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A practical manual on

Fundamentals of Plant Biochemistry and Biotechnology

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Laboratory Manual

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Practical No 1

Aim: Preparation of solution, pH & buffers

Materials required:pH meter,Beakers, Wash bottle, tissue paper, Glacial acetic acid, Sodium acetate.

Theory: The pH meter measures the electrical potential developed by pair ofelectrode pins in a solution. For measurement of pH, an electrode system sensitiveto change in H+ ion concentration of solution is taken. The electrode systemconsists of sequence of electrode whose potential raise with pH (H+ concentrationof the solution).

$$pH = pKa + \log [A^-] / [HA]$$

Procedure

pH measurement:

1. Take some water in a clean beaker.
2. Take the electrode out, wash with distilled water and wipe clean with tissue paper.
3. Tare the meter and dip the electrode in the sample (here water).
4. Note the reading.

Result: pH of the given Sample

Preparation of buffer:

Acetic acid- sodium acetate buffer:

Reagents required:

Acetic Acid 0.2M: 1.5 ml of glacial acetic acid is made up to 100ml withdistilled water.

Sodium Acetate Solution: 0.64 gm. of sodium acetate or 2.72gm of sodiumacetate tri-hydrate is dissolved in 100ml Distilled water.

Procedure:

Pipette out exactly 36.2ml of sodium acetate solution into 100ml ofstandard flask and add 14.8ml of glacial acetic acid, make the volume 100ml usingdistilled water using distilled water. This gives 0.2 M of acetic acid and sodiumacetate buffer. The pH is measured with pH meter.

The pH meter is first standarized with pH buffer. Wash electrode with distilled water and introduced into 0.2M acetic acid-sodium acetate bufferprepared, the pH of solution is 4.6.

Result:

Precautions:

- Weigh the chemical properly

- Mix the contents in around 50mL of distilied water and later scale up to the final volume

Practical No 2

Aim: Qualitative test of carbohydrate

Materials required: test tubes, pipette, beaker, deionized water, benedict's reagent, Fehling's reagent A& B, boiling water,samples (glucose, sucrose & starch).

Principle: Sugar that is capable of acting as a reducing agent is known as reducing sugar because it has a free aldehyde group or a free ketone group. These free groups are oxidized to carboxylic acid. In order for oxidation to occur, the cyclic form must first ring-open to give the reactive aldehyde. Thus any sugar that contains a hemi acetal will be a reducing sugar.

Benedict's test: the reagent that is used in the Benedict 's test is known as Benedict's reagent. It is a clear blue solution containing copper (II) sulphate & sodium tartrate. Copper sulphate provides the cu^{2+} ions and sodium tartarate provides the required alkaline environment. In the test free aldehyde or keto group in the reducing sugar reduce the alkaline cupric oxide to red coloured cuprous oxide.

Fehling's test: It is a test that is done so as to differentiate between reducing and non-reducing sugar. The test was developed by German chemist Hermann van Fehling in 1849. Fehling's solution is a preparation of two separate solution, known as Fehling's solution A and Fehling's solution B. Fehling's solution A is an aqueous solution of copper sulphate (II), which is deep blue. Fehling's solution B is a colourless solution of aqueous potassium sodium tartarate (also known as rochere salt), made in strong alkali, commonly with sodium hydroxide.

The reducing sugar when reacts with Fehling's solution gives yellow or brownish red colour precipitation.

Procedure:

For Benedict's test:

(1) Take a sample in a test tube.
(2) Add few amount of benedict's solution. Then the mixture is shaken well and placed in boiling water for a while.
(3) If the sample is reducing sugar a brick red precipitate will be formed otherwise the mixture would remain clear blue.

For Fehling's Test:

(1) Fehling's solution A and B should add in equal amount and then sample will be added to it.
(2) Then the mixture is shaked well and placed in boiling water for a while.
(3) If a sample is reducing sugar a brick red precipitate will be formed, otherwise the mixture would remain clear blue.

Result and observation

Sample	Reagent Added	Inference	Result

Precaution:

- Chemical should not be wasted.
- The reaction takes place in alkaline environment only.
- In case the mixture is acidic the cu $^{2+}$ ion would be stabilized and will not be easily oxidized thus the reaction will not be completed.

Practical No 3

Aim: Qualitative tests of Protein

Materials Required: Ninhydrin solution, Acetone, HNO_3, NaOH, Spirit lamp, Glasswares

Principle

Ninhydrin test

Ninhydrin is a chemical used to detect ammonia or primary or secondary amines. Amino acid react with Ninhydrin at pH 4, then reduction product is obtained from Ninhydrin to yield a blue coloured substance. Amino acid is extremely sensitive to this test.

