Plant cell and tissue culture

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Points to be covered

- Introduction to PTC,
- Enzyme technology,
- isolation of enzymes,
- immobilization of enzyme,
- cell and plant tissue culture,
- Immobilized plant cells,
- raising mutants in plant cell cultures,
- protoplasts and cell fusion,
- plant cell cultivation and production of secondary metabolites,
- germplasm storage

PLANT TISSUE CULTURE

- Definition Tissue culture is in vitro cultivation of plant cell or tissue under aseptic and controlled environmental conditions, in liquid or on semisolid well defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant.
- Tissue culture relies on three fundamental abilities of plant there are:
- Totipotency
- Dedifferentiation
- competency

- Totipotency -The potential or inherent capacity of a plant cell to develop into an entire plant if suitably stimulated. It implies that all the information necessary for growth and reproduction of the organism is contained in the cell

- Dedifferentiation-Capacity of mature cells to return to meristematic condition and development of a new growing point, follow by redifferentiation which is the ability to reorganize into new organ

- Competency-The endogenous potential of given cells or tissue to develop in a particular way

micropropagation

- The mass multiplication of agricultural, horticultural, medicinal and other desirable plants by tissue culture techniques is known as micropropagation/clonal propagation.

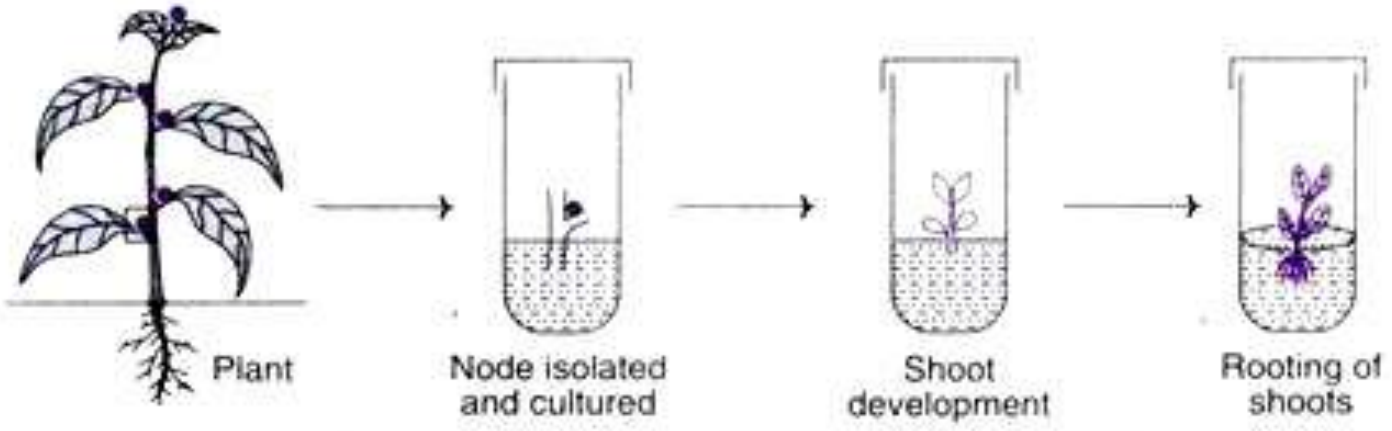


Fig. 47.3 : A diagrammatic representation of micropropagation by single node technique.

Steps involved in the in vitro micropropagation

Cleaning of glassware

Preparation of nutrient medium Selection and sterilization of explant.

Inoculation of aseptic explant in to nutrient medium.

Proliferation of shoots on a multiplication medium.

Transfer of shoots for sub-culturing.

Rooting and hardening of plantlets Field trials.

Procedure for cleaning of glassware

Soak glassware in 10% soap water (teepol) for 1 hour.

Transfer glassware to conc. HCl and keep for 2 hours.

Rinse glassware in tap water. Wash the glassware at least twice with distilled water.

Keep glassware for drying in oven at 100 oC for 1 hour.

Autoclave/ keep glassware in oven at 140-160 oC for 2 hours.

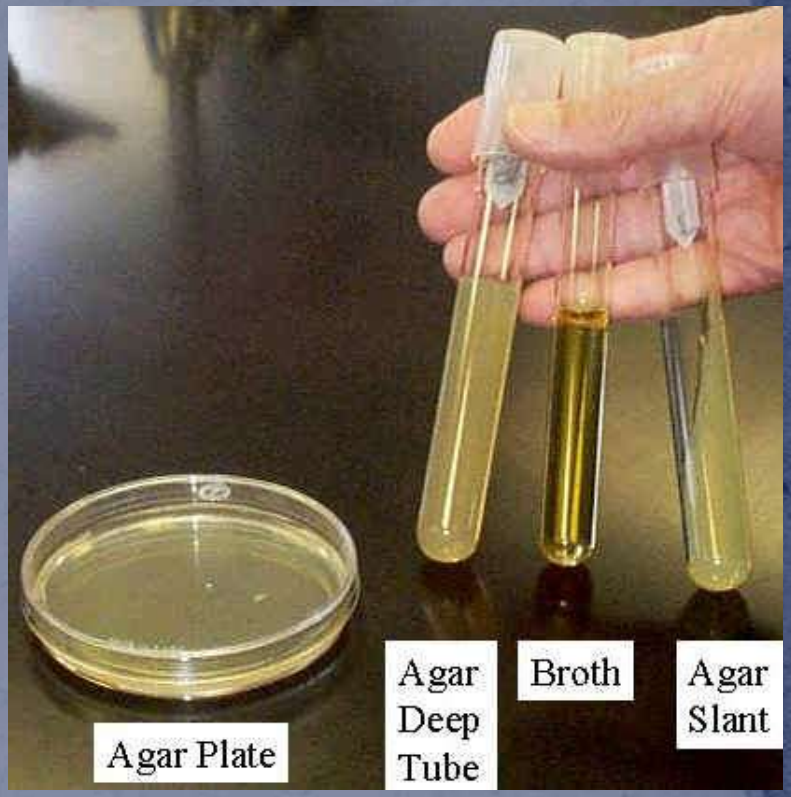
Nutrient medium

Table 43.1 Composition of commonly used plant tissue culture media

Components	Amount (mg l^{-1})				
	White's	Murashige and Skoog (MS)	Gamborg (B5)	Chu(N6)	Nitsch's
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	—	170	—	400	68
$NaH_2PO_4.H_2O$	19	—	150	—	—
KNO_3	80	1900	2500	2830	950
NH_4NO_3	—	1650	—	—	720
$CaCl_2.2H_2O$	—	440	150	166	—
$(NH_4)_2.SO_4$	—	—	134	463	—
Micronutrients					
H_3BO_3	1.5	6.2	3	1.6	—
$MnSO_4.4H_2O$	5	22.3	—	4.4	25
$MnSO_4.H_2O$	—	—	10	3.3	—
$ZnSO_4.7H_2O$	3	8.6	2	1.5	10
$Na_2MoO_4.2H_2O$	—	0.25	0.25	—	0.25

Nutrient medium

- Antibiotics : Stertomycin,kanamycin
- Other organic supplements : Protein, coconut milk,yest,malt extract, orange juice, and tomato juice
- Growth regulators : Auxins,cytokinins
- Water : Demineralized or distilled water
- Solidifying agents : Agar, gelatin.
- pH adjusters : 5 - 6 it is considered to be optimum.



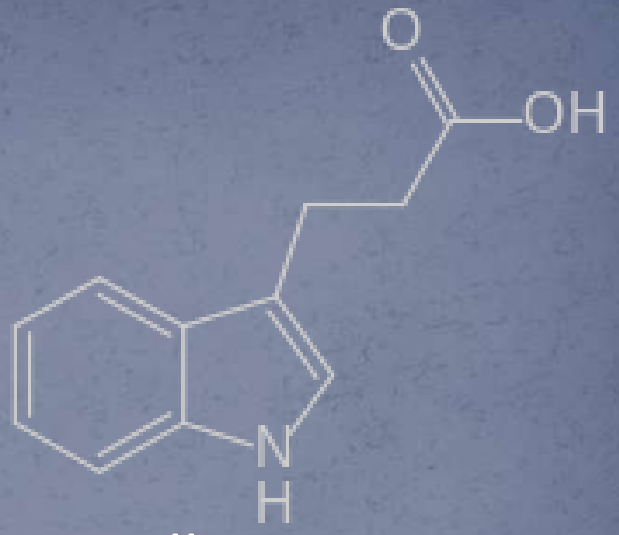
- MS medium, **B5 medium, White's medium**

- White’s medium - is one of the earliest plant tissue culture media
- MS medium - formulated by Murashige and Skoog (MS) is most widely used for many types of culture systems
- B5 medium - developed by Gamborg for cell suspension and callus cultures and at present it’s modified form used for protoplast culture
- N6 medium - formulated by Chu and used for cereal anther culture.
- Nitsch’s medium developed by Nitsch and Nitsch and used for anther culture

Plant growth regulators

- A plant hormone can be defined as a small organic molecule that elicits a physiological response at very low conc.
- PGS plays an important role in the phenotype.
- Act as messenger between environment and the genome.

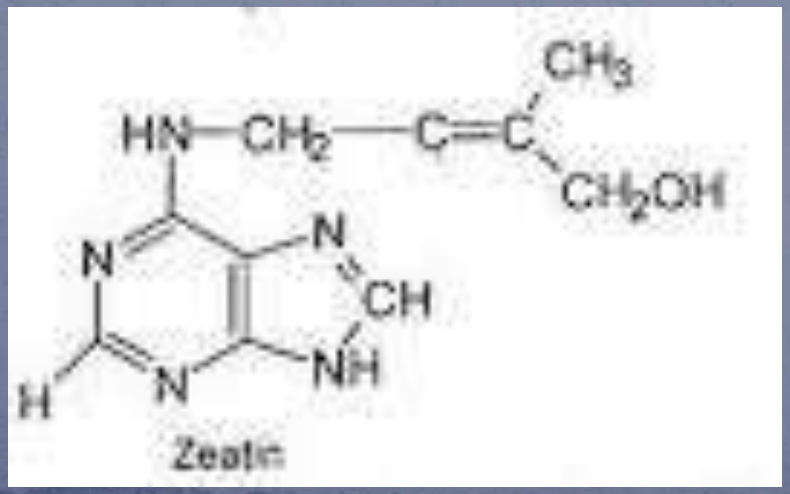
Auxins



- Essential for cell division, cell elongation, cell differentiation, organogenesis and embryogenesis, callus formation, flowering, fruit setting and ripening
- Natural form auxins are IAA, IBA,
- Synthetic form of auxins are NAA, 2, 4-D pCPA
- Used in conc. 0.1-10mg/L in PTC medium
- NAA and 2,4-D are thermostable

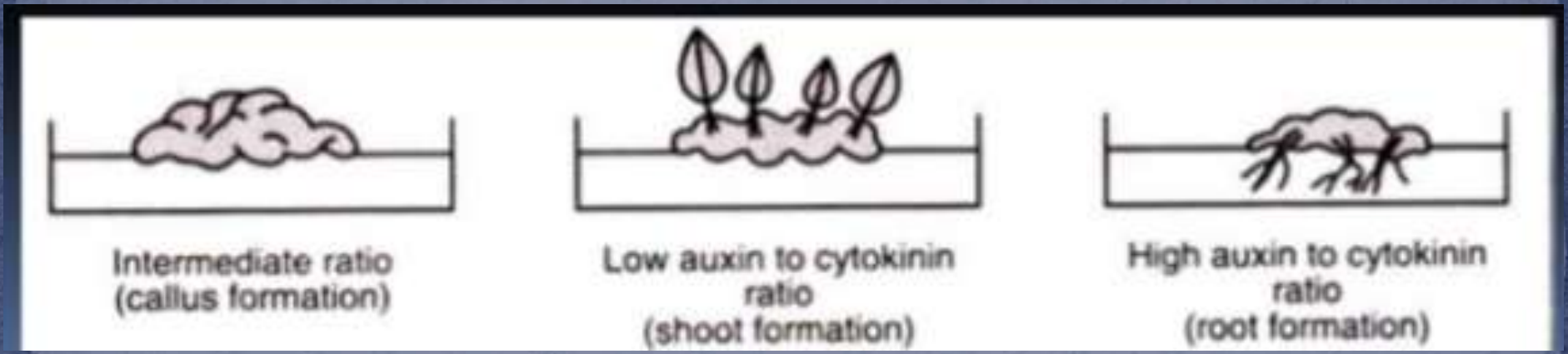
Cytokinins

- Cytokinins promote cell division, root proliferation and influence the cell cycle.
- Embryogenesis and inhibit root formation.
- Synthetic form is 2-ip which is most active cytokinins.
- First synthetic analogue of kinetin isBAP(6-benzyl aminopurin).
- Other- N-isopentenyl aminopurin (2iP) and zeatin (zea)
- conc. 40-80 mg/L

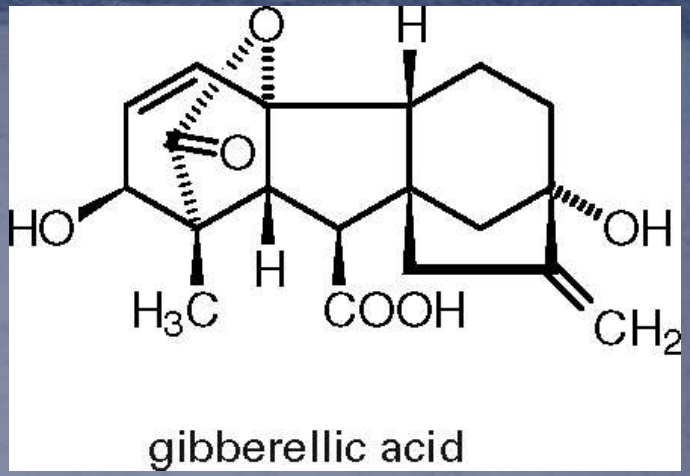


Zeatin

Kinetin


Intermediate ratio
(callus formation)
Low auxin to cytokinin
ratio
(shoot formation)
High auxin to cytokinin
ratio
(root formation)

Gibberellins

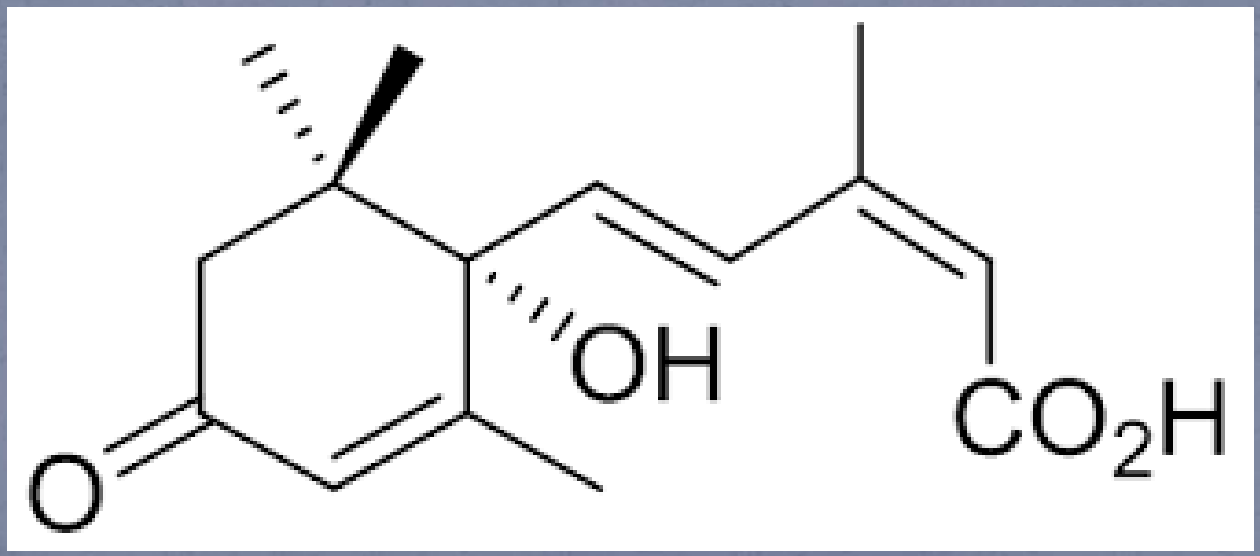


gibberellic acid

- It promotes stem elongation, flowering along with other effects such as internode bulb corm formation and embryo maturation but can inhibit callus growth and root induction.
- GA3 is most common gibberellins.
- Autoclaving reduces activity of GA3 by 90%

Abscisic acid (ABA)

- It inhibits shoot growth and germination of embryo.
- It is thermostable but light sensitive
- It causes acceleration of absission of leaves, dormancy of buds, retardation of germination and bud development.



Ethylene

- C_2H_4 is gaseous hormone synthesized in fungi, bacteria and in cultured cells
- It plays important role in somatic embryogenesis, morphogenesis and plant regeneration from in vitro cultures

Ph of tissue culturemedia

- Ph is adjusted between 5 & 5.8 before gelling and sterilization with the help of dilute NaOH, KOH or HCL.
- Ph below 5 will not gel properly.
- Ph above 6 may be too hard.

Sterilization of media

•The medium is transferred in to flasks and stoppered with cotton plugs, and autoclaved at 120°C to 121°C for 15 to 40 min.

•Pressure used is 15 psi or 1.06 kg/cm2

•The thermo labile substances can alternatively sterilized by filtration sterilization using membrane filters having pour size of not more than 0.2 microns. Eg: Millipore and Seitz filters.

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•The flasks are removed for cooling as early as possible and stored at 10°C prior to use.

BASIC REQUIREMENT FOR A TISSUE CULTURE LABORATORY

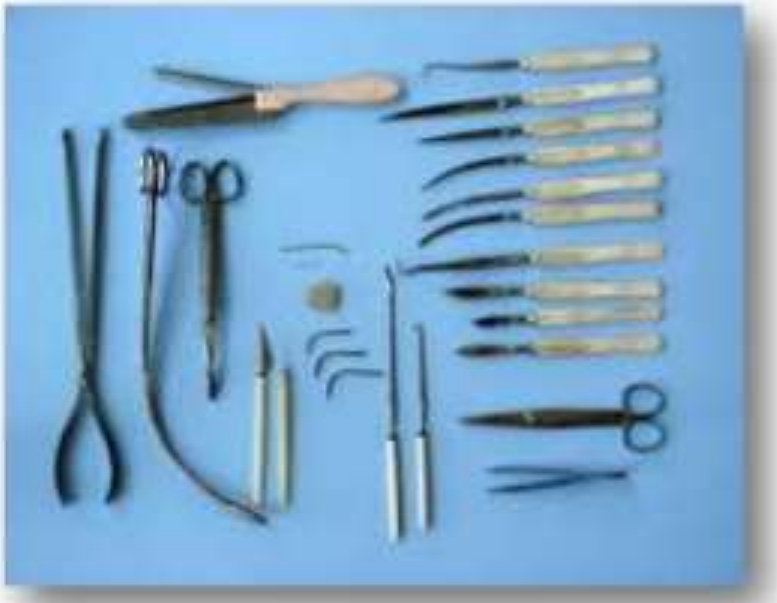
- For the successful achievement, the following general basic facilities are required:
- Equipment & apparatus
- Washing and storage facilities
- Media preparation room
- Sterilization room
- Aseptic chamber for culture
- Culture rooms or incubators fully equipped with temperature, light and humidity control devices
- Observation or recording area well equipped with computer for data processing

Tissue culture laboratory

EQUIPMENT & APPARATUS

- VESSELS & GLASS WARE :
  - All the glassware should be of Pyrex.
  - Large test tubes,flasks,graduated pipettes etc.. are used.

