side chains, can be utilized to identify the different amino acids by their rate of migration in paper chromatography.

In this experiment, we use paper chromatography to identify aspartame, an artificial sweetener, and its hydrolysis products from certain foods.

### **Materials:**

1. 0.1% solutions of aspartic acid, phenylalanine, and leucine
2. 0.5% solution of aspartame (Equal)
3. Diet Coca-Cola
4. 3 M HCl
5. 0.2% ninhydrin spray
6. Butanol: acetic acid: water–solvent mixture
7. Equal sweetener
8. Aluminum foil
9. Whatman no. 1 chromatographic paper: 15x 8 cm; 12x 4 cm
10. Ruler
11. Polyethylene gloves
12. Capillary tubes open on both ends
13. Heat lamp or hair dryer
14. Drying oven, 110°C
15. Wide-mouth jar
16. Iodine crystals
17. Test tubes (100x13 mm)
18. Test tubes (75x10 mm)

## Procedure:

1. Dissolve 10 mg of the sweetener Equal in 1 mL of 3 M HCl in a test tube (100 x 13 mm). Heat the solution with a Bunsen burner, using a small flame (or with a micro burner) to a boil for 30 sec. Do not heat the bottom of the test tube, but heat slightly above the surface level of the solution. As the solution boils, do not let the liquid evaporate completely. Set the solution aside to cool; this is the hydrolyzed aspartame.
2. Label six small test tubes (75x10 mm) as follows: (1) phenylalanine, (2) aspartic acid, (3) leucine, (4) aspartame, (5) hydrolyzed aspartame, (6) Diet Coca-Cola. Place about 0.5 mL samples in each test tube.

3. Use plastic gloves throughout in order not to contaminate the paper chromatogram. Take a strip of Whatman No. 1 chromatographic paper, 8x15 cm and 0.016 cm thick. With a pencil, lightly draw a line parallel to the 8-cm edge 1 cm from the edge. Mark the positions of 6 spots, placed equally, where you will spot your samples

**Spotting:** For each sample, use a separate capillary tube. Then apply a drop of sample to the paper until it spreads to a spot of 1 mm diameter. Further, dry the spot. (If a heat lamp is available, use it for drying.) Do the spotting in the following order: a) Phenylalanine 1 drop; b) Aspartic acid 1 drop; c) Leucine 1 drop; d) Aspartame (in Equal) 1 drop; e) Hydrolyzed aspartame 6 drops; f) Diet Coca-Cola 10 drops.

4. When you do multiple drops of nos. 5 and 6, allow the paper to dry after each drop before applying the next drop (Avoid putting a hole in the paper!). Do not allow the spots to spread to larger than 1 mm in diameter.

5. Pour about 15 mL of solvent mixture (Butanol: acetic acid: water) into a large (1L) beaker. Place a glass rod over the beaker. Using clear tape, affix the chromatographic paper to the rod so that when you lower the rod over the beaker, the paper will dip into the solvent but the pencil marks and the spots will be above the solvent surface.

Cover the rod and beaker with aluminum foil. Place the beaker on a hot plate. Turn to a low setting (e.g., no. 2 out of 10 on the dial) and heat the beaker to about 35°C. Allow the solvent front to advance at least 6 cm (it will take 40–50 min.), but do not allow it to get closer than 1 cm from the top edge. Watch the temperature and make sure it does not rise above 35°C.

While you are waiting for the solvent front to complete its rise, you can do the short chromatography experiment in Part B. Your instructor may choose to make this optional.

6. When the solvent front has advanced at least 6 cm, remove the rod and the paper from the beaker. You must not allow the solvent front to advance up to or beyond the top edge of the paper. Mark immediately with a pencil the position of the solvent front. Under a hood, dry the paper with the aid of a heat lamp or hair dryer. With polyethylene gloves on your hands, spray the dry paper with ninhydrin solution. Be careful not to spray ninhydrin on your hands and do not touch the sprayed areas with bare hands. If the ninhydrin spray touches your skin (which contains amino acids), your fingers will be discolored for a few days. Place the sprayed paper into a drying oven set at 105–110°C for 2–3 min.

7. Remove the paper from the oven. Mark the center of the spots and calculate the Rf values of each spot. Record your observations on the Report Sheet.

8. If the spots on the chromatogram are faded, you can visualize them by exposing the chromatogram to iodine vapor. Place your chromatogram into a wide-mouth jar (or a large beaker, 1-L size) containing a few iodine crystals. Cap the jar (or cover the beaker with aluminum foil) and warm it slightly on a hot plate to enhance the sublimation of iodine. The iodine vapor will interact with the faded pigment spots and make them visible. After a few minutes' exposure to the iodine vapor, remove the chromatogram and mark the spots immediately with a pencil. The spots will fade again with exposure to air. Measure the distance of the center of the spots from the origin and calculate the Rf values.