Xanthoprotic test

Some amino acid contains aromatic group that are derivatives of Benzene. These aromatic group undergoes reaction that have characteristics of benzene and benzene derivatives. One such reaction is nitration of benzene ring with nitric acid . Activated benzene ring of amino acid undergoes nitration. The nitration reaction in the presence of activated benzene ring forms yellow product. This test is application to aromatic amino acid like Tyrosin , Tryptophan, Phenylalanine, and non- aromatic acid like glutamic acid.

Procedure:

Ninhydrin test

- 1ml of sample (ex: filtered wheat flour or crushed seeds solution) solution and 5 drops of 0.2% Ninhydrin solution in acetone was added.
- It was incubated in water bath for 2 minutes.
- Allow it to cool for some time and blue color complex was observed.

Xanthoprotic test

- 2ml of sample was taken in test tube and equal volume of concentrated Nitric acid was added and heated over flame for 2 minutes till there was a colour change.
- It was cooled under running tap water and 40% NaOH was added to make the solution alkaline in nature.
- The colour change in nitro derivative of aromatic structure was observed.

Result:

Precautions:

- Wash the apparatus before and after the experiment.

- Make sure that amount you are taking of samples and liquid drops are the same as mentioned in the procedure.
- Also, make sure that the test tube you are using must be clean and wash very neatly. There must not remain any impurity other than the natural stuff present in the test tube.
- Observe colours at the end of the tests very carefully and note down results on a notepad to avoid future inconvenience.
- Prevents prolonged heating of the test tube otherwise, it will give a false result.
- Carefully handle all the chemicals.

Practical No 4

Aim: Quantitative estimation of glucose

Materials required: Test tube and test tube rack, Measuring cylinder, Distilled water, H_2SO_4, Anthrone reagent, Colorimeter, Water bath,Carbohydrate sample (sucrose solution)

Principle: Anthrone dissolved in 2% concentrated sulphuric acid is used for the quantitative analysis of different carbohydrates .Quantitative determination is only possible where the identity of sugar component is known because colour development varies with different sugars. Nevertheless,the anthrone method is widely used for the determination of starch and soluble sugar in plant material.

Generally, sugar and carbohydrate are extracted from dried and ground plant material. First soluble sugar are extracted with aqueous ethanol, later starch is extracted with an acid. Estimation of total carbohydrates moiety in a sample can be done by anthrone method which is a simple colorimetric method. The preparation of anthrone assay does not require the addition of distilled water and anthrone is directly dissolved in sulphuric acid at 2% concentration. This acid is a powerful dehydrating agent involved in dehydrating sugars leading to formation of furfural which condenses with Anthroneto give a bluish-green complex, which can be measured colorimetrically at 620nm by using a spectrometer (10-keto-9,10-dihydroanthrocene).This is a rapid and continuous method for the determination of hexoses, aldopentoses and hexuronic acid , either in the free form or when present polysaccharide.

Procedure:

1. 200mg of anthrone powder was dissolved to 100ml of chilled 95% sulphuric acid.
2. The standard sucrose solution of concentration 500mg/ml was prepared. From this 0.5 ml of sucrose solution is diluted in distilled water. As a result, the solution curve was plotted for calibrating curve.
3. To all the test tubes 2ml of Anthrone reagent was added.
4. The test tubes were incubated in boiling water bath for 10 minutes. The test tubes were allowed to cool down.
5. In all the test tubes green to dark green color variation was observed.
6. O.D of different aliquots was taken at 620 nm by using UV visible spectrometer.
7. The standard curve was plotted by taking O.D on Y-axis against sugar concentration on X-axis.
8. The amount of sugar present in the sample was obtained from standard plot.

Result:

Precautions:

- Make all the measurements very carefully. All the solutions and samples should be of exact amount as mentioned in the experiment.
- While heating the solution the boiling water bath, do it very carefully. Also bring down the temperature rapidly to room temperature. Take care of the time that you are giving to heat the sample as well as to the accurate temperature.

Practical No 5

Aim: Quantitative estimation of proteins (Bradford Method)

Materials Required: Bradford reagent (Coomassie Blue G250, 95% ethanol, 85% phosphoric acid). BSA standard, Colorimeter, Distilled water

Principle

Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution using the coomassie G250. This method was first described by Marion Bradford in the year 1976. This method was based on the principle of binding of coomassie G250 dye to proteins. The dye exists in three different forms:

(1) Cationic
(2) Anionic
(3) Neutral

Under acidic conditions the dye is in the protonated cationic form. When the dye binds to the proteins in an acidic condition, it is converted to a stable unprotonated blue form, resulting in a spectral shift.