- EQUIPMENT :
  - Scissors,scapels,foreceps Knife are used for explants preparation.
  - A spirit burner for flame sterilization.
  - Hot air oven.
  - A PH meter.
  - A BOD incubator.
  - Laminar air flow chamber.
  - A balance to weigh nutrients.
  - Data collection and recording room.
  - Laminar air flow chamber

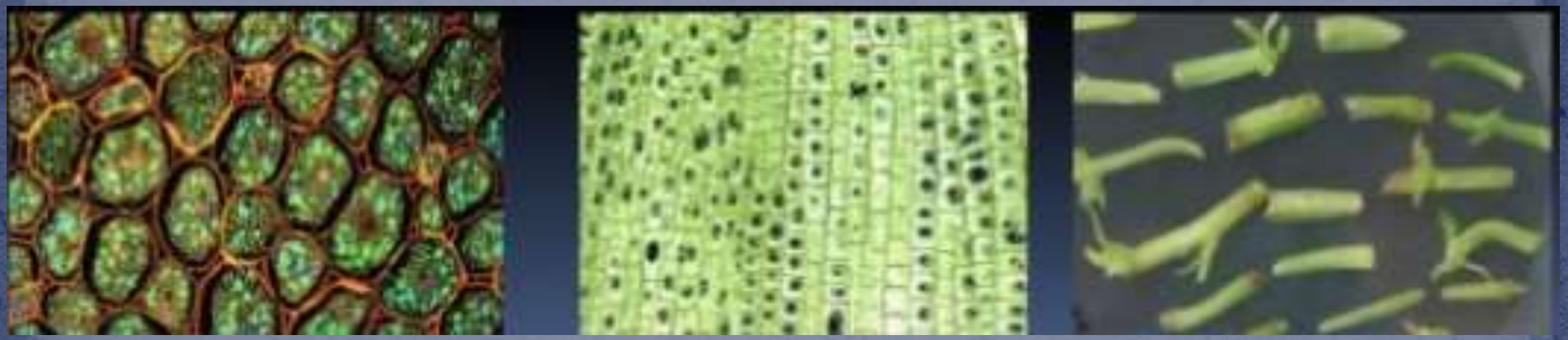


Laminar air flow chamber

DATA COLLECTION & RECORDING ROOM

Explant

- EXPLANTS-Cell,Tissue, or Organs-preferably more rapidly growing tissue or tissue at an early stage of development.



Preparation of explants

- Irrespective of the plant part used as an explant, the explants are cleaned with a liquid detergent in running water and surface sterilized with disinfectant solutions like
  - sodium hypochlorite 0.5 to 5%
  - Calcium hypochlorite 9 to 10%
  - Hydrogen peroxide 10 to 12 %
  - Bromine water 2%
  - Mercuric chloride 0.1 to 1%
  - Benzalkonium chloride 0.01 to 0.1%
  - Ethanol 75 to 95% etc.
- Aseptic explants can also be obtained from aseptic seedling developed from inoculated seeds. For this, seeds are surface sterilized with 0.1% mercuric chloride, rinsed in sterile distilled water and inoculated on basal medium to obtain aseptic seedling in vitro.

Preparation of explant

Sterilization of the plant material (Surface) sterilization

The plant material should be surface sterilized to remove the surface borne micro-organisms.

Water 10% v/v solution of liquid detergent (Teepol) for 10-15 min.

70% ethyl alcohol for 1 min. in front of laminar air flow.

Treatment with 0.1% HgCl2 (W/V) or 5-10% sodium hypochlorite.

inoculation/explantation

- The transfer of explants onto the sterilized nutrient culture medium taken in culture vessels is called inoculation/explantation.
- The inoculation of the explant is carried out in aseptic environment i.e in the laminar flow chamber.

Incubation:

- The culture vessels with inoculated explants are incubated under controlled conditions of temperature, illumination and humidity.
- The cultures are incubated for 3 to 4 weeks during which, the cells of the explant absorb the nutrients, grow and undergo repeated divisions to produce a proliferating undifferentiated mass of cells known as callus or produces shoots or roots directly.
- The explant or callus cultured on different combinations of auxins and cytokinins will produce shoots or roots, called as organogenesis.
- High proportion of auxin and low proportion of cytokinin induce root development from callus called and is known as rhizogenesis.
- Low proportion of auxin and high proportion of cytokinin in the medium induce shoot development from the callus and is called as caulogenesis

- Alternatively, embryo like structures develop from callus and this phenomenon is known as embryogenesis (Somatic embryogenesis).
- The embryos like structures which develop from callus are called embryoids. Sometimes the explant also produces the embryoids directly without callus production.
- Since these embryoids develop from somatic tissue they are referred to as somatic embryos.
- The somatic embryos are transferred to other culture media for development into complete plants.

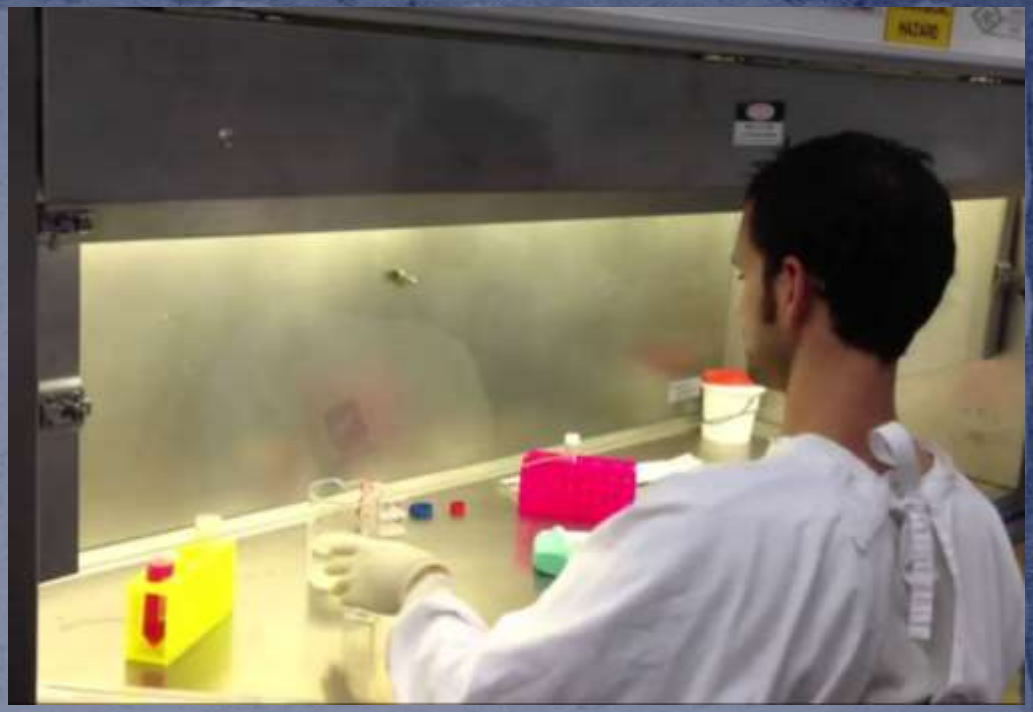
- Cultures are incubated in a culture room where light, temperature and humidity are controlled.
- For some tissues dark is essential while for some both dark and light conditions are required.
- Humidity has also some effect.
- The cultures are incubated on culture rack at 25-28 ^{o}C constant temperature. Culture tubes are placed at 35-40^{o}C inclined position.
- Culture to give a light intensity of 4-10 X 103 lux for 16 hrs.

Incubation requirements

- Humidity for incubation up to 70-75%
- Less humidity (50-55%) causes dehydration of medium and higher humidity (85-90%) may promote microbial growth on cotton plug which may contaminate the medium and culture
- Temperature range- 25-27^{0}C
- Lower temperature preferred during dark period of differentiation of the callus
- Higher amount of gelling agent in the medium decreases the uptake of the nutrients

Subculturing

•Transfer of cell or tissue from old culture medium to fresh culture medium within definite time period.



- It provides sufficient space and nutrients to the growing plantlet.
- Multiplication of the callus.

Rooting

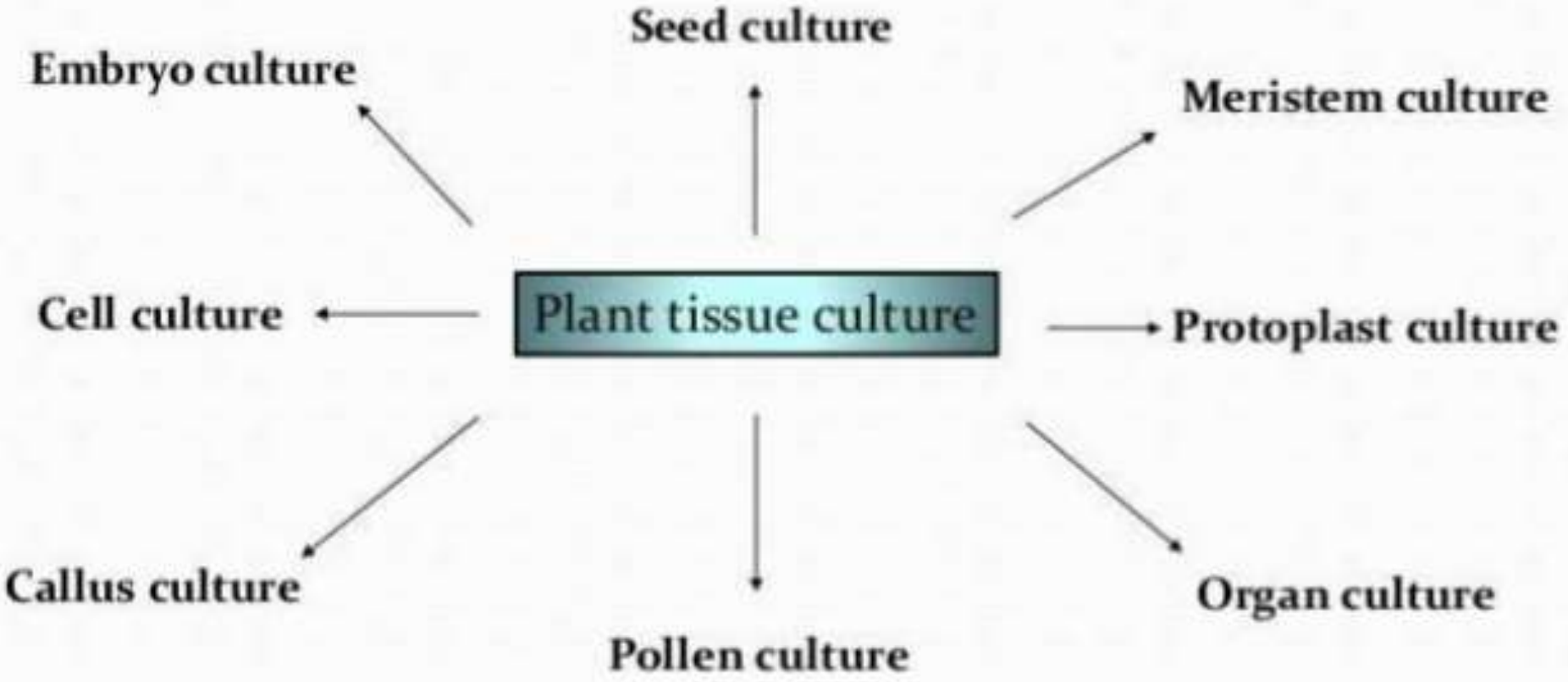
- It is the induction and development of adventitious roots on the proliferated shoots.
- Root formation is induced in a medium with high auxin and low cytokinins concentrations.
- Shoot tip or single node explant is used.
- Culture medium is maintained in a green house/mist chamber.
- Activated charcoal is frequently added to absorb root-inhibiting agents.

Hardening/ Acclimatization

Hardening

- Healthy/elite plantlets are exposed to the natural conditions in a step wise manner.
- It is a gradual acclimatization of in vitro grown plants to in vivo condition.
- The plantlets are transferred to the pots/polythene bag and immediately irrigated with inorganic/nutrient solution.
- Plants are kept in the hardening room where controlled conditions of light, humidity and temperature are maintained.
- Plants are maintained under high humidity for 10-20 days and subsequently transferred in the field so as to grow under natural conditions. The success rate of micropropagation depends on the survival of the plantlets when transferred from culture to the soil (field).

TYPES OF PTC


Seed culture
Embryo culture
Meristem culture
Cell culture
Plant tissue culture
Protoplast culture
Callus culture
Organ culture
Pollen culture

Application of tissue culture

- Rapid propagation.
- Minimum growing space is required.
- Multiplication of medicinal plants.
- Pathogen free plants -meristem culture.
- It is useful in the plants like papaya, coconut, etc.
- Large number of plants can be stored in the small space.
- Problems with seed and vegetative propagation overcome.
- Artificial seeds - do not under go seed dormancy.
- Uniformity of characters.
- Seedless fruit propagated easily.
- In vitro cloning enables genetic manipulation

- Hybrids with desired traits can be obtained by this method.
- Transgenic plants produced by tissue culture technique.
- Rare and endangered plants.
- Early flowering can be induced by tissue culture technique e.g. bamboo.
- There is potential danger of spreading of plants diseases through a diseased material in a large number of plants.
- It is not feasible for some tress, especially for some gymnosperms.
- In some cases multiple shooting takes place but rooting is difficult.
- Contamination in the culture room is a serious problem.
- In some cases shoots show decline in the rate of growth and plant die called vertrification.