### Calculation

$R_f = \text{Distance traveled by the amino acid} / \text{Distance traveled by the solvent front}$

**Table 6.1: Table of Aminoacids and Rf values**

S No	Band Colour	Amino acid	Distance (cm)	Rf values
1				
2				
3				
4				

### Result:

## Experiment-10: Sterilization Techniques

**Aim:** Effective sterilization techniques are essential for working with isolated cell lines for obvious reasons you don't want bugs from the environment growing in your nice culture medium, and equally, cultures must be sterilised before disposal.

### **Wet Heat** (Autoclaving)

The method of choice for sterilisation in most labs is autoclaving; using pressurised steam to heat the material to be sterilised. This is a very effective method that kills all microbes, spores and viruses, although for some specific bugs, especially high temperatures or incubation times are required. Autoclaving kills microbes by hydrolysis and coagulation of cellular proteins, which is efficiently achieved by intense heat in the presence of water. The intense heat comes from the steam. Pressurised steam has a high latent heat; at 100 °C it holds 7 times more heat than water at the same temperature. This heat is liberated upon contact with the cooler surface of the material to be sterilised, allowing rapid delivery of heat and good penetration of dense materials. At these temperatures, water does a great job of hydrolyzing proteins so that those bugs don't stand a chance.

### **Dry Heat** (Flaming, baking)

Dry heating has one crucial difference from autoclaving. You've guessed it there's no water, so protein hydrolysis can't take place. Instead, dry heat tends to kill microbes by oxidation of cellular components. This requires more energy than protein hydrolysis so higher temperatures are required for efficient sterilization by dry heat. For example sterilization can normally be achieved in 15 minutes by autoclaving at 121°C, whereas dry heating would generally need a temperature of 160 °C to sterilize in a similar amount of time.

### **Filtration**

Filtration is a great way of quickly sterilizing solutions without heating. Filters, of course, work by passing the solution through a filter with a pore diameter that is too small for microbes to pass through. Filters can be scintered glass funnels made from heat-fused glass particles or, more commonly these days, membrane filters made from cellulose esters. For removal of bacteria, filters with an average pore diameter of 0.2µm are normally used. But remember, viruses and phage can pass through these filters so filtration is not a good option if these are a concern.

## **Solvents**

Ethanol is commonly used as a disinfectant, although since isopropanol is a better solvent for fat it is probably a better option. Both work by denaturing proteins through a process that requires water, so they must be diluted to 60-90% in water to be effective. Again, it's important to remember that although ethanol and IPA are good at killing microbial cells, they have no effect on spores.

## **Radiation**

UV, x-rays and  $\gamma$  rays are all types of electromagnetic radiation that have profoundly damaging effects on DNA, so make excellent tools for sterilization. The main difference between them, in terms of their effectiveness, is their penetration. UV has limited penetration in air so sterilisation only occurs in a fairly small area around the lamp. However, it is relatively safe and is quite useful for sterilizing small areas, like laminar flow hoods. X-rays and gamma rays are far more penetrating, which makes them more dangerous but very effective for large scale cold sterilization of plastic items (e.g. syringes) during manufacturing.

## **Result:**

## Experiment-11: Estimation of Resistant Starch

**Aim:** To estimate resistant starch.

### Principle:

Starch is the major energy reserve in plants and can be found in grains, roots, tubers, stems, leaves, and fruits. Starch consists of two major glucans: amylose and amylopectin. Amylose is an essentially linear polysaccharide with  $\alpha$ -1,4 linked D-glucopyranose units and a few branches of  $\alpha$ -1,6 linkages, whereas amylopectin is a highly-branched polysaccharide consisting of short linear-chains connected by about 5%  $\alpha$ -1,6 branch linkages. Starch is a major energy source for humans and animals. After being ingested, starch is hydrolyzed to glucose by amylolytic enzymes in the digestive tract. Glucose is then absorbed in the small intestine to be used for energy. A portion of starch, known as *resistant starch (RS)*, cannot be hydrolyzed by enzymes in the small intestine and is passed into the large intestine for fermentation by gut microflora. RS is categorized as a type of dietary fiber because it can provide similar physiological benefits to humans and animals as other dietary fibers. Health benefits of RS include: lowering postprandial plasma glucose concentration and insulin response; increasing insulin sensitivity and preventing Type-2 diabetes; improving serum lipid profile and preventing obesity; and improving colon health. RS can be classified into five different types: physically inaccessible starch (RS1); raw granular starch with the B- or C-type polymorph (RS2); retrograded starch (RS3); chemically modified starch (RS4); and amylose-lipid complex (RS5). When amylose forms single-helical complex with lipids, it becomes resistant to enzymatic hydrolysis

The samples will be incubated with pancreatic  $\alpha$ -amylase and amyloglucosidase (AMG) for 16 h at 37°C. During this time the non-resistant starch will be solubilized and hydrolyzed to glucose by the combined action of these two enzymes. The reaction would be terminated by the addition of an equal volume of ethanol and the RS will be recovered as a pellet on centrifugation. This will later be washed twice with ethanol (50% v/v), and again centrifuged. The RS in the pellet will be dissolved in 2 M KOH by vigorously stirring on an ice-water bath. This solution would be neutralized with acetate buffer and the starch will be quantitatively hydrolyzed to glucose with AMG. The glucose would be quantified with glucose oxidase/peroxidase reagent (GOPOD), which would be giving a measure of the RS content of the sample.