The presence of certain basic amino acids such as Arginine, Histidine, Lycine in the protein are responsible for the development of color in the bradford's assay.

Mechanism

The dye first donates its free electrons to the protein bring treated causing disruptions of its native states and exposing its hydrophobic pockets. These hydrophobic sites on the protein chain then bind to the non-polar region of dye by Vanderwall's force.

The positive amino groups' position with the negative charge of the dye ionic interaction, this ionic interaction gets further strong and stabilizes the blue color. The amount of the color complex present in the solution, estimates the protein concentration which can be measured at 595 nm.

Procedure

1. Reconstitute 1 vial of standard protein (BSA) (10 mg/ml concentration) with 10 ml of distilled water to get the concentration of 10mg/ml.
2. Diluted solution of 120 µl of 10 mg/ml BSA solution with 1880 µl of bradford's reagent was mixed. Bradford reagent (100 mg of Coomassie Blue G250 + 50 mL of 95% ethanol + 100 mL of 85% phosphoric acid and made up to 1 L with distilled water + filtered through whatman no. 1, storage- amber bottle).
3. Pipette out diluted BSA, test sample and distilled water at given in the table and adjust the volume.
4. Now, incubate at room temperature for 10 mins but less than ½ an hour.

5. The absorbance was taken for the standard tubes and samples respectively at 595 nm. A standard curve was drawn by plotting absorbance on Y-axis and concentration (μg/ml) on X-axis.
6. Determination of the concentration of protein of unknown sample has been observed by using standard graph.

Result:

Practical no. 6

Aim Titration methods for estimation of aminoacids

Materials Required: 0.1 N solution NaOH, Formaldehyde, Phenolphthalein indicator, Amino acid solution, glasswares.

Principle: The acid group present in the glycine can be titrated with NaOH. It is not easyin this case because the amino group present will interfere at the end point. Topreventit excess of formaldehyde is used by which the amino group is blocked by th eformation of methylene glycine. Then it is titrated with NaOH using phenolphthaleinindicator.

Procedure:

Estimation of Amino acid

1. Make up the given amino acid solution with distilled water to 100ml in a volumetric flask. Shake the solution well for uniform concentration.
2. Pipette out 20ml of amino acid solution into a clean conical flask.
3. Add5 ml of HCHO and keep it for 2 minutes.
4. Then titrate it against the NaOH solution takenin the burette.
5. Phenolphthalein is used as the indicator.
6. The end point is the appearanceof permanent pale pink colour.
7. Repeat the titration for concordant values.

Blank titration

1. Pipette out 20ml of distilled water in a clean conical flask.
2. Add 5ml of HCHOand keep it aside for a few minutes.
3. Add 1-2 drops of phenolphthalein indicator.
4. Titrateit against the 0.1N NaOH solution taken in the burette.
5. The end point is the appearance of permanent pale pink colour.
6. Repeat the titration for concordant values.

Result:

The amount of amino acid present in the whole of the given solution = _____ g

Titration I (Blank titration)

S. No.	Content on conical flask (ml)	Burette Reading		Concordant Value (ml)
		Initial	Final	

1				
2				
3				

Titration II (Estimation of Amino acid)

S. No.	Content on conical flask (ml)	Burette Reading		Concordant Value (ml)
		Initial	Final	
1				
2				
3				

Calculation I

Volume of NaOH, (Test value) = ml
Volume of NaOH, (Blank value) = ml
Volume of NaOH used to titrate amino acid = Test value – Blank value= ml

CalculationII

Volume of amino acid, V_1= ml
Normality of amino acid, N_1= N
Volume of NaOH, V_2= ml
Normality of NaOH, N_2= N
Strength of amino acid = $V_{(NaOH)} x\ N_{(NaOH)} = V_{(amino\ acid)} x\ N_{(amino\ acid)}$
Amount of amino acid present in 100ml of the given solution=
Equivalent weight x Normality x 100/1000

Precautions

1. Use clean glass wares. Wash the glassware with distilled water before use.
2. Check that there is no air bubble in the burette or pipette.
3. Avoid inconsistent burette reading

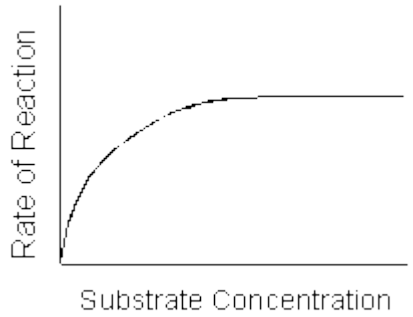
Practical No 7

Aim: Effect of pH, temperature and substrate concentration on enzyme action.