Enzyme technology

- For thousands of years natural enzymes made by microorganisms have been used to make products such as cheese, bread, wine and beer.
- Enzymes are now used in a wide range of industrial processes.
- **The study of industrial enzymes and their uses is called Enzyme technology.**
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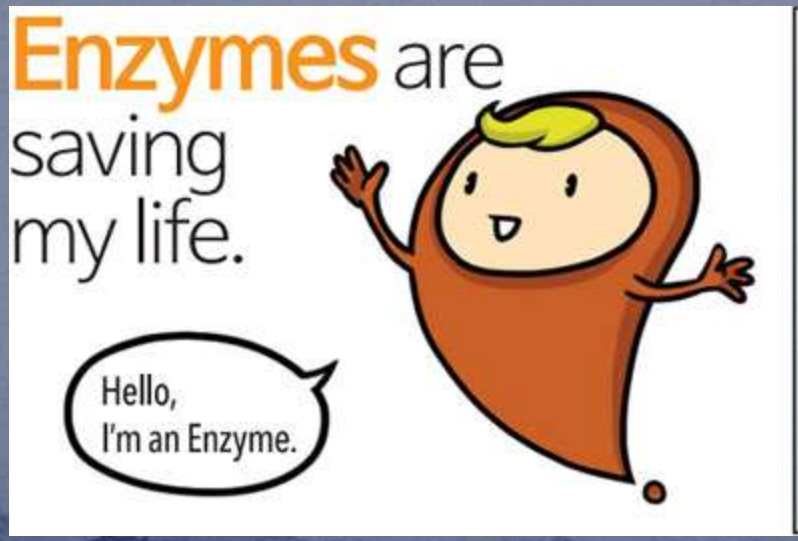
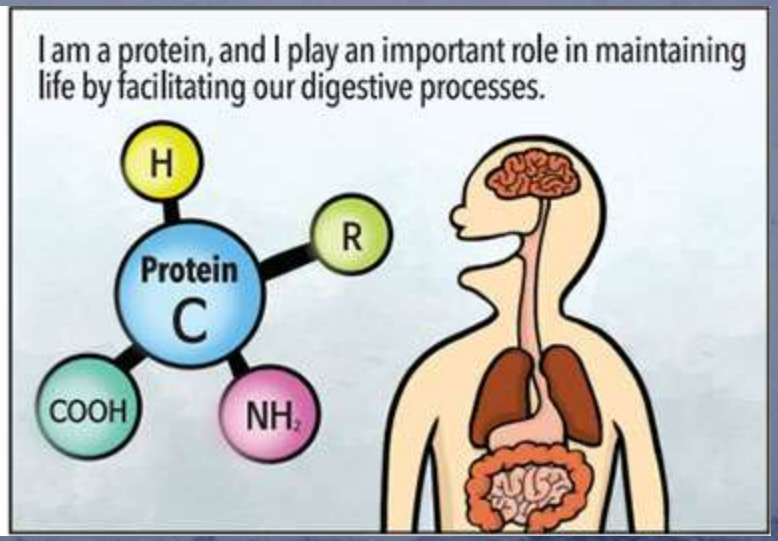
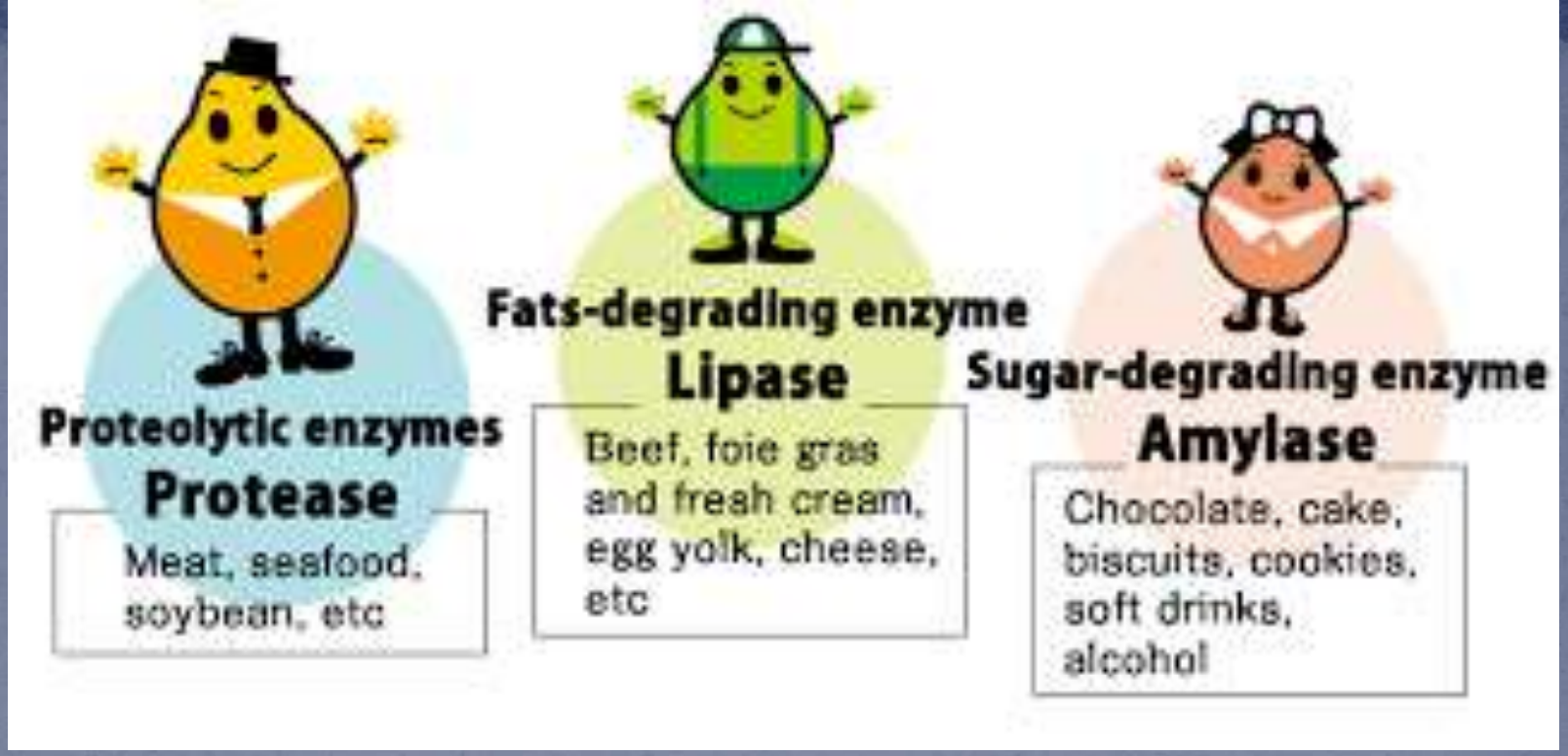
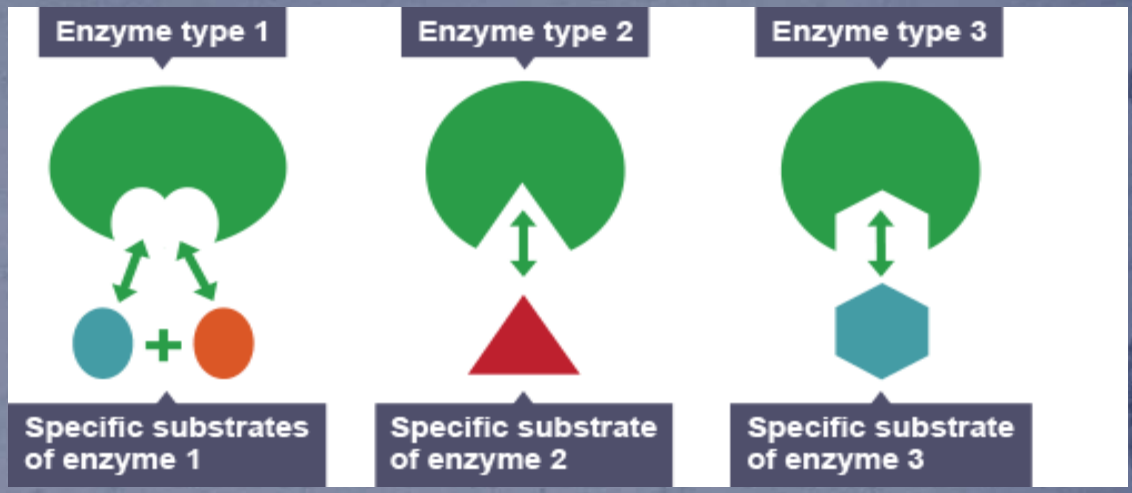






- Enzymes are the catalyst of biological system.
- They are protein that speed up biochemical reaction without being consumed or changed by the reaction by the process of catalysis.
- They accelerate the course of chemical reaction by several orders through a substantial decrease in the activation energy.

Properties of Enzymes



- Reduce activation energy.
- Increase rate of reaction.
- No change in equilibrium of reaction.
- Enzyme itself remain unchanged.
- Substances upon which enzyme work are called as substrates.
- Each enzyme will only fit and act upon a specific set of substrates.

Sources of enzymes

- Biologically active Enzymes may be extracted from any living organisms.
- Microbes are preferred to plants and animals as sources of enzymes because: They are generally cheaper to produce.
- Their enzyme contents are more predictable and controllable.

ENZYME	SOURCE	INDUSTRIAL USE
Animal Enzymes		
Catalase	Liver	Food
Chymotrypsin	Pancreas	Leather
Trypsin	Pancreas	Leather
lipase	Pancreas	Food
Rennet	Abomasum	Cheese
PLANT Enzymes		
Alpha and Beta Amylase	Malted Barley	Brewing
Bromelain	Pineapple latex	Brewing
Ficin	Fig latex	Food
Lipoxygenase	Soyabeans	Food
Papain	Papaya latex	Meat
Actinidin	Kiwi Fruit	Food

ENZYME	SOURCE	INDUSTRIAL USE
Bacterial Enzyme		
Alpha and Beta Amylase	Bacillus sp	Starch
Asparaginase	E coli	to treat **leukaemia**
Glucose isomerase	Bacillus	Fructose syrup
Catalase	Aspergillus	Food
Lactase	Aspergillus	Dairy
Rennet	Mucor	Cheese
Yeast Enzymes		
Invertase,Raffinase	Sacchromyces	Confectionary and food
Lipase	Candida	Food
Lactase	Kluveromyces	Dairy

Application of Enzymes:

- **In detergent Industry**: Proteases,Lipases, Amylases, cellulases.
- **IN Textile Industry**: Amylases, catalases, lactases.
- **In Genetic Engineering** Nucleases, Restriction Enzymes, DNA ligase, Reverse transcriptase etc
- **In Biofuels** : Cellulases convert cellulose fibres from feedstocks like corn into sugars. These sugars are subsequently fermented into ethanol by microbes.
- **Leather industry** : uses proteolytic and lipolytic enzymes in leather processing which involves three steps like soaking phase, dehairing / dewooling phase and bating phase.
- **In Pharma/chemical industry** In R and D, nucleic acid manipulations carried out by Restriction enzyme and DNA polymerase. Diagnostic procedures uses enzymes such as luciferase and glucose oxidase.
- **In study of various biochemical pathway:** Chymotrypsin: Aids digestion.

The advantages and disadvantages of using enzymes are directly related to their properties

Advantages	Disadvantages
They are specific in their action and are therefore less likely to produce unwanted byproducts.	They are highly sensitive to changes in physical and chemical conditions surrounding them
They are biodegradable and therefore cause less environmental pollution.	Easily denatured by even a small increase in temp and are highly susceptible to poisons and changes in pH. Therefore the conditions in which they work must be tightly conrolled.
They work in mild conditions,i.e. low temps, neutral pH and normal atmospheric pressure, and therefore are energy saving.	The enzyme substrate mixture must be uncontaminated with other substances that might affect the reaction.

Requirements for the production of microbes in fermenters :

- Oxygen is needed for aerobic respiration of (some) micro-organisms – others are strict anaerobes and oxygen must be excluded
- A source of Carbohydrate is needed as an energy source for respiration to release energy needed for growth.
- A source of Nitrogen is needed for protein synthesis – Ammonia (NH_3) and urea ($(NH_2)_2CO$) are both widely used as (cheap) sources of useable nitrogen

Isolating the Enzyme:

- **Pure enzymes** are needed for commercial use; therefore microbes must be grown in aseptic conditions , free from contaminants - such as unwanted chemicals - and other microbes.

Extracellular enzymes are present in the culture outside the microbial cells, since they have been secreted. They are often soluble in water, so they can readily be extracted from the culture medium and purified.

To obtain an intracellular enzyme, the microbe cells are harvested (by filtration or centrifugation) from the culture and are then broken

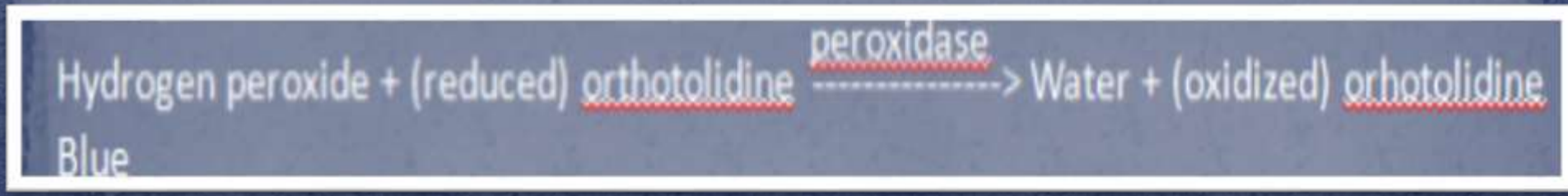
The mixture is next centrifuged to remove large cell fragments and the enzymes are precipitated from solution by a salt or alcohol.

The required enzyme must then be purified by techniques such as electrophoresis or column chromatography. This last process is complicated and expensive, so these enzymes are only used when no other alternative is available.

Intracellular Enzyme	Extracellular Enzyme
More difficult to isolate.	Easier to isolate.
Cells have to be broken apart to release them.	No need to break cells-secreted in large amounts into medium surrounding cells.
Have to be separated out from cell debris and a mixture of many enzymes and other chemicals	Often secreted on their own or with a few other enzymes
Often stable only in environment inside intact cell.	More stable
Purification/downstreaming processing is difficult/expensive	Purification/downstreaming processing is easier/cheaper

Medical uses of enzymes (Diagnostic):

- (Diagnostic) Reagent strips have been designed to perform rapid and semi-quantitative analysis for glucose .
- A Clinistix contains molecules of two enzymes fixed onto the end of a plastic strip.
- When this is dipped into a sample, the first, **glucose oxidase**, converts any glucose molecules, by reaction with atmospheric oxygen, into gluconic acid and hydrogen peroxide.
- The second enzyme, **peroxidase**, then enables the hydrogen peroxide to react with an indicator to give a purple colour.



- A colour chart on the strip will match the shade of purple to the glucose concentration
- The idea of fixing an enzyme to a plastic support is the basic principle of biosensors - mobile, cheap and accurate sensors which can monitor a number of biochemicals in blood, urine and also in food and soil.

Industrial use of Enzymes (using the whole microbe):

- Industrial use of Enzymes (using the whole microbe) Historically, three examples of the industrial use of microbes (and their enzymes) are:
  - Brewing.
  - Vinegar production.
  - Yoghurt production.

Industrial use of Enzymes (not using the whole microbe):

- Industrial use of Enzymes (not using the whole microbe) Softening of Leather by the help of Enzyme Trypsin and proteases obtained by dog excreta and extract from the pancreases of slaughtered animals.
- Washing powders: Pancreatic extract such as trypsin and alkaline protease are suitable enzymatic washing agent and spot remover.
- Cheese making: By the help of enzyme Rennin (formerly extracted from dead calves' stomachs, but now produced from Bacteria).

Producing a pure product more cheaply-reusing the Enzyme:

- Producing a pure product more cheaply-reusing the Enzyme By attaching the enzyme molecules to an inert surface (such as plastic beads) and then bringing the surface into contact with a solution of the substrate.
- This method has the advantage of enabling the enzyme molecules to be used over and over again, with the result that a lot of product can be made from a relatively small amount of Enzyme.
- An Ex of continuous production using an immobilized Enzyme is Fructose syrup production from glucose using Glucose isomerse.

Improving the Enzyme stability:

- Improving the stability of an enzyme refers to its ability to retain its tertiary structure so that it continues to be effective under a wide range of conditions.
- Most enzymes are relatively unstable and work only within narrow ranges of temp and pH.
- They quickly become denatured when subjected to unnatural environments.
- Organisms evolve to produce enzymes that are adapted to their environmental conditions
- Thermophilic bacteria produce thermostable enzymes that do not denature at high temperatures ie 65-75 degree C.
- The gene for thermostable enzyme can be isolated from a natural bacteria and transferred to a microbe ‘host’ that can be used in the industrial process.

What Is Enzyme Immobilization ?

Enzyme immobilization may be defined as a process of confining the enzyme molecules to a solid support over which a substrate is passed and converted to products.

What Is An Immobilized Enzyme?

An immobilized enzyme is one whose movement in space has been restricted either completely or to a small limited region.

Why Immobilize Enzymes?

- Protection from degradation and deactivation.
- Re-use of enzymes for many reaction cycles, lowering the total production cost of enzyme mediated reactions.
- Ability to stop the reaction rapidly by removing the enzyme from the reaction solution.
- Enhanced stability.
- Easy separation of the enzyme from the product.
- Product is not contaminated with the enzyme.

What are the applications of enzyme immobilization?

- Industrial production:E g. Antibiotics, beverages, amino acids etc.
- Biomedical applications: treatment, diagnosis and drug delivery
- Food industry: production of jams, jellies and syrups
- Research: HRP in blotting experiments, proteases for cell lysis
- Production of biodiesel: from vegetable oils
- Waste water management: treatment of sewage & industrial effluents
- Textile industry: scouring, bio-polishing and desizing of fabrics
- Detergent industry: immobilization of lipase for effective dirt removal

An Ideal Carrier Matrices For Enzyme Immobilization

- Inert.
- Physically strong and stable.
- Cost effective.
- Regenerable.
- Reduction in product inhibition.

CLASSIFICATION OF CARRIERS

Inorganic Carriers

•High pressure stability.

• May undergo abrasion

Examples:

1. Commercialy SiO_2 available materials-
   - Porous glass.
   - Silica.
2. Mineral materials - (clays)

Celite ,Centonite

Organic Natural Carriers

•Favourable compatibility with proteins.

Examples:

1. cellulose derivatives-
   - DEAE-cellulose
   - CM-cellulose.
2. Dextran.
3. Polysacharides

Agarose, Starch

Pectine ,Chitosan

Organic Synthetic Carriers

•High chemical and mechanical stability.

Examples:

1. Polystyrene
2. Polyvinylacetate
3. Acrylic polymers

Natural polymers

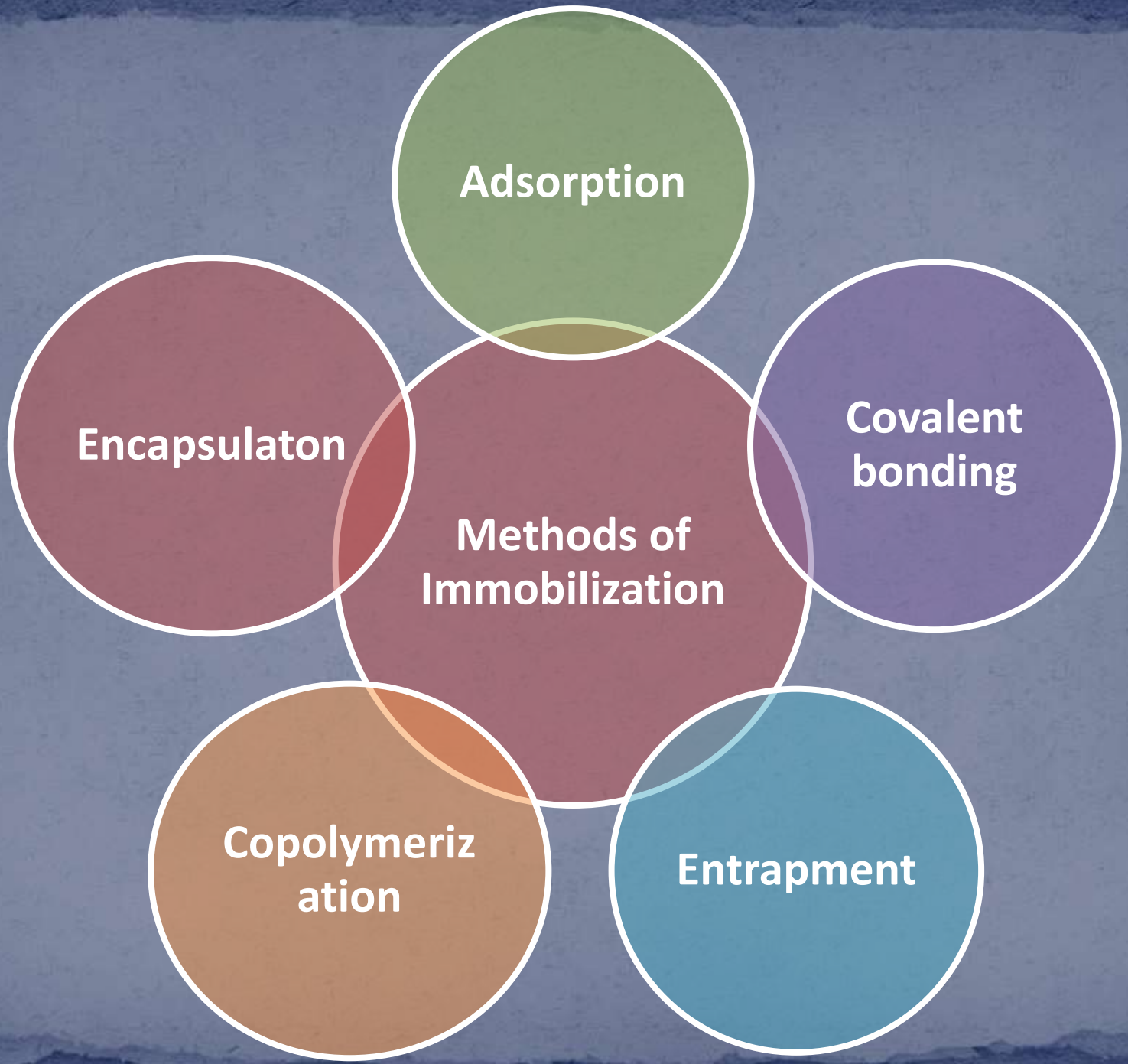
- Alginate: derived from algal cell wall (calcium or magnesium alginate)
- Chitosan and chitin: enzyme bins to the — OH groups
- Collagen: protenaceous support
- Carrageenan: a sulfated polysaccharide obtained from algae
- Gelatin: partially hydrolyzed collagen, good water holding capacity
- Cellulose: cheapest support available
- Starch: good water holding capacity
- Pectin: good water holding capacity