**Materials:**

1. Powdered rice grain
2. Shaking water bath
3. Test tubes
4. Magnetic stirrer
5. Centrifuge

**Reagents:**

1. Sodium maleate buffer (0.1 M, pH 6),
2. Sodium acetate buffer (1.2 M, pH 3.8 and 0.1 M, pH 4.5)
3. Potassium hydroxide (2M)
4. Aqueous ethanol (Industrial Methylated Spirits or IMS)

**Preparation of Reagents:**

1. Sodium maleate buffer (0.1 M, pH 6) – Dissolve 23.2 g of maleic acid in 1600 mL of distilled water and adjust the pH to 6.0 with 4 M (160 g/lit) sodium hydroxide. Add 1.47 g of calcium chloride dehydrate and dissolve. Adjust the volume to 2 L.
2. Sodium acetate buffer (1.2 M) – Add 69.6 ml of glacial acetic acid (1.05 g/ml) to 800 ml of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide. Adjust the volume to 1 lit with distilled water.
3. Sodium acetate buffer (0.1 M) – Add 5.8 ml of glacial acetic acid to 900 mL of distilled water and adjust to pH 4.5 using 4 M sodium hydroxide. Adjust the volume to 1 L with distilled water.
4. Potassium hydroxide (2M) – Add 112.2 g KOH to 900 ml of deionized water and dissolve by stirring. Adjust volume to 1 L. Store in a sealed container.
5. Aqueous ethanol (or IMS) (approx. 50% v/v) – Add 500 ml of ethanol (95% v/v or 99% v/v) or industrial methylated spirits (IMS; denatured ethanol; ~ 95% v/v ethanol plus 5% v/v methanol) to 500 ml of H<sub>2</sub>O. Store in a well-sealed bottle.

## **Procedure:**

1. Weigh about 100 mg of the sample individually into screw cap tube, add 4.0 ml of pancreatic  $\alpha$ -amylase solution (10 mg/ml) containing AMG (3 U/ml) and subsequently incubate at 37°C with continuous shaking (200 strokes/min) for exactly 16 h.

***CRITICAL STEP: Horizontal shaking is better and it's critical that samples should be immersed in solution.***

2. Disperse 4.0 ml of 99% (IMS)/alcohol into tube with vigorous stirring and then centrifuge at 1,500g for 10 min.

3. The supernatants would be decant and pellets would be re-suspended in 2 mL of 50% IMS/alcohol, vortex vigorously and add 6 ml of 50% IMS/alcohol, vortex and again centrifuge at 1,500g for 10 min.

4. Above step 4 is repeated twice.

***CRITICAL STEP: Complete removal of alcohol from the tubes is necessary***

5. The pellet would be kept for resistant starch determination whereas the centrifuged washings will be combined with the original decanted supernatant and used for solubilized starch determination. The pellet would be re-suspended with 2 ml of 2 M KOH.

6. After continuous stirring for 20 min in an ice bath, 8 mL of 1.2 M sodium acetate buffer (pH 3.8) and 0.1 ml of AMG (3300 U/ml) would be added and the tubes would be placed in a water bath at 50°C for 30 min.

***CRITICAL STEP: Proper stirring by placing in ice is critical, pellet should be completely dissolved.***

7. At the end of the treatment, the solution would be brought to a total volume of 100 ml (optional) with distilled water, mixed and centrifuged at 1500 rpm for 10 min.

8. 0.1 ml aliquot of supernatant would be treated with 3.0 ml of D-glucose (oxidaseperoxidase) assay kit (GOPOD) reagent (Megazyme, Ireland), vortex-mixed and kept at 50°C for 20 min.

***CRITICAL STEP: Working GOPOD can be stored in 4°C, if turned pinkish should be discarded.***

9. Blank (0.1 ml of 100 mM sodium acetate buffer pH 4.5) and 3.0 mL of GOPOD and glucose standards 0.1 mL of D-glucose (1 mg/ml) and 3.0 ml of GOPOD, would be incubated concurrently.

10. The absorbance would be read with a spectrophotometer at 510 nm. The total starch contents are calculated as the sum of digestible starch and resistant starch.

### **Calculation:**

Resistant Starch (g/100 g sample) (samples containing < 10% RS)=  $\Delta E F 10.3/0.1$   
 $1/1000 100/W 162/180 = \Delta E F/W 9.27$

Where:  $\Delta E$  = absorbance (reaction) read against the reagent blank.

$F$  = conversion from absorbance to micrograms (the absorbance obtained for 100  $\mu$ g of D-glucose in the GOPOD reaction is determined and  $F = 100$  ( $\mu$ g of D-glucose) divided by the GOPOD absorbance for this 100  $\mu$ g of D-glucose.