Effect of substrate concentration:

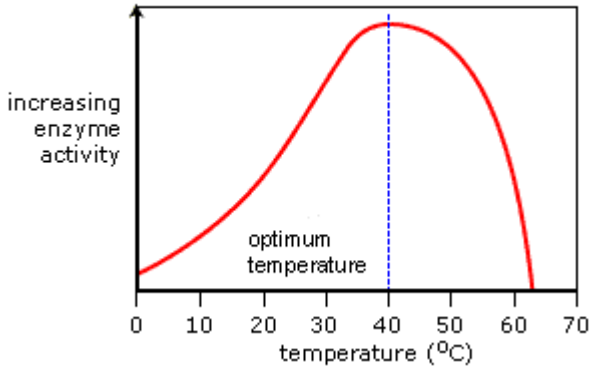
Reaction velocity of an enzymatic process increases with constant enzyme concentration and increase in substrate concentration. The velocity (V) is expressed in micromoles of substrate converted per minute. As the concentration of substrate increases, the velocity of the reaction increases. Continued increase in substrate concentration may lead to a reduction in rate of the reaction and leads to flattened curve. The maximum velocity obtained from an enzymatic reaction is called as V_{max}. V_{max} represents the maximum reaction rate possible in the presence of excess substrate. Though V_o approaches but never reaches V_{max}. At lower concentration of substrate, V_o increases almost linearly with an increase in [S]. The substrate concentration at which V_o is half maximum is K_m, the Michaels constant. The relationship between substrate concentration and reaction rate can be expressed quantitatively through

$V_o = V_{max}[S] / K_m + [S]$.



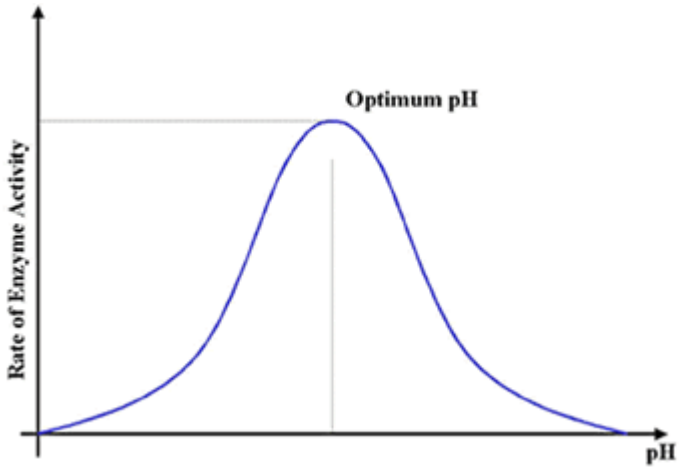
Effect of temperature:

Velocity of enzymatic reaction increases with temperature of the medium which they are most efficient and the same is termed as optimum temperature. As temperatures increases it leads to denaturation; a molecular arrangement which causes a loss of the active sites of the enzyme surfaces and results in a loss of efficiency.



Effect of pH:

Like temperature, all enzymes have a optimum pH, at which the enzymatic activity will be at maximum. Many enzymes are most efficient in the region of pH 6-7, which is the pH of the cell. Outside this range, enzyme activity drops off very rapidly. Reduction in efficiency caused by changes in the pH is due to changes in the degree of ionization of the substrate and enzyme. Highly acidic or alkaline conditions bring about a denaturation and subsequent loss of enzymatic activity. Some exceptions such as pepsin (with optimum pH 1-2), alkaline phosphatase (with optimum pH 9-10) and acid phosphatase (with optimum pH 4-5) are even noticed.



Practical No 8

Aim: TLC demonstration for separation of amino acids.

Chromatography:

Chromatography is by far the most useful general group of techniques available for the separation of closely related compounds in a mixture. Here the separation is effected by differences in the equilibrium distribution of the components between two immiscible phases, viz., the stationary and the mobile phases. These differences in the equilibrium distribution are a result of nature and degree of interaction of the components with these two phases. The stationary phase is a porous medium like silica or alumina, through which the sample mixture percolates under the influence of a moving solvent (the mobile phase). There are a number of interactions between the sample and the stationary phase and these have been well exploited to effect the separation of compounds.