Synthetic polymers

- They are ion exchange resins / polymers
- They are insoluble supports with porous surface
- The porous surface trap and hold the enzymes / cells
- Example:
  - DEAE cellulose
  - Polyvinyl chloride (PVC)
  - UV activated Polyethylene glycol (PEG)

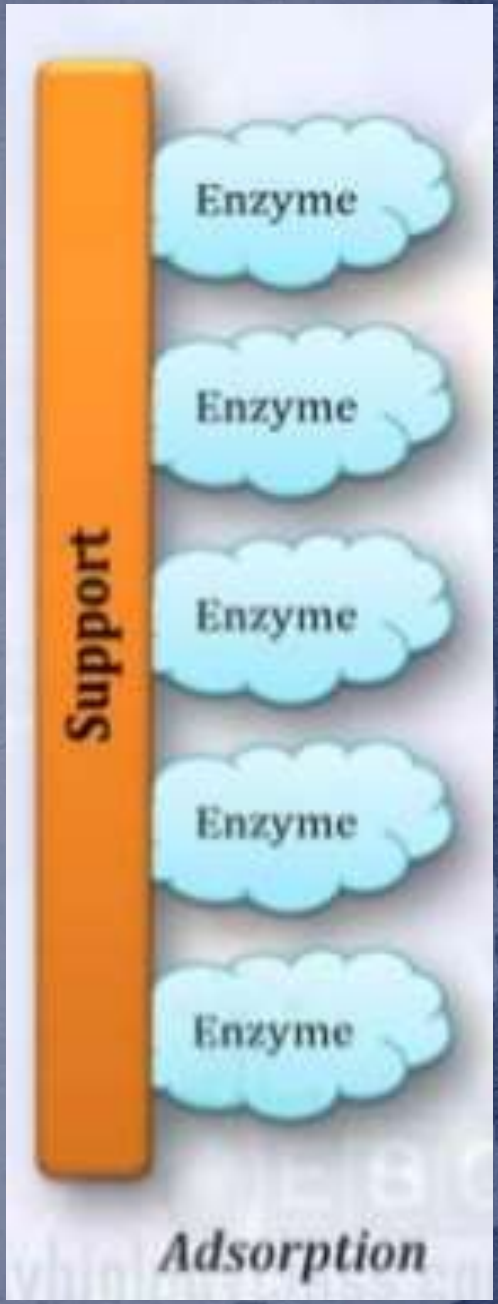
Inorganic materials

- Zeolites
- Ceramics
- Diatomaceous earth (Trade name celite]
- Silica
- Glass
- Activated carbon
- Charcoal


Adsorption
Encapsulaton
Covalent bonding
Methods of Immobilization
Copolymeriz ation
Entrapment

Adsorption

- Oldest method of enzyme immobilization
- Simplest method of enzyme immobilization
- Nelson & Griffin used charcoal to adsorb invertase
- Enzymes are adsorbed to external surface of support
- Support/ carrier may be :
  - 1. Mineral support (aluminum oxide, clay)
  - 2. Organic support (starch)
  - 3. Modified sepharose and ion exchange resins

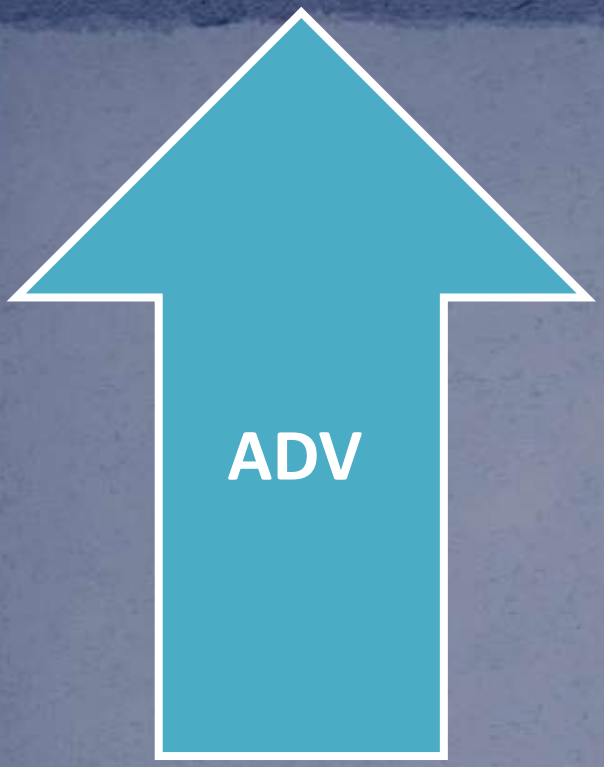


Adsorption continued…

- Weak bonds stabilize enzymes to the support/ carrier
- Bonds involved are low energy bonds such as:
  - Ionic interaction
  - Hydrogen bonds
  - Van der Waal forces
- Carrier particle size must be small (for appreciable surface bonding)
- Particle size used: 500 A to 1 mm diameter
- No pore diffusion limitations (since enzyme are immobilized externally)

Methods of adsorption:

- 1. Static process: Immobilization to carrier by allowing the solution containing enzyme to Contact the carrier (without stirring)
- 2. Dynamic batch process: Carrier is placed in the enzyme solution and mixed by stirring or agitation
- 3. Reactor loading process: Carrier is placed in the reactor, then enzyme solution is transferred to reactor
- 4. Electrode position process: Carrier is placed proximal to an electrode in an enzyme bath and the current is put on, the enzyme migrates to the carrier and deposited on the surface



-Easy to carry out

-No reagents are required

-Minimum activation steps involved

-Comparatively cheap method

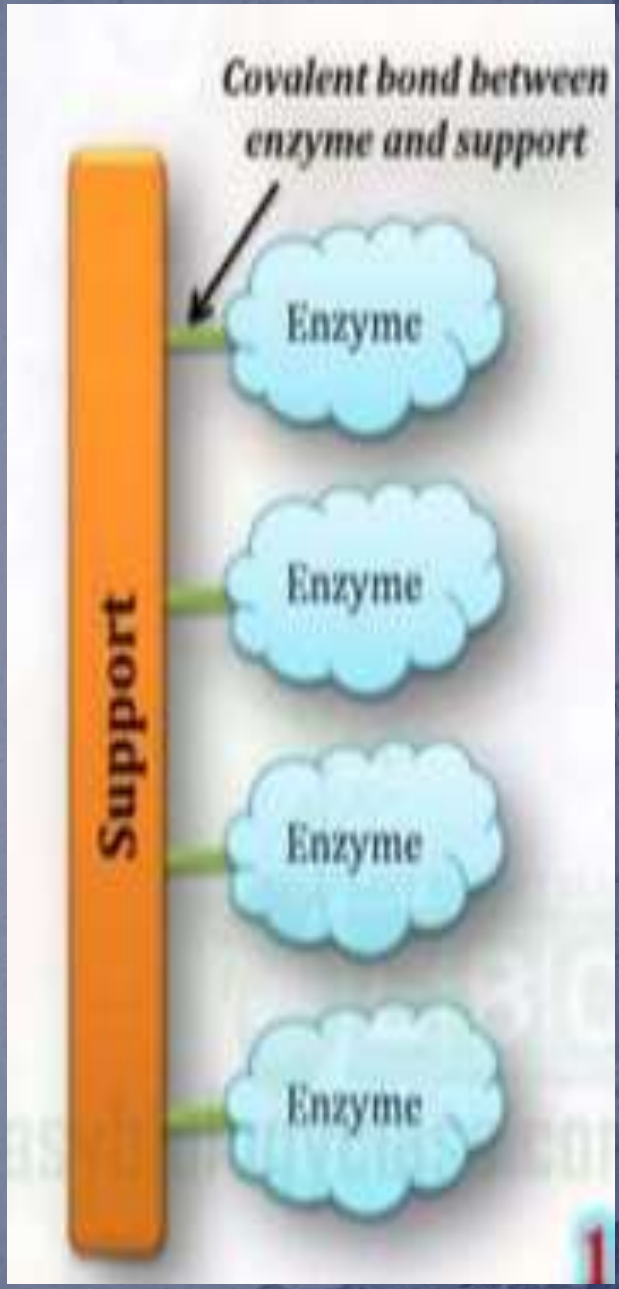
-Less disruptive to protein than chemical methods

-Desorption of enzymes from the carrier

- Efficiency is less

Covalent bonding

- Involves the formation of covalent bonds between enzyme and support Widely used method of enzyme immobilization
- Chemical groups in enzymes that forms covalent bonds with support are:
  - Amino groups, Imino groups
  - Hydroxyl groups
  - Carboxylic group
  - Thiol groups and Methylthiol groups
  - Guanidyl groups and Imidazole groups
  - Phenol rings



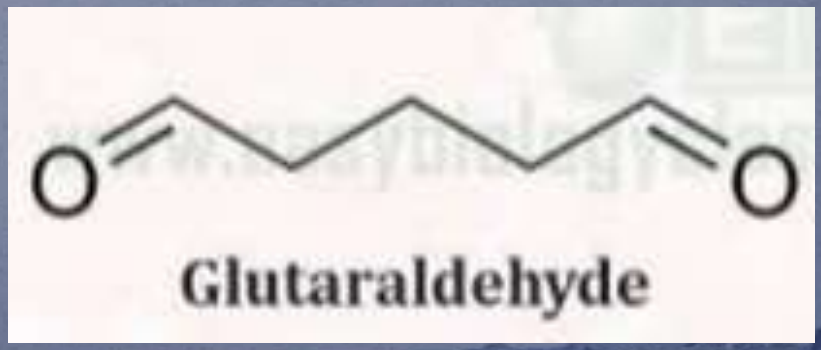
Covalent bonding continue..

- Important functional groups of enzyme that provide chemical groups to form covalent bonds with support/ carrier are:
- Alpha carboxyl group at ‘C’ terminal
- Alpha amino group at ‘N’ terminal
- Epsilon amino groups of Lysine and Arginine
- Beta and gamma carboxyl groups of Aspartate and Glutamate
- Phenol ring of Tyrosine
- Thiol group of Cysteine
- Hydroxyl groups of Serine and Threonine
- Imidazole group of Histidine
- Indole ring of Tryptophan

- Carriers / supports used for covalent bonding:
  Carbohydrates:
  - Eg. Cellulose,
  - DEAE cellulose,
  - Agarose
  - Synthetic agents: Eg. Polyacrylamide
  - Protein carriers
  - Amino group bearing carriers: Eg. amino benzyl cellulose
  - Inorganic carriers: Porous glass, silica
  - Cyanogen bromide (CNBr)-agarose and CNBr Sepharose ‘
  - Hydroxyl and Amino groups form covalent bonds more easily

Methods of covalent bonding

- 1. Diazoation: Bonding between amino group of support and thyrosil or histidyl group of enzyme
- 2. Peptide bond: between amino / carboxyl groups of support and enzyme
- 3. Poly functional reagents: Use of a bi-functional or multifunctional reagent (glutaraldehyde) which forms bonding between the amino group of the support and amino group of the enzyme



Advantages

- Strong linkage of enzyme to the support
- No leakage or desorption problem
- Comparatively simple method
- A variety of support with different functional groups available
- Wide applicability

Disadvantages

- Chemical modification of enzyme leading to functional conformation loss
- Enzyme inactivation by changes in the conformation
- This can be overcome through immobilization in the presence of enzyme substrate or a competitive inhibitor

Entrapment

- In entrapment, the enzymes or cells are not directly attached to the support surface, but simply trapped inside the polymer matrix.
- Enzymes are held or entrapped within the suitable gels or fibres.

- Matrix used will be water soluble matrix: polyacrylamide gels ,Cellulose triacetate, Agar, Gelatin, Carrageenan, Alginate

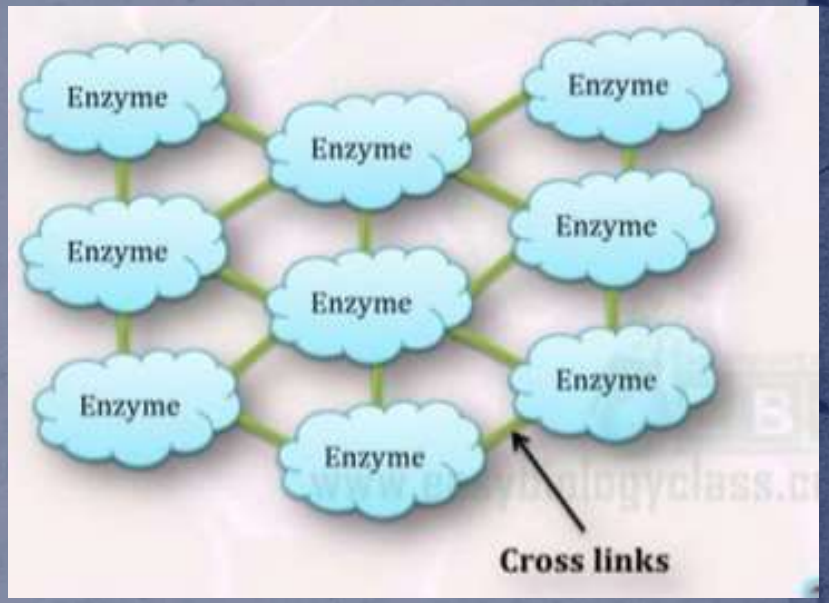
- Form and nature of matrix varies ‘ Pore size of matrix is adjusted to prevent loss of enzyme
- Possibility of leakage of low molecular weight enzymes Agar and carrageenan have large pore sizes
- Pore size can be adjusted with the concentration of the polymer
- Entrapment of enzyme can be used for sensing application Not much success in industrial process
- Easy to practice at small scale

- Methods of en trapment:
- 1. Inclusion in the gels: enzymes trapped in gels
- 2. Inclusion in fibers: enzymes supported on fiber format
- 3. Inclusion in microcapsules: Enzymes entrapped in microcapsules formed by monomer mixtures such as polyamine, calcium alginate

- Advantages:
  - Fast Cheap (low cost matrix available]
  - Mild conditions are required Less chance of conformational changes in enzyme
- Disadvantages:
  - Leakage of enzyme
  - Pore diffusion limitation Chance of microbial contamination

Cross linking (Copolymerization)

- covalent bonding between various groups of enzymes via polyfunctional reagents
- If No matrix or support are involved
- Commonly used polyfunctional reagents: Glutaraldehyde, Diazonium salt
  - Technique is cheap and simple but not often used with pure proteins
  - It is widely used in commercial preparations
- Demerit- reagent can denature enzyme

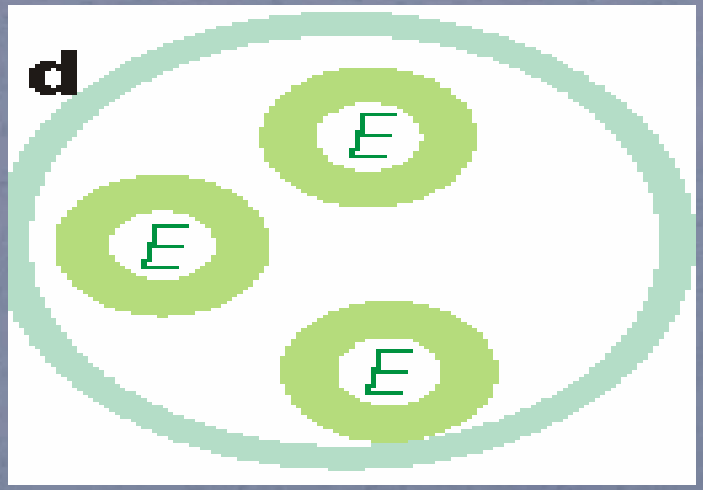


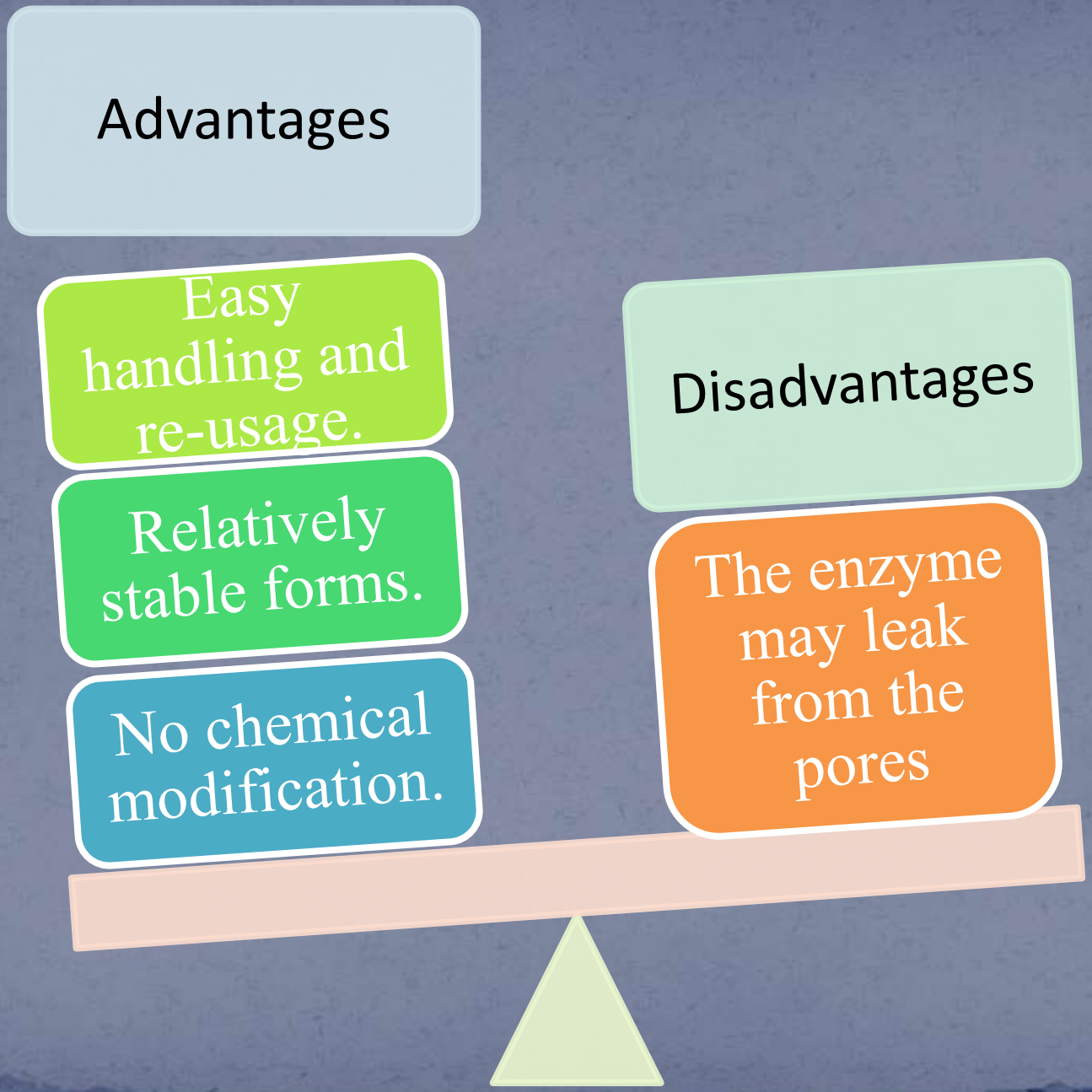
- **Advantages of cross linking:-**
- 1. Very little desorption(enzyme strongly bound)
- 2. Higher stability (i.e. ph, ionic & substrate concentration)

- **Disadvantages of cross linking:-**
- 1. Cross linking may cause significant changes in the active site.
- 2. Not cost effective.