$100/0.1$  = volume correction (0.1 ml taken from 100 ml).

$1/1000$  = conversion from micrograms to milligrams.

$W$  = dry weight of sample analyzed = "as is" weight [(100 moisture content)/100]

$100/W$  = factor to present RS as a percentage of sample weight.

$162/180$  = factor to convert from free D-glucose, as determined, to a hydro-D-glucose as occurs in starch.

$10.3/0.1$  = volume correction (0.1 ml taken from 10.3 ml) for samples containing 0-10% RS where the incubation solution is not diluted and the final volume is ~ 10.3 ml.

### **Results:**

## **Experiment-12: Extraction of Oil and Estimation of its Characteristic Features**

**Aim:** To extract oil and estimate its characteristics features

### **Principle:**

Fats and oils belong to a group of biological substances called lipids. They are constructed of building blocks called triglycerides resulting from combination of one unit of glycerol and three units of fatty acids. They are insoluble in water but soluble in most organic solvents. They have lower densities than water, and may have consistencies at ambient temperature of solid, semi-solid or clear liquid. When they are solid-appearing at normal temperature, they are referred to as “fats” and when they are liquid at the ambient temperature, they are called oils. The principle is based on the extraction of oil using non-polar solvent viz., ether, hexane, petroleum ether (40-60 oc), chloroform:methanol (2:1). And it involves repeated extraction of oil. The solvent is then distilled off completely. The oil is dried, weighed and the % of oil is calculated.

### **Method of extraction and estimation of oil:**

Extraction of oil from an oil-seed is generally carried out using the following two methods:

- (a) Soxhlet method
- (b) Cold percolation method

Here in the present experiment the protocol for Soxhlet method of oil extraction is given.

### **Soxhlet method of oil extraction:**

#### **Materials:**

1. soxhlet extractor assembly
2. Absorbent cotton
3. Whatman filter paper

#### **Reagent:**

Hexane or diethyl ether/petroleum ether (40-60oC).

**Procedure:**

1. Fold a piece of filter paper and make into a sample packet in such a way to hold the seed powder (2-5g) depending on oil content.
2. Place the sample packet into the extractor of soxhlet apparatus, after placing some cotton at the bottom. A piece of cotton is placed at the top to evenly distribute the solvent as it drops on the sample during extraction.
3. Add organic solvent, two and a half times the capacity of the extractor and extract oil for a period of six hours, or for a longer period till the solvent in the extractor becomes colorless.
4. Put off the heaters and allow it to cool. Flash evaporate the solvent or evaporate the ether on a water bath until no odour of ether remains. Keep it in oven at 70 degree for 10 min. Cool at room temperature.
5. Weigh the flask after removing moisture. Repeat heating until constant weight is recorded.

**Calculations:**

% Oil in the sample =  $\text{Weight of oil (g)} \times 100 / \text{Weight of sample (g)}$

**Results:**

## Experiment-13: Estimation of Acid Value of Oil

**Aim:** To estimate the acid value of extracted oil.

### Principle:

The acid value is the number of milligrams of potassium hydroxide required to neutralize the free fatty acids present in 1 g of fat or oil. The acid value of oil is dependent on the amount of free fatty acids present. This in turn is dependent on the degree of hydrolysis of oil or nature of processing which the oil may have undergone. Refined oils are often referred to as neutralized *i.e.* they have low acid value. Raw and crude oils are naturally hydrolyzed and consequently have a high acid value.

Free fatty acids are usually present in small quantity in oil along with the triglycerides, the proportion increase during storage. The fat/oil on storage may become rancid due to peroxide formation by microorganisms in presence of atmospheric oxygen. The amount of free fatty acids gives an indication of age and quality of fat. The free fatty acids in oil are estimated by titrating it against standard potassium hydroxide in the presence of phenolphthalein indicator.

### Reagents:

1. 0.1N KOH
2. Neutral solvent: Mix equal volume of 95% ethanol and solvent ether. Acid or alkali is added to make it neutral depending on the pH.
3. 0.1N potassium hydrogen phthalate for standardization of KOH or 0.1N oxalic acid.
4. 1% phenolphthalein as indicator.

### Procedure:

1. Accurately weigh 1-10 g oil in a clean dry 100 ml conical flask.
2. Add 25 ml of neutral solvent solution and add few drops of phenolphthalein and warm it.
3. Titrate the warm content against 0.1N potassium hydroxide.
4. Shake constantly until a pink colour which persists for 30 sec is obtained.
5. Run a blank following the similar procedure as for the test sample.