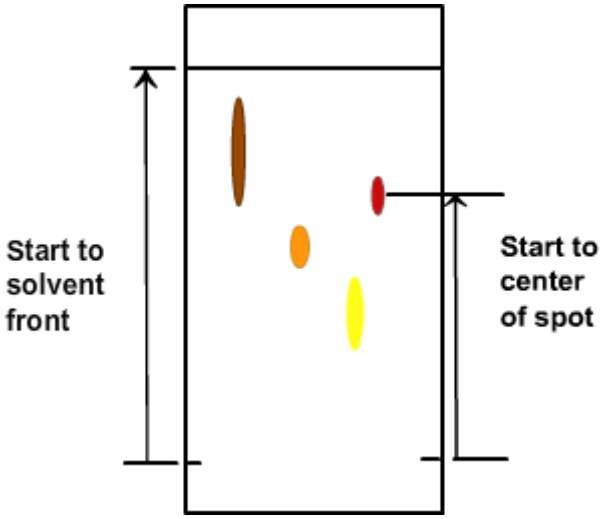
Thin layer chromatography [TLC]:

Thin layer chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data. Thin layer chromatography is a technique used to separate and identify compounds of interest. A TLC plate is made up of a thin layer of silica adhered to glass or aluminum for support. The silica gel acts as the stationary phase and the solvent mixture acts as the mobile phase. In the ideal solvent system the compounds of interest are soluble to different degrees. Separation results from the partition equilibrium of the components in the mixture.
In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is applied near one end of the TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is called as the development of TLC plates. The separation depends on several factors; (a) solubility: the more soluble a compound is in a solvent, the faster it will move up the plate. (b) attractions between the compound and the silica, the more the compound interacts with silica, the lesser it moves, (c) size of the compound, the larger the compound the slower it moves up the plate.

The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value.

The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value.

$$Rf = \frac{distance\ moved\ by\ the\ substance\ from\ origin}{distance\ moved\ by\ solvent\ from\ origin}$$



Chromatographic Separation of Amino acids:

The present experiment employs the technique of thin layer chromatography to separate the amino acids in a given mixture.

All 20 of the common amino acids [standard amino acids] are a-amino acids. They have a carboxyl group and an amino group bonded to the same carbon atom (the α- carbon). They differ from each other in their side chains, or R groups, which vary in structure, size, and electric charge. The interaction of the amino acids with the stationary phase like silica varies depending on their 'R' groups. The amino acid that interacts strongly with silica will be carried by the solvent to a small distance, whereas the one with less interaction will be moved further. By running controls [known compounds] alongside, it is possible to identify the components of the mixture.

Since amino acids are colourless compounds, ninhydrin is used for detecting them. To identify this, after development, the TLC plate is sprayed with ninhydrin reagent and dried in an oven, at 105°C for about 5 minutes. Ninhydrin reacts with α- amino acids that results in purple coloured spots [due to the formation of the complex - Rheuman's purple] [http://vlab.amrita.edu/?sub=3&brch=63&sim=156&cnt=1]. Rf values can be calculated and compared with the reference values to identify the amino acids. [The Rf value for each known compound should remain the same provided the development of plate is done with the same solvent, type of TLC plates, method of spotting and in exactly the same conditions].

Practical No 9

Aim: Sterilization techniques

Materials Required: Autoclave, spirit lamp, glass beadsterilizer, filters, ethanol, laminar air hood.

Principle and procedure

Wet heat (Autoclaving)

The method of choice for sterilisation in most labs is autoclaving; using pressurised steam to heat the material to be sterilised. Sterilisation of plant media and autoclavable plastic wares, can normally be achieved in 15 minutes by autoclaving at 121.6° C and15 psi pressure. This method is also useful for sterilization of glassware, cotton, forceps, scalpels etc

Dry heat (Flaming, Glass bead sterilization)

Inoculation loop can be sterilized by passing an ethanol dipped loop over the flame for a few seconds. Glass bead sterilization is used to keep the metal instruments such as scalpels, scissors, forceps sterilized in the laminar air flow. Temperature of the glass bead sterilizeris around 250°C- 265°C.

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 0.2um is normally used.

But 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. 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 sterilising 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.

Laminar air hood

Laminar airflow hoods are used in commercial and research tissue culture settings. A horizontal laminar flow unit is designed to remove particles from the air. Room air is pulled into the unit and pushed through a HEPA (High Efficiency Particulate Air) filter with a uniform velocity of 90 ft/min across the work surface. The air is filtered by a HEPA filter so nothing larger than 0.3 micrometer, which includes bacterial and fungal spores, can pass through. This renders the air sterile. The positive pressure of the air flow from the unit also discourages any fungal spores or bacteria from entering. Depending on the design of the hood, the filters are located at the back or in the top of the box. A UV lamp is attached on the top and initial sterilization for 15- 20 min is carried out using UV lamps followed by air flow through HEPA filter.

Practical No 10

Aim: Preparation of MS nutrient medium

Materials required: glassware, chemicals, pH meter, distilled water, autoclave.