MicrocapsuleType Entrapmet/ Encapsulation/Membrane Confinement

- It involves enclosing the enzymes within semi -permeable polymer membranes e.g. semi permeable collodion or nylon membranes in the shape of spheres.




Advantages
Easy handling and re-usage.
Relatively stable forms.
No chemical modification.
Disadvantages
The enzyme may leak from the pores

Limitations Of Enzyme Immobilization

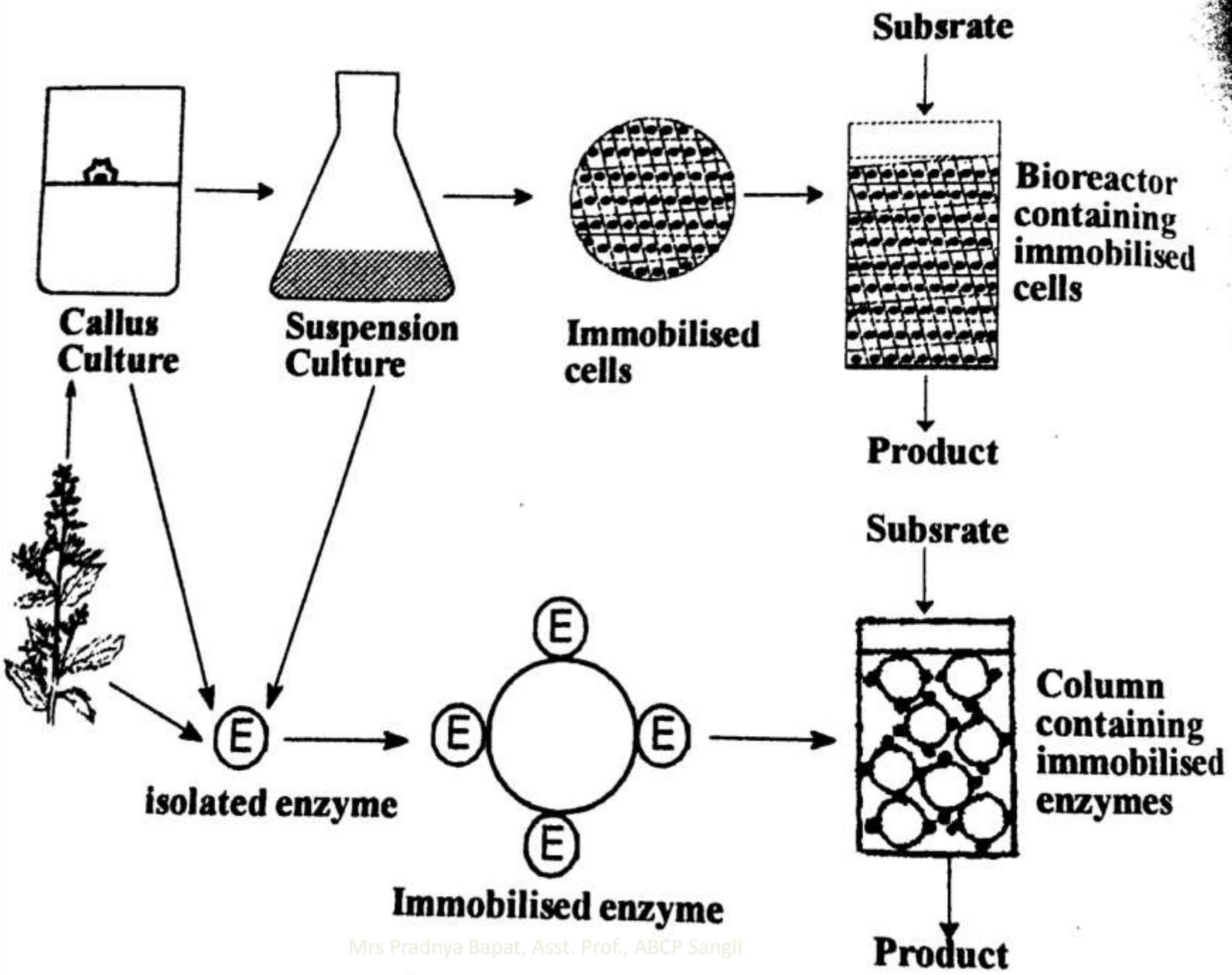
- Cost of carriers and immobilization.
- Changes in properties (selectivity).
- Mass transfer limitations.
- Problems with cofactor and regeneration.
- Problems with multienzymes systems.
- Activity loss during immobilization.

Cell immobilization

- Closely resembles the invivo environment of plant→ secondary metabolite production in plants.
- Production is affected by correlation with morphological organization, cytodifferentiation ,decline in culture growth rate and enhanced accumulation.
- Less growth→ more production of sec. metabolite

Immobilization of cells

- Well developed method for the utilization of enzymes from microbes
- Effective method when:
  - Individual enzymes become inactive during immobilization
  - Isolation and purification of enzyme is not cost effective
- Here enzymes will be active and stable for a long period of time Method of cell immobilization are same as described for enzyme immobilization
- Adsorption method is the oldest method [use of woodchips as a carrier]

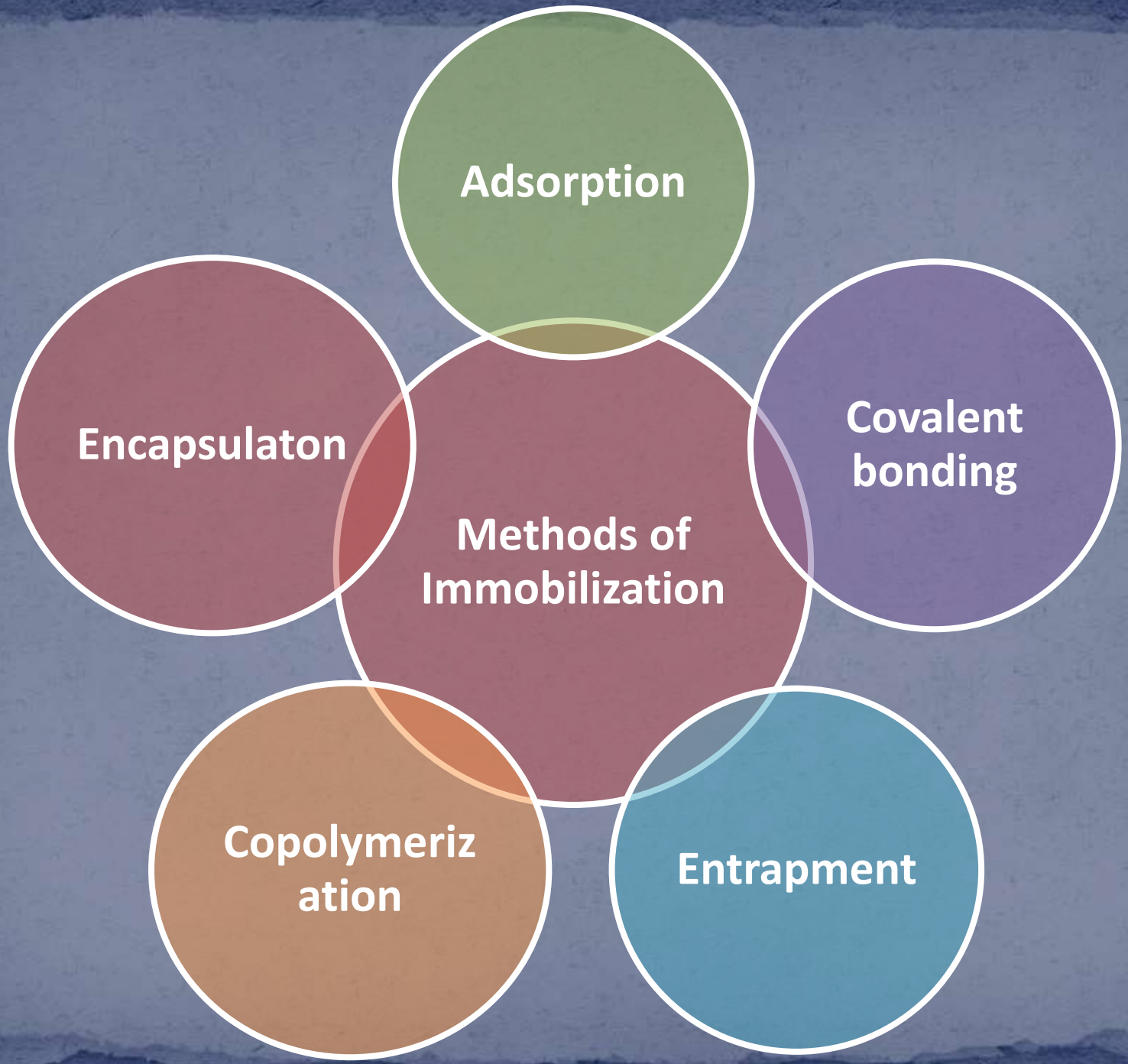

Subsrate
Callus Culture
Suspension Culture
Immobilised cells
Bioreactor containing immobilised cells
Product
Subsrate
E
isolated enzyme
E
E
E
E
Immobilised enzyme
Column containing immobilised enzymes
Product

Advantages

- Multiple enzymes can be introduced to a single step
- Extraction and purification of enzymes are not required
- Enzymes are stable for long time Native conformation of enzyme is best maintained
- Cell organelles like mitochondria and chloroplasts can be immobilized

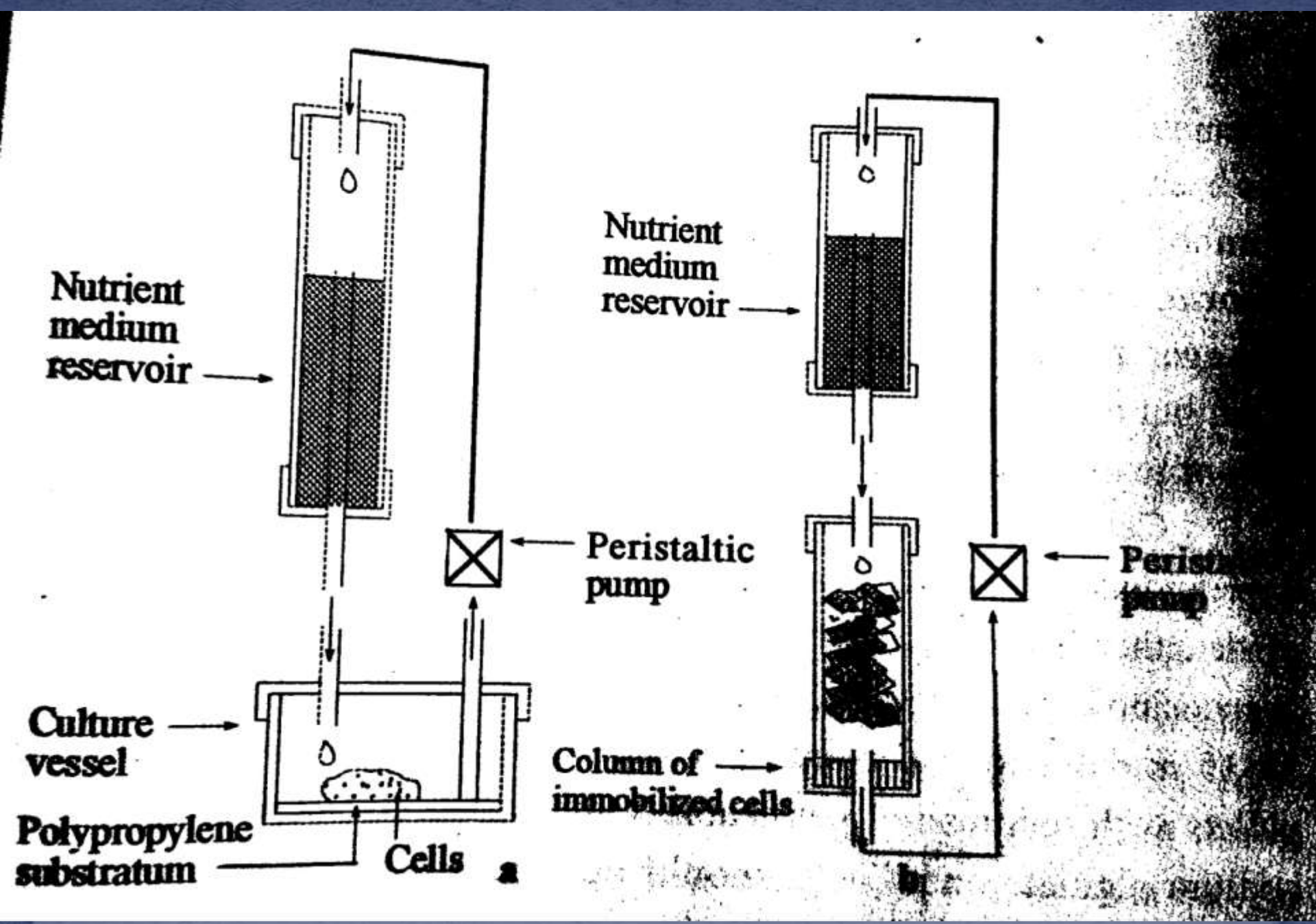
Disadvantages

- Concentration of enzymes will be less
- Production of unwanted enzymes and unwanted products
- Modification of end products by other enzymes


Adsorption
Encapsulaton
Methods of Immobilization
Covalent bonding
Copolymeriz ation
Entrapment

Immobilization of cells: Methods, Support materials, Cells and Reaction

Method	Support Material	Cells	Reaction
Adsorption	Gelatin	*Lactobacilli*	Lactose ⟹ lactic acid
	Porous glass	*Saccharomyces*	Glucose ⟹ ethanol
	Cotton fibers	*Zymomonas*	Glucose ⟹ ethanol
	DEAE Cellulose	*Nocardia*	Steroid conversion
Covalent bonding	Cellulose + cyanuric chloride	*S. cerevisiae*	Glucose ⟹ ethanol
	Titanium oxide	*Acetobacter*	Vinegar
Cross linking	Glutaraldehyde	*E. coli*	Fumaric acid
Entrapment	Aluminium alginate	*Candida tropicalis*	Phenol degradation
	Calcium alginate	*S. cervisiae*	Glucose ⟹ ethanol
Encapsulation	Polyester	*Streptomyces sps.*	Glucose ⟹ fructose
	Alginate polylysine	Hybridoma cells	Monoclonal antibodies


Nutrient medium reservoir
Peristaltic pump
Culture vessel
Polypropylene substratum
Cells
a
Nutrient medium reservoir
Column of immobilized cells
b

(1) Supply precursors of secondary products in large quantities but in low concentrations,

(2) Perform sequential chemical treatments on the cells and

(3) Harvest metabolites from the nutrient medium. The latter two manipulations are possible in semicontinuous batch reactors.