### Calculations:

Acid value (AV) =  $\frac{(\text{Titre value} - \text{blank value}) \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample}}$

Weight of the sample

The free fatty acid (FFA) is calculated as oleic acid equivalent using the equation

% FFA = AV/1.99 or 1 ml of N/10 KOH = 0.0282 g of oleic acid (on the basis of oleic acid)

Say Normality of KOH is in

*i.e.* N gm equivalents/100 ml

Since acidity of KOH is 1

= N moles/1000 ml

= N 56.1 g/1000 ml

= N 56.1 1000mg/1000 ml

Or 1000ml contains = N 56.1 1000 mg of KOH

Say net titre value is V, and then amount of KOH consumed in the reaction is:

$N \times 56.1 \times 1000 \times V \text{ mg}$

1000

Say this value has been obtained for W g of sample

So, Acid value for 1 g of sample will be =  $(V \times N \times 56.1) / W$

### Results:

## **Experiment-14: Estimation of Iodine Value of an Oil Sample**

**Aim:** To estimate the iodine value of extracted oil.

### **Principle:**

In chemistry, it is the mass of iodine in grams that is consumed by 100 gm of a chemical substance. An iodine solution is violet in color and any chemical group in the substance that reacts with iodine will make the color disappear at a precise concentration. The amount of iodine solution thus required to keep the solution violet is a measure of the iodine sensitive reactive groups. Iodine value is a measure of unsaturation. The higher the iodine value, the higher the level of unsaturation and more reactive the oil is. Non-drying oils have low iodine values (<125) whereas drying oils have high iodine values (>150). Oils with iodine value between 125 and 150 are known as semi-drying oils. Solid fats have low iodine value (typically <60).

If oil consists of triglycerides with a high percentage of unsaturated fatty acids such as linolenic acid then that oil will be reactive and is known as drying oil. This means that when applied to a non-porous surface it will dry to a film in a certain time. Non-drying, Drying and semi-drying oils find uses in surface coating applications where drying properties are essential e.g. paints, resins, printing inks. Iodine value of oil is defined as number of grams of iodine absorbed by 100 gm of oil or fat. This contains oil both saturated and unsaturated fatty acids such as oleic and linoleic acids as glyceryl esters (triacyl glycerols). Iodine gets incorporated into the fatty acid chain wherever the double bond exists to form addition (saturated) compounds.

Present experiment makes use of iodine monobromide (Hanus solution) to saturate a known weight of oil. The unreacted iodine monobromide is then reacted with potassium iodide which converts it into  $I_2$ . The  $I_2$  concentration is then determined by titration with standard sodium thiosulfate using starch as an indicator.

### **Reagent:**

1. 0.1 N sodium thiosulfate
2. 15% potassium iodide

3. 0.3N Hanus solution (IBr) in glacial acetic acid
4. 1% starch solution
5. 0.1 N potassium dichromate ( required for standardization of sodium thiosulfate)

**Procedure:**

1. Weigh 50-100 mg oil into an iodine flask and dissolve in 25 ml of carbon tetrachloride.
2. Add 25 ml of Hanus solution from a burette and keep the flask in dark for 45 min. with occasional shaking.
3. Add 10 ml of 15% potassium iodide solution mix thoroughly and add 25 ml of water, to washing much down any free iodine on the stopper.
4. Titrate against 0.1 N sodium thiosulfate till the color of the titre becomes pale yellow. From this point onwards color fades very slowly from pale yellow to colorless.
5. To get a definite color change at the end point, 1 ml of starch indicator is added in the solution which turns it blue black. Titration is then continued till the end point is obtained when colour suddenly changes from blue black to colorless.
6. A blank determination is carried out without oil, using exactly the same quantity of carbon tetrachloride and keeps it for the same duration in Hanus solution and determine amount of used IBr.

**Calculation:**

$$\text{Iodine value} = \frac{(V_2 - V_1) \times \text{Normality of thiosulphate} \times 12.69}{\text{Weight of oil}}$$

V<sub>2</sub> = Volume of thiosulphate used for blank

V<sub>1</sub> = Volume of thiosulphate used for oil sample.

**Note:**

1. One ml of 1.0 N Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> = 0.1269 g of iodine.
2. Starch forms an insoluble complex with iodine. The titer problem precludes the addition of the indicator at the start of the titration and this is why the indicator is to be

*added just prior to the end point when the color of the solution has changed to pale yellow.*

**Results:**

# **Experiment-15: Protein Characterization Using by Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS-PAGE)**

**Aim:** Protein Characterization Using by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

## **Principle:**

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a technique used in

biochemistry and molecular biology to separate proteins according to their electrophoretic mobility (a function of length of polypeptide chain or molecular weight as well as higher order protein folding, posttranslational modifications and other factors). The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures and dithiothreitol (DTT) or 2-mercaptoethanol (beta-Mercaptoethanol/BME) which reduces the disulfide bonds, and applies a negative charge to each protein in proportion to its mass. Without SDS, different proteins with similar molecular weights would migrate differently due to differences in folding, as differences in folding patterns would cause some proteins to better fit through the gel matrix than others.

Adding SDS solves this problem, as it linearizes the proteins so that they may be separated strictly by length (primary structure, or number of amino acids). The SDS binds to the protein in a ratio of approximately 1.4 g SDS per 1.0 g protein (although binding ratios can vary from 1.1-

2.2 g SDS/g protein), giving an approximately uniform mass:charge ratio for most proteins, so that the distance of migration through the gel can be assumed to be directly related to only the

size of the protein. A tracking dye may be added to the protein solution to allow the experimenter to track the progress of the protein solution through the gel during the electrophoretic run.