Principle:

The basic nutritional requirements of cultured plant cells as well as plants are very similar. However, the nutritional composition varies according to the cells, tissues, organs and protoplasts and also with respect to particular plant species. The appropriate composition of the medium largely determines the success of the culture. A wide variety of salt mixtures have been reported in various media. A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and other organic supplements. When referring to a particular medium, the intention is to identify only the salt composition unless otherwise specified. Any number and concentration of amino acids, vitamins, growth regulators and organic supplements can be added in an infinite variety of compositions to a given salt composition in order to achieve the desired results.

Procedure:

1. Dehydrated M S Basal medium (Murashige and Skoog 1962) is used after dissolving in deionized water, to which agar (0.75%) is added.
2. pH of the media to be adjusted between 5.6-5.8 using 1N HCl and 1 N NaOH.
3. For liquid cultures agar is not added.
4. Approximately, 30 ml of the media is poured in 300 ml capacity glass bottles with polypropaline cap each and autoclaved at 121.6°C and 15 PSI pressure for 16 minutes.

Composition of M S Medium

Ingredients	**milligrams/litre**
Potassium nitrate	1900.00
Ammonium nitrate	1650.00
Calcium chloride.$2H_2O$	440.00
Magnesium sulphate	180.69
Potassium phosphate monobasic	170.00
Manganese sulphate.H_2O	16.90
Boric acid	6.20
Potassium iodide	0.83
Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulphate.$7H_2O$	8.60

Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Ferrous sulphate.$7H_2O$	27.80
EDTA disodium salt.$2H_2O$	37.30
myo - Inositol	100.00
Thiamine hydrochloride	0.10
Pyridoxine hydrochloride	0.50
Nicotinic acid (Free acid)	0.50
Glycine (Free base)	2.00
Sucrose	30000.00
TOTAL gm/litre	34.54

Precautions:

- Regular stirring is to be done while dissolving the agar.
- Media should be dissolved in lower volume of around 800 ml and then volume should be made up to 1000ml.

Practical No 11

Aim: Callus induction from various explants, micro-propagation, hardening and acclimatization.

Materials required: Prepared media with phytohormones, laminar air flow, scalpel and forceps, ethanol.

Principle:

For successful initiation of callus culture and micro propagation, three important criteria should be accomplished:

1. Aseptic preparation of plant material,
2. Selection of suitable nutrient medium supplemented with appropriate ratio of auxins and cytokinins and
3. Incubation of culture under controlled physical condition.

Procedure:

1. Wipe down and turn on the laminar air flow 15 minute before doing work in the hood. Flames –sterilize the instruments.
2. The young, actively growing nodal segments (2-3 cm), apical buds or leaves are taken as explants and washed thoroughly under running tap water.
3. Later surface sterilised in 0.1% bavistin (systemic fungicide) solution(w/v) for 15min followed by rinsing 3-4 times with distilled water.
4. Finally, the explants are surface sterilized under laminar air flow cabinet, with 0.1% $HgCl_2$ (w/v) for 2-5 min and thoroughly rinsed with sterilized distilled water.
5. This sterilized nodal explants are inoculated in plantgrowth medium (ex- Murashige and Skoog, 1962) solidified with agar (0.75%) and supplemented with different combinations of phytohormones (generally cytokinins for shoot multiplication and a combination of cytokinins and auxins for callus induction). pH of the media is adjusted between 5.6-5.8 and autoclaved at 121.6°C and 15 PSI pressure for 16 minutes.
6. Cultures are maintained in the growth chamber with 16h/8h (light/dark) photoperiod at 25 ± 2 °C with light intensity of 25 μmol m^{-2} s^{-1} by cool-white fluorescent lamps.
7. Rooting of in-vitro multiplied shoot lets are induced in plant growth medium supplemented with different concentrations of auxins.
8. The plantlets thus generated were hardened primarily in portray containing vermicompost: coco peat (1:1) inside a poly tunnel for 8- 10 days followed by

secondary hardening for 20 days in poly bags containing FYM: garden soil: sand (1:1:1) inside green house.

Result:

Precautions:

- Wear a lab coat and keep long hair tied back.
- Wipe down working surfaces and hands with ethanol.
- Use sterile equipment.
- Stay as organized as possible—label everything and set up all of your materials before getting started.
- Inspect all equipment and media for visible contamination before use.
- If you must completely remove a lid from a tube, plate or bottle, set it down within the hood with the open surface facing up. Otherwise, keep tubes, plates or bottles closed as much as possible.
- Do not pass your hands/arms over any open bottle, plate or tube.