Table 10.6 : Various types of gels used for immobilization

Gel	Advantages / disadvantages	Concentration
Agar	Non toxic and freely permeable of inactivated cells	(2 %)
Polyacrylamide	Adverse effect on cell viability	-
Gelatin	form cross linked gelatin foam with 25 % glutaraldehyde	(20%)
K-Carrageenam	Forms gel in the presence of K^+ ions	3 %

- Net/ foam entrapment→ polyurethane

- Tubular hollow fibre membrane bioreactor:
- Cells entrapped in Tubular hollow fibres made up of cellulose acetate/ Si polycarbonate→ continuous/ batch culture→ in chemostat/ turbidostat fermenter

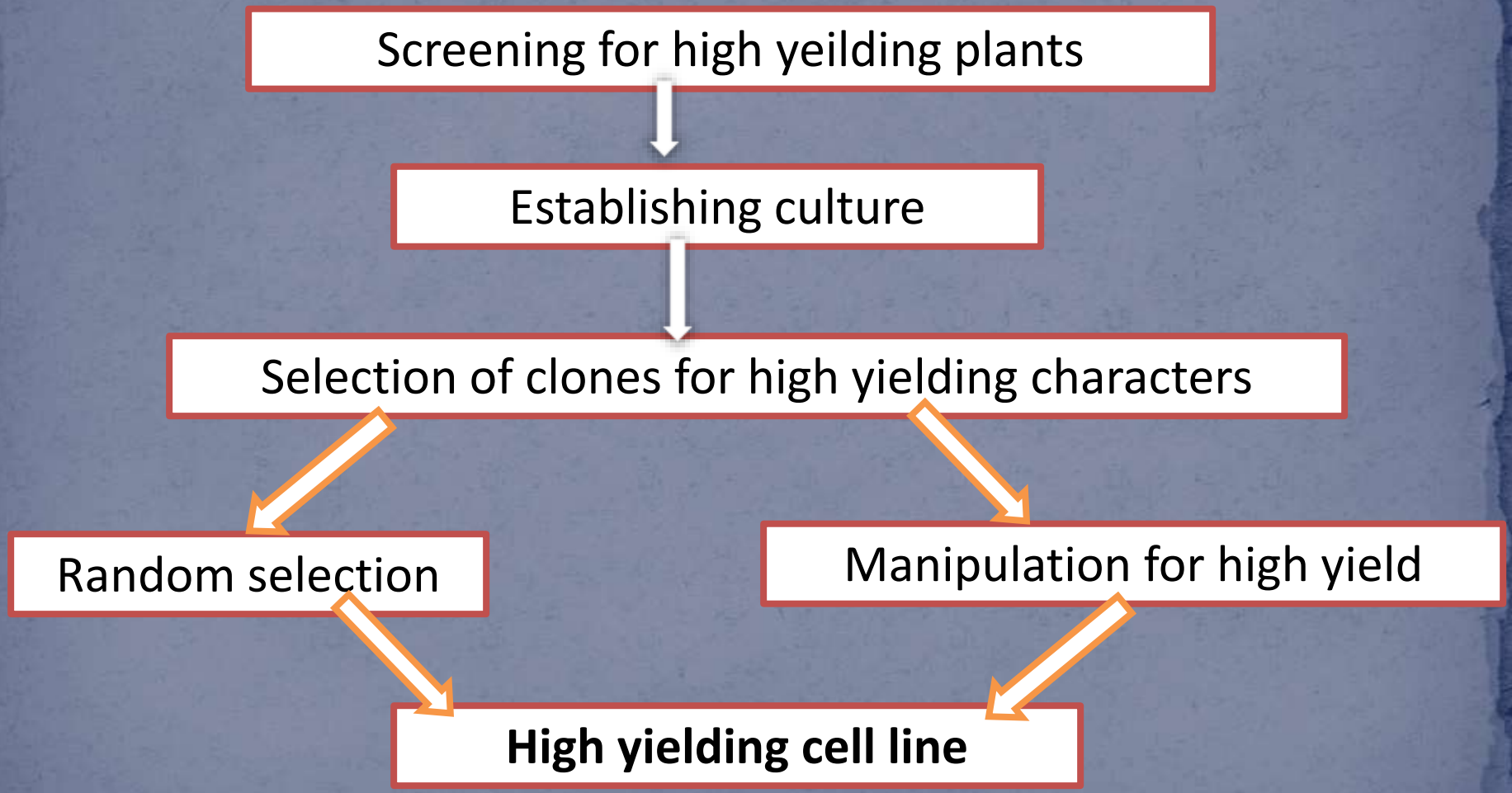
Applications

- Large scale production of secondary metabolites
- Long term storage/ transport of cells and protoplasts
- Can be used in cell- cell recognition phenomena
- Study of biochemical response of plant cells to infection

Table 10.7 : Summary of the application of cell immobilization

Species	Cell type	Immobilized substratum
Catharanthus roseus	cells	Calcium algenate
Digitalis lanata	cells	Calcium alginate
Cannabia sativa	cells	Calcium alginate
Glycine max	-	Hollow fibres
Datura innoxia	cells	Matting, calcium alginate
Capsicum frutescens	cells	Reticulate polyurethane
Mucuna pruriens	cells	Calcium alginate
Papaver somniferum	cells	Calcium algenate
Daucus carota	Protoplast	Agarose + lectins
Spinacia oleracea	Chloroplast	Calcium alginate
Persea americana	Mitochondria	-

Production of secondary metabolite

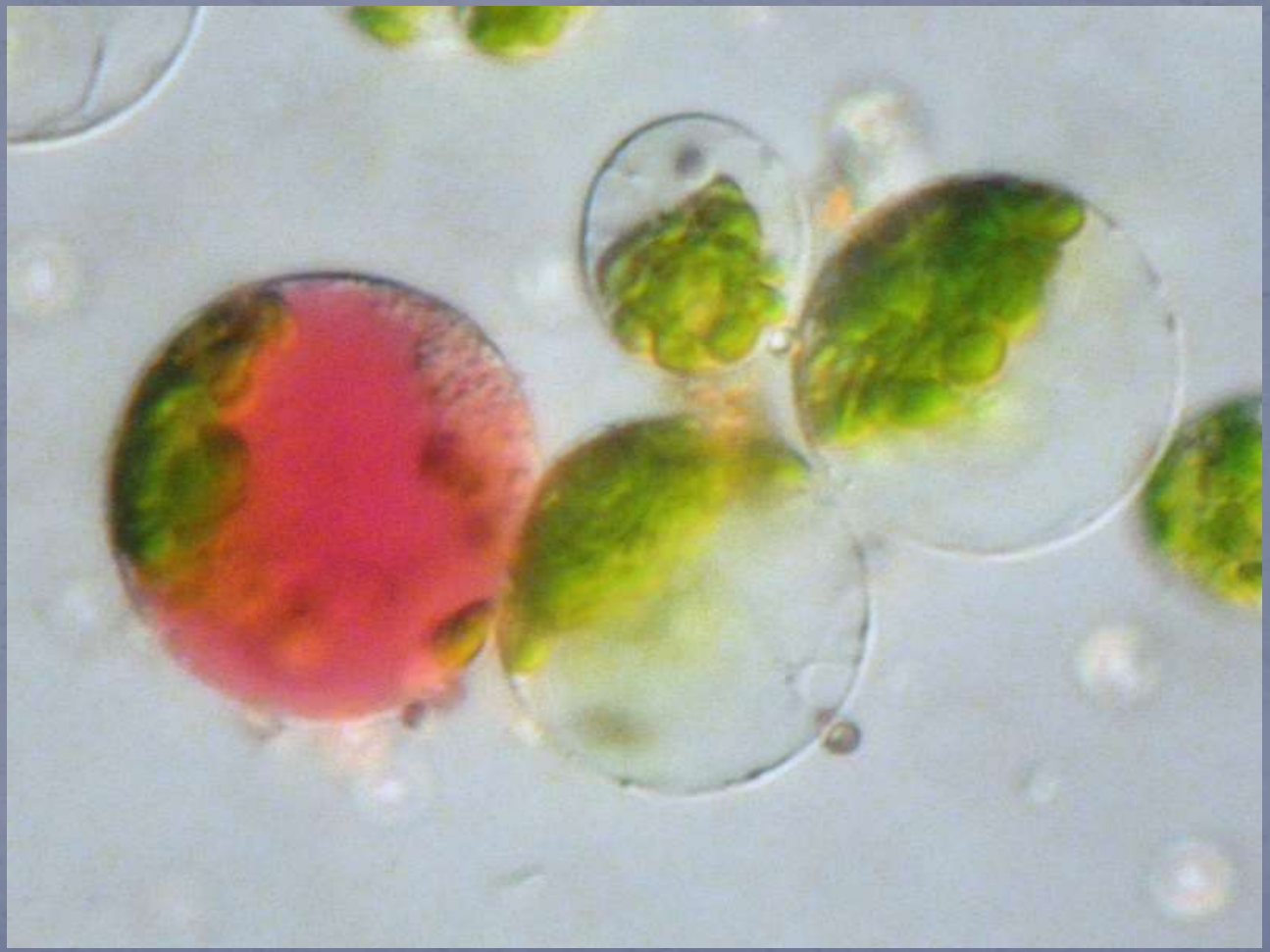


Examples

- Shikonin production
- From Lithospermum erythrorhizon
- Shikonin is used as traditional dye in herbal medicines and in lipsticks
- Procedure: mass production is done in LS (Linsmaier and Skoog) medium followed by second stage envolving transfer of cells into Whites medium

- Cultured Ginseng cell mass
- From Panax ginseng
- Used as adpotgenic agent, general tonic, as food additive
- Procedure: Callus and root tissue in culitvated MS medium. Crude saponin concentraon are in callus 21%, in redifferntiated roots 27.4%, in crown gall19% and in natural roots 4.1%

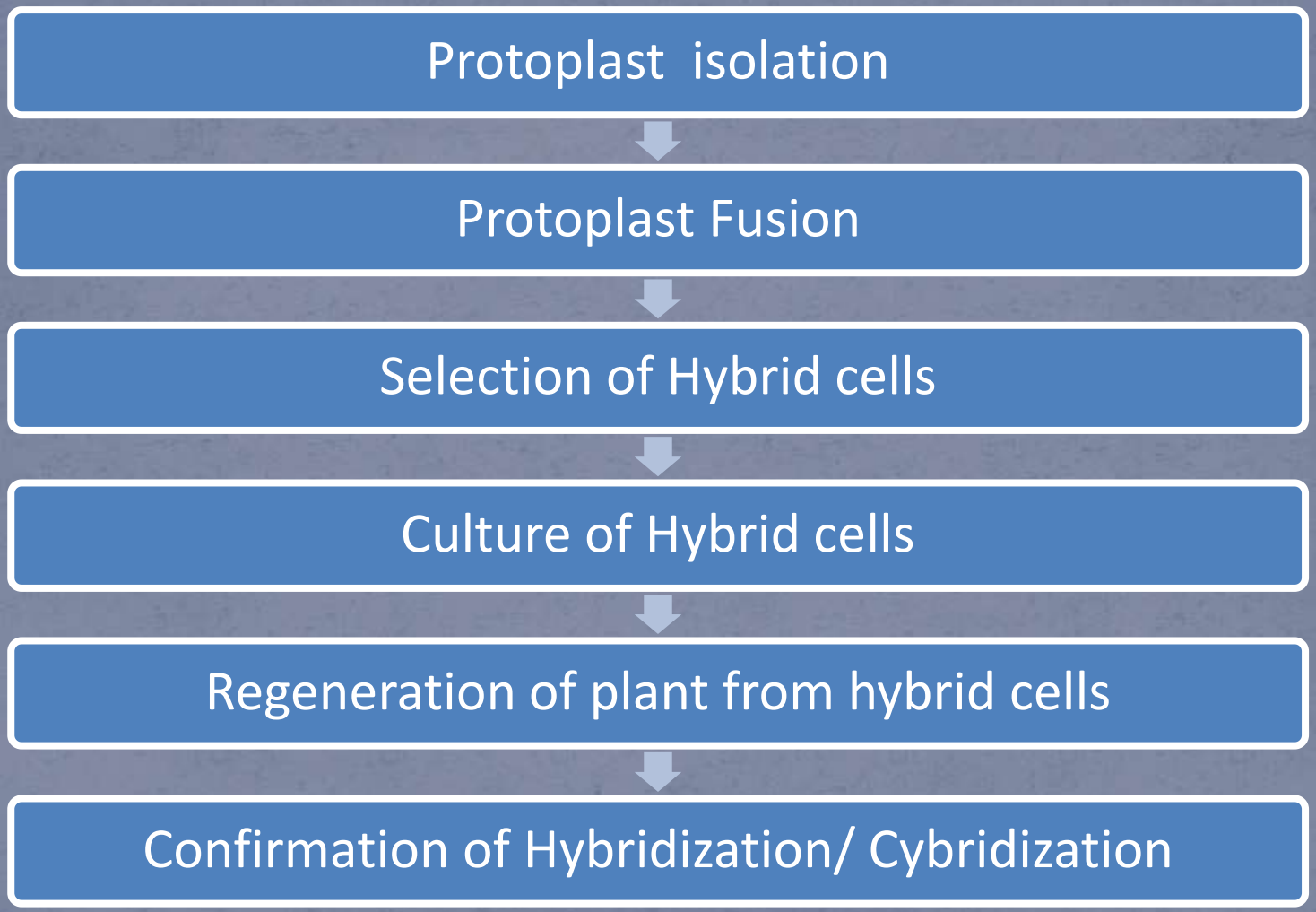
- Fused protoplast (left) with chloroplasts (from a leaf cell) and coloured vacuole (from a petal).



WHAT IS PROTOPLAST ?

- Protoplast are cells without a cell wall. They are naked cells which are potentially capable of cell wall regeneration , growth and division.
- Plant protoplasts have a great potential in securing genetic recombination through somatic hybridization.

PROCEDURE


Protoplast isolation
Protoplast Fusion
Selection of Hybrid cells
Culture of Hybrid cells
Regeneration of plant from hybrid cells
Confirmation of Hybridization/ Cybridization

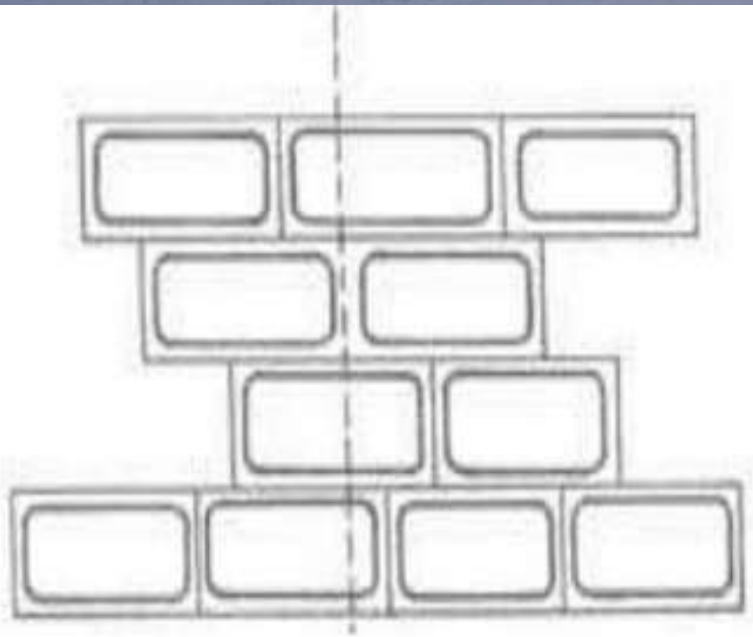
ISOLATION OF PROTOPLASTS

- Protoplasts can be isolated from a variety of plant tissues using either mechanical or enzymatic method of isolation.
- The plant material which is often chosen as a source of protoplasts in dicots in leaf mesophyll.
- Important part of isolation is removal of cell wall without causing damage to protoplast.

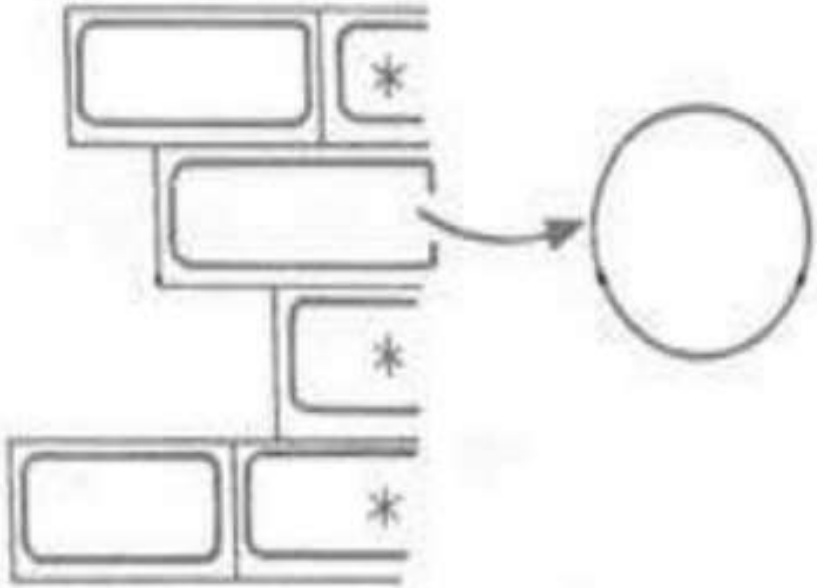
METHODS OF ISOLATION OF PROTOPLAST

- Mechanical isolation
- enzymatic isolation

ISOLATION OF PROTOPLAST BY MECHANICAL METHOD



A. Tissue is cut along the dotted line



B. Release of Protoplasts from Damaged cells

- ADVANTAGES : It is suitable method for the isolation of protoplasts from vacuolated cells. E.g. onion bulbs, radish roots.

- DISADVANTAGES :
  - # Poor yield # unsuitable for the isolation of protoplasts from meristematic cells
  - unsuitable for the isolation of protoplasts from less vacuolated cells
  - Tedious method

ISOLATION OF PROTOPLAST BY ENZYMATIC METHOD

- Sterilization of leaves
- Peeling of the epidermis
- Enzymatic treatment
- Isolation and cleaning of the protoplasts

- Commonly used enzymes

Type of cell	Enzyme
Plant cells	Cellulase, pectinase, xylanase
Gram-positive bacteria	Lysozyme (+EDTA)
Fungal cells	Chitinase

- Cellulase, hemicellulase, pectinase are commonly used at pH- 5.5-5.8 for 3-6 Hrs

Sterilization of leaves

- Fully expanded leaves are sterilized by the following procedure
  - dipping in 70% ethyl alcohol for about a minute and then with 2% solution of sodium hypochlorite for 20-30 minutes
  - rinsing with sterile distilled water three times.

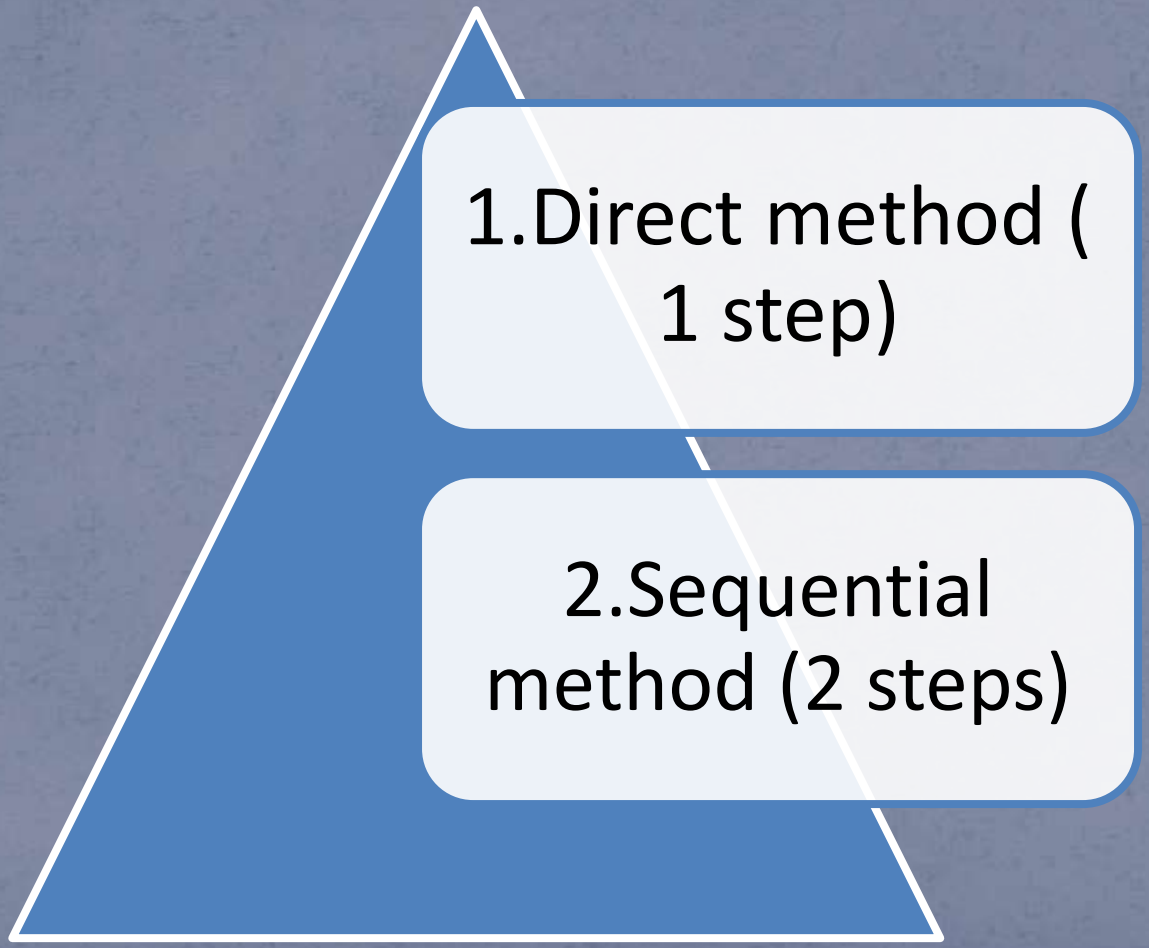
Peeling of the epidermal layer :

- The lower epidermis is carefully peeled off and the stripped leaves are cut into small pieces.
- This operation must be carried out under aseptic conditions
- Mesophyll protoplasts can be isolated from the peeled leaf segments while epidermis yields epidermal protoplasts

ADVANTAGES OF ENZYMATIC METHOD OVER MECHANICAL METHOD

- the cells are intact and are not injured in enzymatic method as in case of mechanical methods of isolation.
- Isolation of protoplasts from various tissues is possible on a large scale in enzymatic method.
- In enzymatic method , protoplasts could be obtained from non – vacuolated meristematic cells in which cell plasmolysis does not occur readily.