The denatured proteins are subsequently applied to one end of a layer of polyacrylamide gel submerged in a suitable buffer. An electric current is applied across the gel, causing the negatively charged proteins to migrate across the gel. Depending on their size, each protein will move differently through the gel matrix: short proteins will more easily fit through the pores in the gel, while larger ones will have more difficulty. After a set amount of time (usually a few hours though this depends on the voltage applied across the gel; higher voltages run faster but tend to produce somewhat poorer resolution), the proteins will have differentially migrated based on their size; smaller proteins will have traveled farther down the gel, while larger ones will have remained closer to the point of origin. Thus proteins may be separated roughly according to size (and therefore, molecular weight). Following electrophoresis, the gel may be stained (most commonly with Coomassie Brilliant Blue or silver stain), allowing visualization of the separated proteins, or processed further (e.g. Western blot). After staining, different proteins will appear as distinct bands within the gel. It is common to run "marker proteins" of known molecular weight in a separate lane in the gel, in order to calibrate the gel and determine the weight of unknown proteins by comparing the distance traveled relative to the marker.

Gel electrophoresis is usually the first choice as an assay of protein purity and characterization due to its reliability and ease. The presence of SDS and the denaturing step causes proteins to be separated solely based on size.

### **Materials:**

1. 30% Acrylamide/bis (29:1) solution
2. 4 x Resolving gel buffer: 1.5 M Tris-HCl (pH 8.8), 0.4% SDS
3. 4 x Stacking gel buffer: 0.5 M Tris-HCl (pH 6.8), 0.4% SDS
4. 1 x Electrophoresis buffer: 25 mM Tris-HCl (pH 8.3), 250 mM glycine, 0.1% SDS
5. 3 x protein loading buffer: 0.19 M Tris-HCl (pH 8.0), 3% glycerol, 6% SDS, 300 mM DTT, and 0.25% bromophenol blue;
6. 10% Ammonium persulfate (APS)

7. TEMED
8. Gel staining buffer: 1 g of Coomassie Brilliant Blue dissolved in 400 ml of solution containing  
45% methanol, 45% H<sub>2</sub>O, and 10% acetic acid
9. Destaining buffer: 25 % methanol, 10% acetic acid, and 65% H<sub>2</sub>O
10. Gel Drying Buffer: 40% ethanol, 4% glycerol, and 56% H<sub>2</sub>O
11. Protein samples
12. Protein standards (Protein markers)

### **Procedure:**

#### **1. Preparation 40 ml of 15% resolving gel solution as following:**

- 9.68 ml of H<sub>2</sub>O
- 20 ml of 30% Acrylamide/bis solution
- 10 ml of 4 x resolving gel buffer
- 300 µl of 10% APS
- 20 µl of TEMED

First assemble the gel caster, and add the 15% gel mix to gel caster. Overlay the solution gently with water or 1-butanol saturated with H<sub>2</sub>O, and allow to be polymerized. You should see the gel-H<sub>2</sub>O interface disappear and then reappear when the gel is polymerized;

#### **2. Preparation 20 ml of 5% stacking gel solution as following:**

- 11.5 ml of H<sub>2</sub>O
- 3.3 ml of 30% Acrylamide/bis solution
- 5 ml of 4 x stacking gel buffer
- 200 µl of 10% APS
- 10 µl of TEMED

Add the gel mix to the gel caster, and insert the comb to the gel solution; you should wait until gel is fully polymerized. Once the gel is polymerized, the comb can be removed gently. Then assemble the gel into the electrophoresis apparatus.

#### **3. Loading of samples:**

Mix two volumes of your samples with 1 volume of 3 x sample loading buffer, and heat the samples over 90 0C for 5 min, and load 20 µl of your samples to each well of the gel. Also add the protein standards in two lanes on each gel.

#### **4. Running the gels:**

Apply power at 100-150 V until the dye has reached the top of the resolving gel, and then increase the power into 200 V. When the dye reaches the bottom of the resolving gel, turn off the power supplier. Disassemble the gel apparatus, and stain the gel for about one hour at Gel staining buffer. Destain your gel at the destaining buffer until the protein bands are clearly seen in the gel, and dry the gel if it is possible.

#### **5. Determination of molecular weight of the protein:**

The individual protein bands can be analyzed from the banding pattern observed after staining the acrylamide gel by comparing their respective positions with respect to the bands in the protein marker lane.