Practical No 12

Aim: Demonstration on isolation of DNA

Materials Required: Extraction buffer, Microfuge tubes, Mortar and Pestle, Liquid Nitrogen, Absolute Ethanol (ice cold), 70 % Ethanol (ice cold), 7.5 M Ammonium Acetate 65° C water bath Chloroform : Iso Amyl Alcohol (24:1) Water (sterile), TE Buffer (10 mM Tris, pH 8, 1 mM EDTA).

Composition of extraction buffer (For 5 ml)

component	Stock concentration	Final Concentration	Volume taken from stock
Tris	1M	0.1 M	500 µl
EDTA	0.5M	20 mM	200 µl
NaCl	5M	1.4 M	1.40 ml

+ CTAB 0.1g (2%) + PVP 0.1g (2%) (Heatat 65° Ctill dissolved)+ β-mercaptoethanol 10µl (0.2%)

Principle:

Isolation of DNA using CTAB exploits that polysaccharides and DNA have different solubilities in CTAB depending on the concentration of sodium chloride. At higher salt concentrations, polysaccharides are insoluble, while at lower concentrations DNA is insoluble. Consequently, by adjusting salt concentration in lysates containing CTAB, polysaccharides and DNA can be differentially precipitated.Polyphenols are compounds that contain more than one phenolic ring (e.g., tannin), a structure that binds very efficiently to DNA. They are naturally occurring in plants, but are also generated when plants have tissue damage (browning). Upon the homogenization of plant tissues, polyphenols are synthesized by liberated polyphenol oxidase. The addition of polyvinyl pyrrolidone prevents the interaction of DNA and phenolic rings by binding up the polyphenols.

Procedure:

1. Weigh 200 mg leaves.
2. Crush with liquid nitrogen.
3. Add 1 ml extraction buffer.
4. Mix well and incubate at 65oC for 30 min.
5. Cool down to room temperature.
6. Add equal volume of chloroform : isoamylalcohol (24:1).
7. Centrifuge at 10000 rpm for 10 min. at 4˚C.
8. Take out upper aqueous phase in fresh tube.
9. Add 0.6 volume of chilled isopropanol.
10. Incubate at -20o C for 1 hour.
11. Centrifuge at 12000 rpm for 15 min. at 4˚C.
12. Discard supernatant and add 1ml 70% ethanol.

13. Centrifuge at 10000 rpm for 10 min. at 4˚C.
14. Discard supernatant and air dry pellet at room temperature.
15. Add 50 µl of TE (10:1) and store at 4˚C for overnight.

Result:

Precautions:

- Material finely ground in liquid nitrogen should be immediately transferred into the extraction buffer.
- In chloroform : isoamyl alcohol extraction, the aqueous phase should be carefully removed and organic phase re-extracted to ensure full recovery of DNA. If no separation is observed between the two phases, may be due to high concentration of DNA and /or cell debris in aqueous phase, dilution with more digestion buffer and re-extraction is the solution.
- Care should be taken to do the operations as gently as possible. Vortexing, pipetting using fine tips etc. should be avoided to prevent the shearing of DNA.
- DNA should not be over dried as resuspension in TE become difficult.
- All the glassware, plastic ware, pestles and mortars etc. should be decontaminated properly. Care should be taken to prevent cross-contamination.
- Blank extraction controls are carried out along with normal extractions to check for any contamination.

Practical No 13

Aim: Demonstration of gel electrophoresis techniques.

Materials required: TAE buffer, Agarose gel (1% in TAE buffer), loading dye, casting tray, gel electrophoresis unit, trans illuminator, ethedium bromide solution, distilled water.

Principle:

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones.In order to visualize nucleic acid molecules in agarose gels, ethidium bromide or SYBR Green are commonly used dyes. Illumination of the agarose gels with 300-nm UV light (under trans illuminator) is subsequently used for visualizing the stained nucleic acids.

Procedure:

1. Prepare a 1 % solution of agarose by melting 1 g of agarose in 100 mL of 1X TAE buffer in a microwave for approximately 2 min.
2. Allow to cool for a couple of minutes then add 2.5 µl of ethidium bromide, stir to mix.
3. Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface.
4. Load the following into separate wells - 10 µL 1kb ladder, 5 µL sample + 1 µL 6x Loading dye.
5. Run the gel for 30 min at 100 V.
6. Expose the gel to UV light (under transilluminator) and photograph.
7. Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation.

Result:

Precautions:

- Temperature of the agarose solution should be around 55-65° C at the time of casting. Avoid pouring it very hot or cold.
- Ethidium bromide is a mutagen and should be handled as a hazardous chemical (Always wear gloves while handling).

Practical No 14

Aim: DNA fingerprinting

Materials Required:PCR unit, Gel electrophoresis Unit, Sourthern Blot Unit. DNA extraction chemicals and kit, restriction enzyme.