Enzymatic treatment :



- Direct method : Here simultaneous treatment with macerase (pectinase) and cellulose enzymes is carried out.

  0.5% macerase and 2% cellulose enzyme in 13% sorbitol or mannitol at pH 5.4
- Sequential method :
  - 1 step – Sample is treated with macerase (pectinase) enzyme for isolation of cells
  - 2 step – Isolated cells are treated with cellulose enzyme for protoplast isolation In both cases , peeled leaf segments are placed with the lower surface downwards in a petridish containing enzyme mixture

PURIFICATION:

1.Sedimentation

- the crude protoplasts suspension is centrifuged at low speed (50-100g for 5 min).
- The intact protoplasts form a pellet and supernatant containing cell debris can be pipetted off.
- The pellet is gently resuspended in fresh culture media plus mannitol and rewashed. This process is repeated two or three times to get relatively clean protoplast preparation.

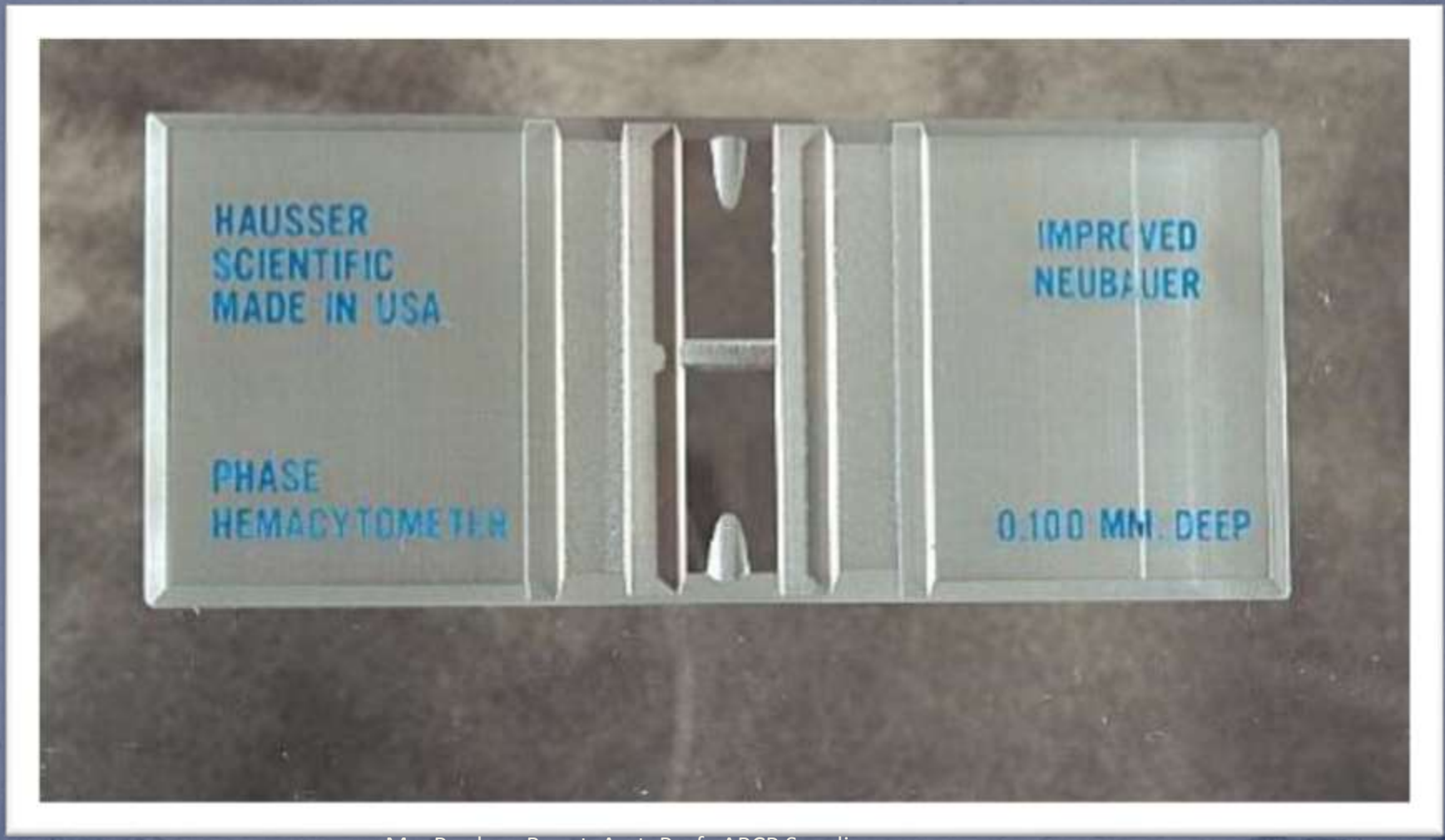
Floatation

- Protoplasts being lighter (low density) than other cell debris, gradients may be used, which will allow the protoplasts to float and the cell debris to sediment.
- A concentrated solution of **mannitol, Sorbitol** and **sucrose (0.3-0.6M)** can be used as a gradient and crude protoplasts suspension may be centrifuged in this gradient at an appropriate speed.
- Protoplasts can be pipetted off from the top of the tube after centrifugation.

Visualisation & viability of Protoplast

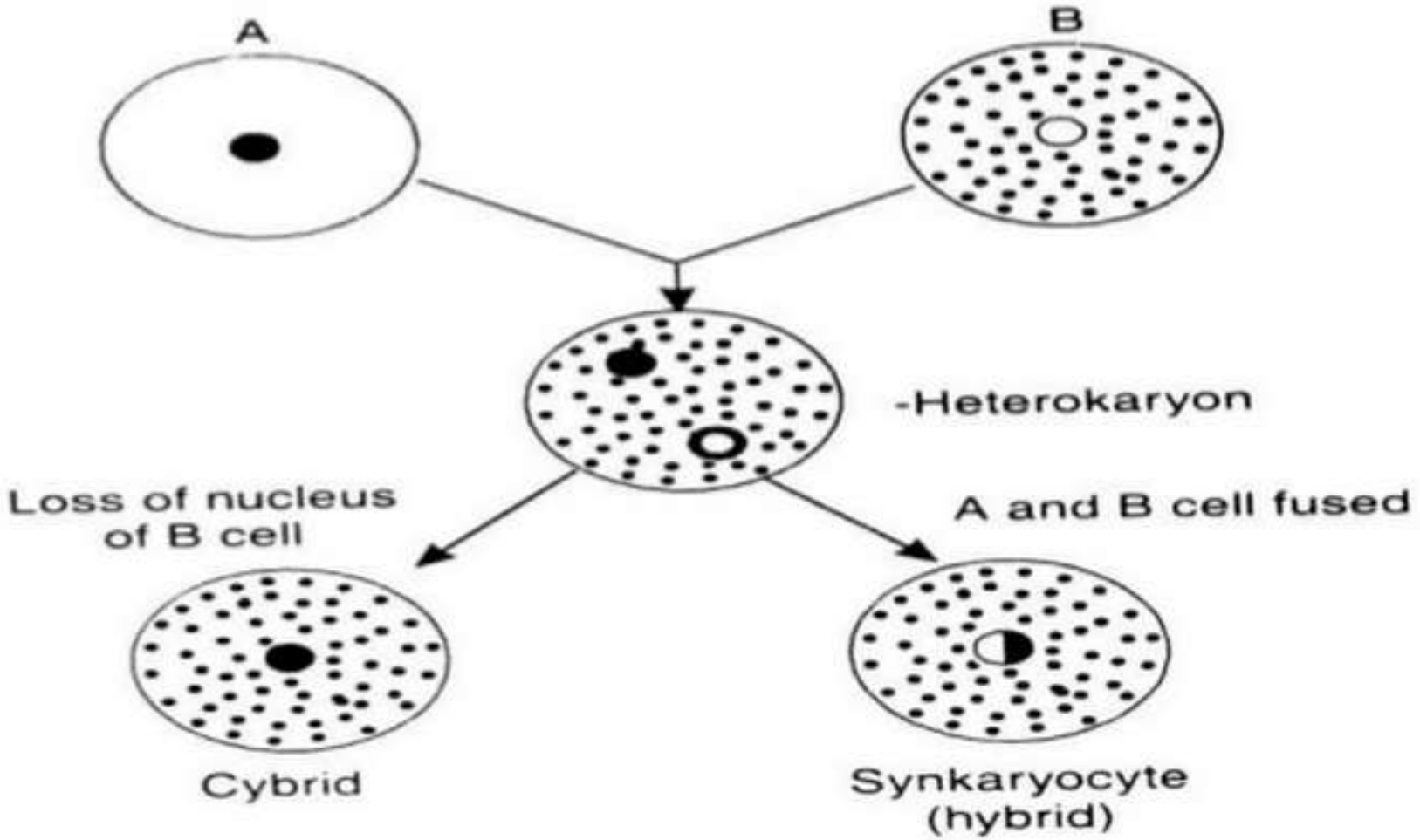
- Visualization under light microscope-spherical and absence of birefrigence. Stains- Tinapol(yellow), calcoflour(white) can be used
- Viability-FDA(living green), Phenosafrani n (0.1 %, dead protoplasts , which turn red), CPW (Calcofluor white) 0.1 % V/V(It detects onset of cell wall regeneration around plasma membrane of viable protoplasts in the form of a ring of fluorescence.)

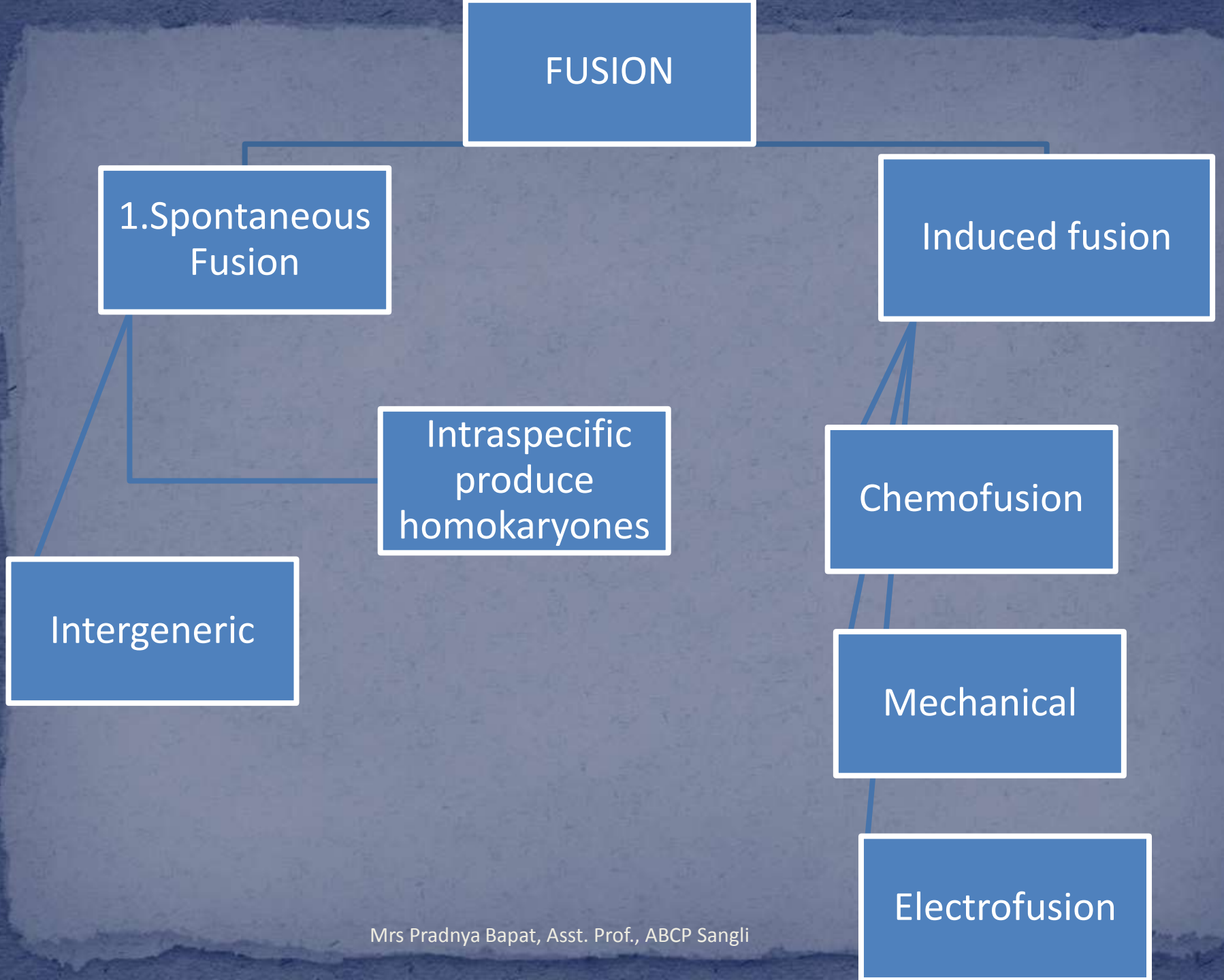
Counting of Protoplast



FUSION

- THE HYBRIDS AND CYBRIDS Fusion of cytoplasm of two protoplasts results in coalescence of cytoplasms.
- Thenuclei of two protoplasts may or may not fuse together even after fusion ofcytoplasms.
  - The binucleate cells are known as **heterokaryon or heterocyte**
  - When nuclei are fused the cells are known as **hybrid** or **synkaryocyte** .
  - Only cytoplasms fuse and genetic information from one of the two nuclei is lostis known as **cybrid** i.e. cytoplasmic hybrid or heteroplast .


A
B
-Heterokaryon
Loss of nucleus
of B cell
A and B cell fused
Cybrid
Synkaryocyte
(hybrid)


FUSION
1.Spontaneous Fusion
Induced fusion
Intraspecific produce homokaryones
Intergeneric
Chemofusion
Mechanical
Electrofusion

Spontaneous fusion

- During isolation of protoplasts for culture, when enzymatic degradation of cellwalls is affected, some of the protoplasts, lying in close proximity, may undergo fusion to produce homokaryons or homokaryocytes, each with 2-40 nuclei.
- The occurrence of multinucleate fusion bodies is more frequent, when protoplasts are prepared from actively dividing cells.

Induced Fusion

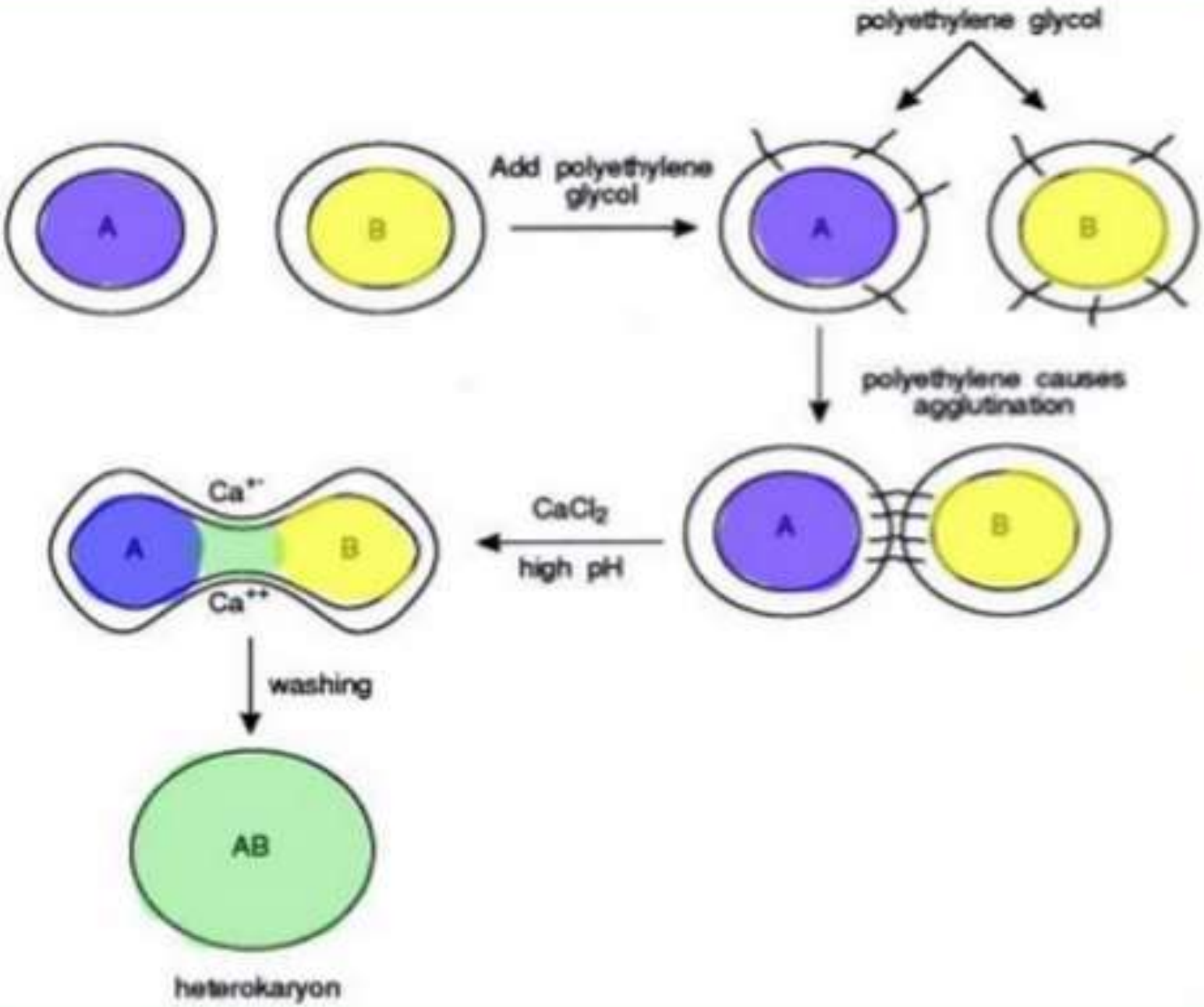
- Chemofusion - fusion induced by chemicals
- Types of fusogens
  - PEG
  - NaNo3
  - Ca 2+ ions
  - Polyvinyl alcohol
  - Lysozyme
  - Dextran
  - Fatty acids and esters.