## **Experiment-16: Estimation of vitamin A content**

**Aim:** To estimate vitamin A content.

### **Principles:**

Carotenoids, also called tetraterpenoids, are organic pigments that are produced by plants and algae, as well as several bacteria and fungi. The only animals known to produce carotenoids are aphids and spider mites, which acquired the ability and genes from fungi. There are over 600 known carotenoids; they are split into two classes, xanthophylls (which contain oxygen) and carotenes (which are purely hydrocarbons, and contain no oxygen). All are derivatives of tetraterpenes, meaning that they are produced from 8 isoprene molecules and contain 40 carbon atoms. In general, carotenoids absorb wavelengths ranging from 400, 550 nanometers (violet to green light). This causes the compounds to be deeply colored yellow, orange, or red. Plant carotenoids are the primary dietary source of provitamin A worldwide, with  $\beta$ -carotene as the best-known provitamin A carotenoid.

The sample is extracted in acetone which dissolves both the fat and water soluble pigments. The acetone extract is then taken in petroleum ether layer. The fat soluble carotenoid pass from acetone to the petroleum ether leaving all the rest of the pigment pigments in acetone. Different methods have been proposed for the analysis of carotenoids including  $\beta$ -carotene. Methodology for the estimation of  $\beta$ - carotene from a variety of food material is outlined below.

### **Reagent:**

1. Water saturated n-butanol: *Mix n-butanol and water in ratio of 6:2 (v/v) and shake vigorously. Then allow to stand till it separates into two phases; the upper clear layer is water saturated n-butanol.*

### **Procedure:**

1. Disperse 10g of sample in 50 ml water-saturated n-butanol to make a homogenous suspension.
2. Shake gently and allow to stand overnight (16 h) at room temperature in dark.

3. Shake the suspension again and filter through Whatman filter paper No. 1
4. Make the volume of filtrate to 100 ml.
5. Measure the absorbance (A) of the clear filtrate at 440 nm in Spectronic-20/spectrophotometer using saturated n-butanol as a blank.

**Calculation:**

The amount of  $\beta$ -carotene from the following equation:

$$\beta\text{-carotene content (ppm)} = 0.0105 + 23.5366 \times A$$

**Results:**

## **Experiment-17: Estimation of vitamin C content**

**Aim:** To estimate vitamin C content.

### **Principle:**

Vitamin C is a highly water-soluble natural acidic bioactive compound having strong reducing properties and well known as a wonder worker because of wide range of roles played. This method determines the vitamin C concentration in a solution by a redox titration using an indophenol dye. Ascorbic acid which has strong reducing property reduces 2,6-dichloro indophenol (DCPIP) dye to a colourless, leuco base in acidic medium. In this reaction ascorbic acid is oxidised to dehydro ascorbic acid while indophenol dye, which is violet in color, acts as oxidising agent and gets decolourised after being reduced the end point is an appearance of faint pink colour which persists for few seconds. The method is suitable for use with vitamin C tablets, fresh or packaged fruit juices and solid fruits and vegetables.

### **Materials:**

1. Sample containing vitamin C
2. Standard ascorbic acid
3. 2,6-dichloro indophenol dye (DCPIP)
4. Sodium bicarbonate
5. Metaphosphoric acid
6. Mortar and pestle
7. Burette and stand
8. 100 mL or 200 mL volumetric flask
9. Micropipette
10. 10 mL and 100 mL measuring cylinders
11. 250 mL conical flasks
12. Whatmann No.1 filter papers
13. Water bath with thermostat
14. Centrifuge

### **Reagents:**

1. **2,6-dichloro indophenol dye solution:** Dissolve 125 mg of sodium salt of dye and 105 mg NaHCO<sub>3</sub> in 100 ml hot distilled water (about 80°C). Cool, filter and make the volume to 500 ml with distilled water.
2. **Stabilizing medium: (3% w/v):** Weigh 15 g of metaphosphoric acid (HPO<sub>4</sub>), stir after adding 50 ml of distilled water and make the volume to 500 ml and keep it in cold.
3. **Standard ascorbic acid solution (1 mg per ml):** Dissolve 100mg of ascorbic acid in 100 ml of stabilizing medium.

## **Methods:**

### **Extraction of ascorbic acid from sample**

1. Extract approx. 10g of sample in stabilizing medium (1:5) in mortar pestle. In case of citrus fruits (Lemon etc), juice is directly extracted by squeezer.
2. Centrifuge the homogenate. Take the supernatant and make the volume to 100 ml with stabilizing medium.
3. Filter it, if needed, through whatman filter paper No.1 and suitable aliquot is used for estimation of ascorbic acid.

### **Standardization of dye**

Take 1 ml of standard ascorbic acid (1mg per ml) in 50 ml conical flask and titrate with DCPIP dye solution to faint pink color which should persist for 15 sec. note the titre value.

***CAUTION:** Iodine stains both skin and clothing so proper care is advised. If staining does occur, alcohol may remove skin stains and cleaners are available for fabric stains.*

### **Estimation of ascorbic acid in sample**

Take known volume (5-10 ml) of phosphoric acid extracted sample in 50 ml conical flask and titrate with standard dye to faint pink color which should persist for 15 sec.