Principle:

Nearly 0.1% of DNA is unique to the individual that makes all the difference between two individuals. These differences are a consequence of mutations during evolution. As single change in nucleotide may make a few more cleavage site of a given nucleotide or might abolish some existing cleavage site. Thus, if DNA of any individual is digested with a restriction enzyme, fragments pattern (sizes) will be produced and will be difference in cleavage site position. This is the basics of DNA fingerprinting. DNA Fingerprinting is used by scientists to distinguish between individuals of the same species using only samples of their DNA. The process of DNA fingerprinting was invented by Sir Alec Jeffrey at the University of Leicester in 1985.

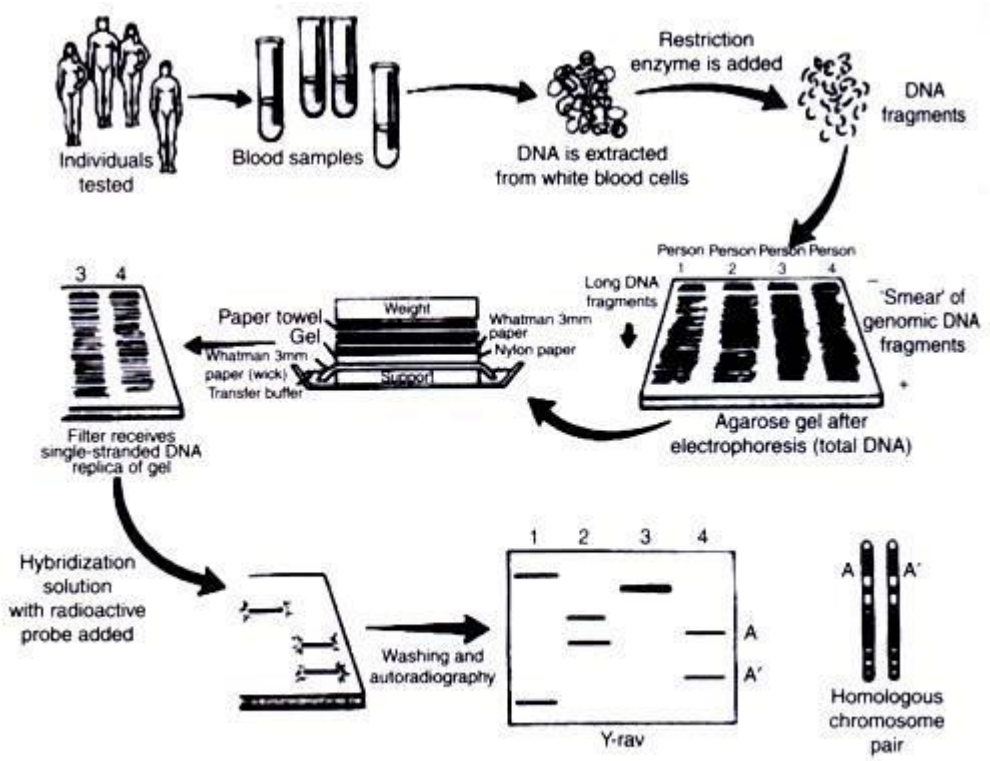
Procedure:

Unless they are identical twins, individuals have unique DNA. DNA fingerprinting – The name used for the unambiguous identifying technique that takes advantage of differences in DNA sequence. The process of DNA fingerprinting begins by isolating DNA from – blood, semen, vaginal fluids, hair roots, skin, skeletal remains, or elsewhere.

After we isolate the DNA and amplify it with PCR. Treat the DNA with restriction enzymes – cut DNA at specific sequences – Everyone's DNA is different, so everyone's DNA will cut at different sites .This results in different sized fragments. The different sized fragments are called restriction fragment length polymorphisms, or RFLPs. We can observe the different sized fragments in an experiment that separates DNA based on fragment size called Gel Electrophoresis.

Fragments of DNA from restriction enzyme cleavage are separated from each other when they migrate through a support called an agarose gel. The size-based separation of molecules of DNA separate based on size when an electric current is applied to an agarose gel.

The separated DNA fragments are then drawn out of the gel using a nylon membrane. The nylon membrane is treated with chemicals that break the hydrogen bonds in DNA and separate the strands. The single stranded DNA is cross linked to the nylon membrane – By heat or UV light. Incubate the nylon membrane with a radioactive probe of single stranded DNA complementary to the VNTRs.The radioactive probe shows up on photographic film – Because as it decays it gives off light – The light leaves a dark spot on the film. Different individuals have different patterns of bands – these make up the fingerprint.



DNA fingerprinting