***CAUTION:** The importance of the room temperature being constant and not too high is because otherwise the vitamin C content may have been altered, since vitamin C is heat labile. Moreover, if the temperature varied, the measured results might have varied also*

## **Calculation:**

$$\text{Amount of ascorbic acid (mg per/100g)} = \frac{X \text{ mg} \times V2 \times Z}{V1 \times Y \text{ ml} \times \text{wt of the sample}} \times 100$$

Where,

X = mg of standard ascorbic acid

V1 = Titre value of standard ascorbic acid against dye

V2 = Titre value of sample against dye

Y = amount of aliquot taken (ml) for estimation

Z = Total volume made up of extracted sample

**NOTE:** *Ascorbic acid is susceptible to oxidation by atmospheric oxygen over time. For this reason, the samples should be prepared immediately before the titrations. However, if the samples have to be prepared several hours earlier, oxidation can be minimised by the addition of a small amount of oxalic acid (eg 1 g oxalic acid per 100 mL of sample solution)*

**Results:**

## **Experiment-18: Use of Soxhlet extractor (to extract fats and oils from seeds, fibres, etc.)**

**Aim:** Extraction of fats and oils from seeds, fibres using Soxhlet Extractor.

**Principle:** In the laboratory, a Soxhlet extractor is used for extraction. The fat extractor uses the solvent reflux and siphon principle to continuously extract the solid matter by pure solvent, which saves the solvent extraction efficiency and high efficiency. The solid material is ground prior to extraction to increase the area of solid-liquid contact. The solid material is then placed in a filter paper holder and placed in an extractor. The bottom end of the extractor is connected to a round bottom flask containing a solvent, and is connected to a reflux condenser. The bottom flask is heated to boil the solvent, the vapor rises through the branch pipe of the extractor, is condensed and drops into the extractor, and the solvent is contacted with the solid for extraction. When the solvent surface exceeds the highest point of the siphon, the solvent containing the extract is siphoned back. The flask, thus extracting a portion of the material, is repeated such that the solid material is continuously taken as a pure solvent and the extracted material is concentrated in the flask.

### **Procedure:**

1. A Soxhlet extraction procedure usually involves the following steps.
2. The sample material is packed in filter paper and placed in the thimble.
3. Vapors of a fresh solvent, produced in a distillation flask, pass through the thimble containing the material to be extracted and are liquefied in the condenser.
4. When the liquid reaches the overflow level in the thimble, a siphon aspirates the solution, and the liquid falls back into the distillation flask, carrying the extracted solutes into the bulk liquid. The separation of solute from solvent takes place in the distillation flask.
5. The solute is left in the flask and fresh solvent vapors pass back into the solid bed of sample material.
6. The operation is repeated until complete extraction is achieved. It may take 2-4 hours for complete extraction.

## **Experiment-19: Identification of fats and oil**

**Aim:** Identification of fats & oils like organic compounds

**Principles:** Saponification is a process by which triglycerides are reacted with sodium or potassium hydroxide to produce glycerol and a fatty acid salt called "soap." The triglycerides are most often animal fats or vegetable oils.

**Procedure:** There are two simple methods to identify fats and oils.

- a) The solution of fat will stain paper and make it translucent.
- b) **Saponification test:** To about 2 ml of the test solution, add few drops of NaOH solution and heat to boil. Cool it in air. A semi-solid (soap) mass is produced which forms lather with water.

**Result:**

## **Experiment-20: Determination of moisture and fat in butter**

**Aim:** To determine moisture and fat in butter

**Principle:** Commercial butter is about 80% butterfat and 15% water; traditionally made butter may have as little as 65% fat and 30% water. Butter contains a good amount of saturated fat, and a little amount of unsaturated fats.

### **Determination of moisture**

1. Procedure for Determination of Moisture
2. Place the sample of butter in sample bottle
3. Keep the sample bottle on water bath at 35°C
4. Stir the sample constantly until it softens (don't allow to melt)
5. Tare an Al beaker and weigh into it accurately 10g of butter sample
6. Hold the beaker with tong and heat gently on a wire gauze in a mild flame
7. Slowly the butter will turn brown in color
8. Allow the sample to cool in air
9. Weigh the Al beaker with sample
10. % of moisture = loss in weight/wt. of sample x 100

### **Determination of fat**

1. To the dried sample obtained above, add 50 ml petroleum ether and stir thoroughly with glass rod
2. Remove the glass rod and set aside for 4 minutes
3. Decant the ether dissolved fat without disturbing the settled residue
4. Repeat the extraction 2-3 times using 25 ml of petroleum ether
5. Warm the Al beaker holding on a wire gauze and continue heating till the residue is free from the solvent and appears powdery
6. Cool and dry in air
7. Fat % = loss in wt/wt. of sample x 100

**Result:**

**Reference:**

- 1) Sadasivam S and Balasubramanian T (1985). Practical Manual (Undergraduate), Tamil Nadu Agriculture University, Coimbatore, p.2.
- 2) Sadasivam S, Manickam A (2008). Biochemical Methods, New Age International Publishers, 3<sup>rd</sup> Edition, ISBN: 978-81-224-2140-8.