- The following treatments have yielded success in producing somatic hybrid plants.
- $NaNO_3$ treatment :
  - Isolated protoplasts cleaned by **floating in 10% sucrose solution**. Transfer of protoplast in **5.5% $NaNO_3$ solution** → incubation→ centrifugation @2000rpm. protoplast kept in water bath for 30^0C for 30 min during which fusion occurs.
  - This treatment results in low frequency of heterokaryon formation , particularly when mesophyll protoplast are used.

- 2. High pH and high Ca++ treatment : Isolated protoplast incubated in solution of **0.5M mannitol** containing **0.05M CaCl2, pH 10.5 and temp. 37ºC (30-40 min.**) aggregation of protoplast and fusion usually occurs with in 10 min. By this methods , 20-50% of the protoplasts involved in fusion.

- 3. PEG treatment : This has the become the method of choice, due to its high success rate. Isolated **protoplasts** in culture medium **(1ml) +** equal volume **(1ml) of 28-56% PEG** in a tube. PEG enhances fusion of protoplasts in several species. Tube is **shaken(5 min)** and allowed to **settle(10 min)**. Settled protoplasts washed several times with culture medium. This method is widely used for protoplast fusion.

- MOA-PEG is potent Cell Agglutinator and also acts as Membrane Modifier. PEG causes dehydration effect on cell membrane and binds with the phospholipids within cell membrane→ membrane fusion.
- Advantage : a) high frequency of heterokaryon formation b) low toxicity to cells. c) reduced formation of binucleate heterokaryon


polyethylene glycol
A
B
Add polyethylene glycol
A
B
polyethylene causes agglutination
A
B
CaCl2
high pH
Ca++
A
B
Ca++
washing
AB
heterokaryon

Mechanical Fusion

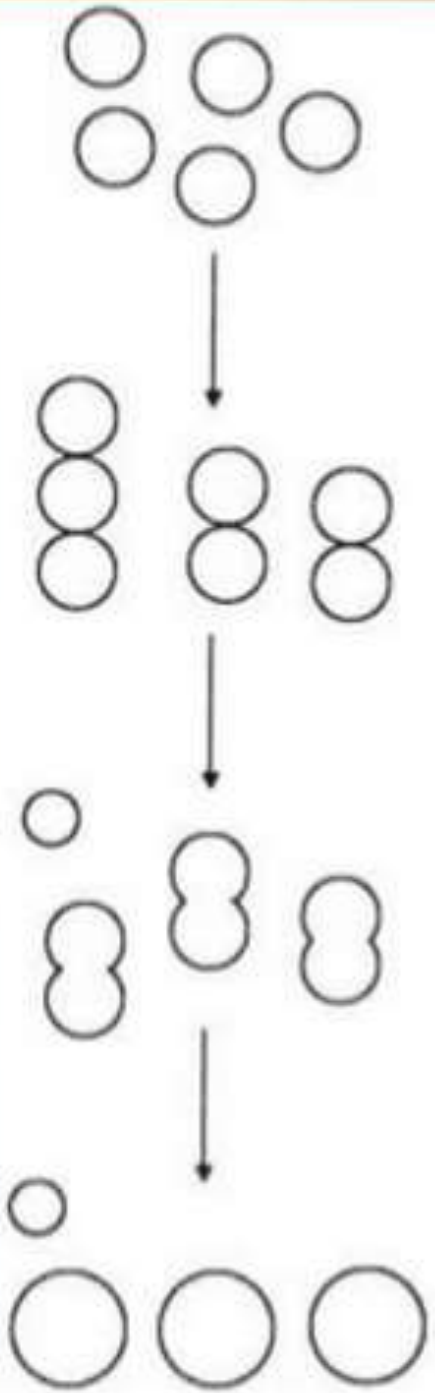
- Physical fusion of protoplasts under microscope by using micromanipulator and perfusion micropipette.

Mrs Pradnya Bapat, Asst. Prof., ABCP Sangli

Electrofusion

- Fusion induced by **electrical stimulation.**
- If Protoplasts are placed into a small culture vessel containing electrodes and a potential difference is applied, then the protoplasts will line up between the electrodes. If now an extremely short, electric shock is applied, protoplasts can be induced to fuse.

- Pearl chain of protoplasts is formed by **low** strength electric field (**100V/cm**)
- Fusion of protoplasts of pearl chain is induced by the application of high strength electric field (**1000V/cm**) for few microsecond.
- So the plasma lemma distribution and organization disturbances leading to the fusion of two protoplasts and the fusion of these protoplast is done in device **electroporator**



protoplasts in suspension

Low intensity AC current to protoplast suspension

cells line up as 'strings of pearls'

apply short DC pulse

750-1000V/cm for short duration 20-50 usec

breakdown of membrane at contact point

fusion

Identification and Selection of somatic hybrid cells

- Hybrid identification- Based on difference between the parental cells and hybrid cell with respect to pigmentation
- Cytoplasmic markers
  - Fluorochromes like FITC (fluoroscein isothiocyanate) and RITC (Rhodamine isothiocyanate) are used for labelling of hybrid cells
- Presence of chloroplast
- Nuclear staining
  - Heterokaryon is stained by carbol-fuschin, aceto- carmine or aceto-orcein stain

selection procedures

- **Auxin Autotrophy:**
- The selection of the hybrids of Nicotiana glauca and N. langsdorffi is based on auxin autotrophy of the hybrid cells (Fig 13.3). The parental protoplast or cell requires an auxin compound in order to proliferate, whereas hybrid callus tissue needs no such requirement because the cells are auxin autotrophic. Therefore, somatic hybrid cells can be isolated selectively by growth on auxin free culture medium. Auxin autotrophy of the hybrid cell is expressed only as a result of the genetic combination of the two parental protoplasts.

- **Use of Uncommon Amino Acids:**
- Attempts have also been made to utilize uncommon amino acids as selective agents.
- Conavaline which is present in some legume, inhibits division of soya bean and pea cells but sweet clover and alfalfa are unaffected.
- Heterokaryon obtained by the fusion of protoplast from soya bean with those from any one of the resistant plant will divide in presence of the conavaline.

- **Use of Phytotoxin:**
- Some of the well-known fungal toxin may be used in selecting the fusion product. For an example, the protoplast of cultured soya bean cells resistant to HmT toxin produced by Helminthosporium maydis race T, whereas the leaf protoplasts of Zea mays are sensitive to this toxin. It has been observed that fusion products of soya bean and Zea mays survive on toxin containing medium. On this it is suggested that toxin may be a useful selective agent in fusion experiment.

- **Use of Herbicides:**
- Plants possess differences in their capacity to metabolize herbicides. This property can be utilized effectively for selection. For an example, rice plants are resistant to propanil (3, 4-dichloropropionanilide). This resistance is based on the ability of rice cells to metabolize propanil.

CULTURE OF PROTOPLAST

- The protoplasts which are obtained after cleaning have to be suspended in a suitable medium in order to allow them to reform a cell wall and initiate divisions.

- For this purpose, they are allowed to grow in proper medium .

- NUTRIENT MEDIA -The nutrient media used for the culture of protoplasts are generally modified to contain **reduced levels of inorganic substances** .
- The Murashige and skoog ‘s medium is modified by reducing the levels of inorganic substance concentration of iron and zinc can also be lowered.
- **Osmotic stabilizers and plant growth substances** (cytokinin like kinetin , auxin like IAA)are two important ingredients in the protoplast culture medium.

METHODS OF CULTURE

- The methods employed in protoplast culture are the modifications of the methods used for the culture of plant cells are :
- Droplet culture
- Plating method
- Micro culture chambers
- Feeder layers

DROPLET CULTURE

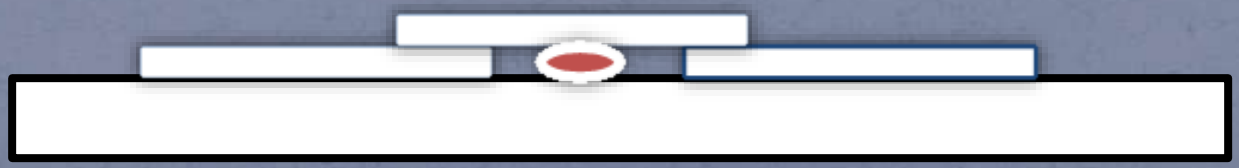
- Protoplasts are suspended in a liquid medium at a **density of about 105/ml** either in conical flasks or plastic Petri dishes .
- The droplet culture technique consists of placing approximately **50 ml of droplets** containing protoplasts in plastic Petri dishes.
- The plastic Petri dishes are sealed and incubated at **25-30^{o} C at low light intensities or in the dark.**

PLATING METHOD

- Protoplasts are suspended in a liquid medium in a Petri dish at double the concentration and mixed gently but quickly with an equal volume of the medium containing double the agar concentration .
- The Petri dishes are sealed and incubated upside down in continuous light at 23 to 25 C.

MICROCULTURE CHAMBERS

- This method requires the culturing of 30- 50 microleter of medium containing one or more protoplasts on a microscopic slide , which is enclosed by a cover glass resting on two other cover glasses placed on either side of the drop . The cultures are sealed with sterile paraffin oil and incubated in light at 23 to 25 degree celcius .



FEEDER LAYERS

- Non -dividing but metabolically active ,X – irradiated protoplasts embedded in nutrient agar support , the growth of protoplasts plated at very low densities above them.
- Feeder layer or nurse culture are also used where the fast growing protoplasts aid the recalcitrant species.

CELL WALL REGENERATION

- Protoplasts which are cultured in an appropriate medium show rapid respiration ,synthesis of RNA protein and polysaccharides , increase in size , formation of numerous cytoplasmic strands etc.
- Cell wall regeneration takes place by deposition of cellulose microfibrils on the surface of plasma membrane.
- The newly deposited cell wall is composed of loosely organized to form a typical plant cell wall.
- The first cell division after the formation of a new cell usually occurs between two to seven days after the culture.

REGENRATION OF PLANTS

- The regenration of protoplast derived callus into whole plant is either through embryogenesis or through organogenesis.
- Cell colonies formed from protoplasts can be transferred to fresh nutrient media for further growth and multiplication.
- In many species , shoot and root differentiation and plantlet formation can be induced in protoplast – derived callus tissues with the help of auxins and cytokinins .

Advantages of protoplast technology

- Production of novel interspecific and intergenic hybrid – can produce hybrids from sexually incompatible crosses. Eg. Pomato (Hybrid of potato and tomato)
- But hybrid are sterile and produce nonviable seed. hence protoplast fusion in same genus or closely related genus are more likely to be successful.
- Production of fertile diploids and polypoids from sexually sterile haploids, triploids and aneuploids
- Transfer gene for disease resistance, abiotic stress resistance, herbicide resistance and many other quality characters
- Production of somatic hybridization- Production of heterozygous lines in the single species which cannot be propagated by vegetative means
- Studies on the fate of plasma genes
- For study of virus replication and functions encoded by the virus genome

Limitations of Somatic hybridization

- Poor regeneration of hybrid plants
- Non-viability of fused products
- Not successful in all plants.
- Production of unfavorable hybrids
- No confirmation of expression of particular trait in somatic hybrids

Germplasm conservation

- Germplasma= total net content of genes
- The very objective of germplasm conservation (or storage) is to preserve the genetic diversity of a particular plant or genetic stock for its use at any time in future.
- A global body namely International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation.

Approaches for germplasm conservation

- In situ conservation
- Ex- situ conservation

In situ conservation

- The conservation of germplasm in their natural environment by establishing biosphere reserves (or national parks/gene sanctuaries
- This approach is particularly useful for preservation of land plants in a near natural habitat along with several wild relatives with genetic diversity.
- The in-situ conservation is considered as a high priority germplasm preservation programme.

Limitations of in situ conservation

- The risk of losing germplasm due to environmental hazards
- The cost of maintenance of a large number of genotypes is very high.

Ex-Situ Conservation:

- The genetic materials in the form of seeds or from in vitro cultures (plant cells, tissues or organs) can be preserved as gene banks for long term storage under suitable conditions.
- For successful establishment of gene banks, adequate knowledge of genetic structure of plant populations, and the techniques involved in sampling, regeneration, maintenance of gene pools etc. are essential.

Germplasm conservation in the form of seeds

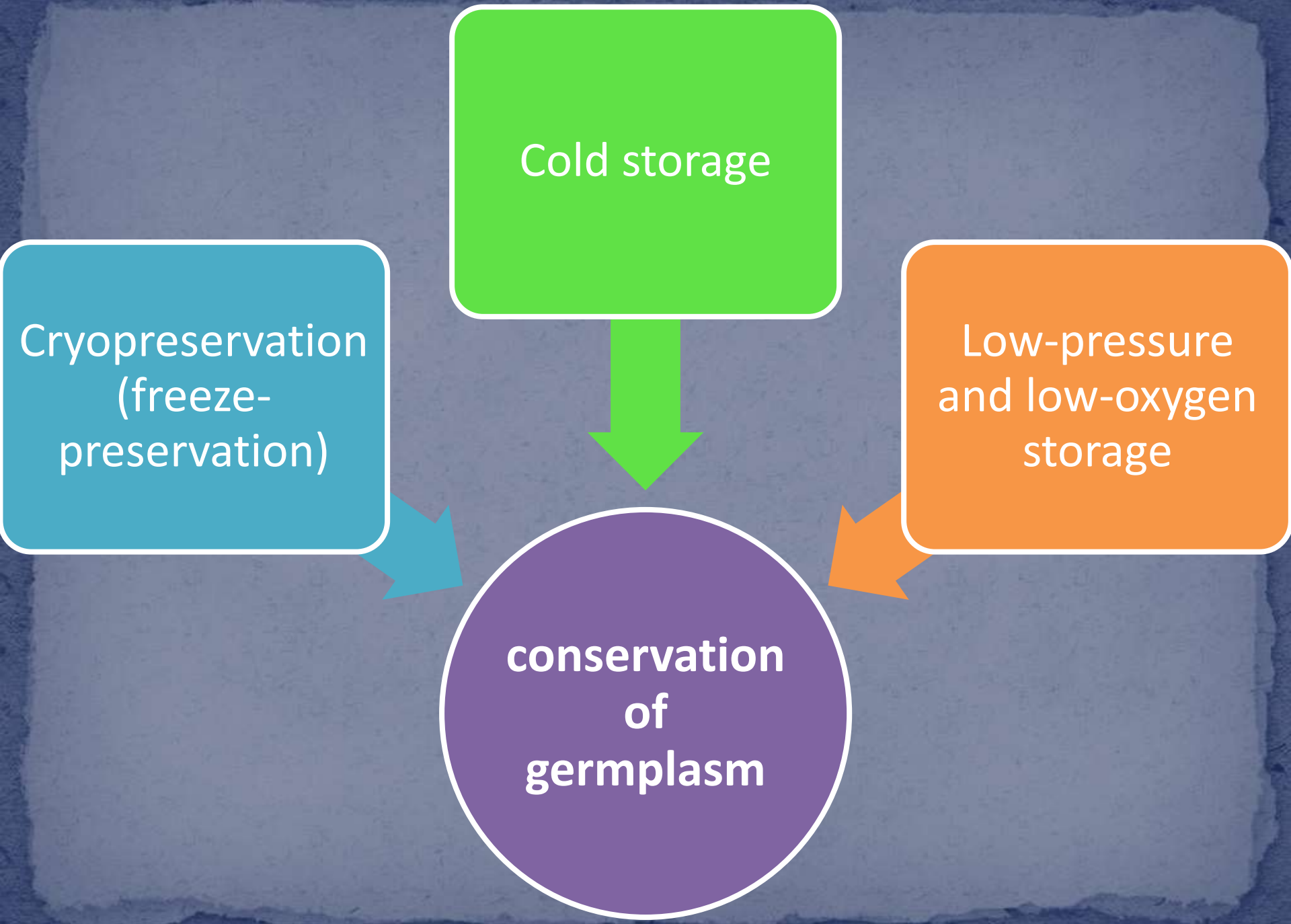
- Usually, seeds are the most common and convenient materials to conserve plant germplasm.
- This is because many plants are propagated through seeds, and seeds occupy relatively small space.
- Further, seeds can be easily transported to various places.

Limitations

- Viability of seeds is reduced or lost with passage of time.
- Seeds are susceptible to insect or pathogen attack, often leading to their destruction.
- This approach is exclusively confined to seed propagating plants, and therefore it is of no use for vegetatively propagated plants e.g. potato, Ipomoea, Dioscorea.
- It is difficult to maintain clones through seed conservation.

In vitro methods

- In vitro methods employing shoots, meristems and embryos are ideally suited for the conservation of germplasm of vegetatively propagated plants.
- The plants with recalcitrant seeds and genetically engineered materials can also be preserved by this in vitro approach.


Cold storage
Cryopreservation (freeze-preservation)
Low-pressure and low-oxygen storage
conservation of germplasm

Cryopreservation:

- Cryopreservation (Greek, krayos-frost) literally means preservation in the frozen state.
- The principle involved in cryopreservation is to bring the plant cell and tissue cultures to a zero metabolism or non-dividing state by reducing the temperature in the presence of cryoprotectants.
- **Cryopreservation broadly means the storage of germplasm at very low temperatures-**
  - Over solid carbon dioxide (at -79°C)
  - Low temperature deep freezers (at -80°C)
  - In vapour phase nitrogen (at -150°C)
  - In liquid nitrogen (at -196°C)

- Among these, the most commonly used cryopreservation is by employing liquid nitrogen. At the temperature of liquid nitrogen (-196°C), the cells stay in a completely inactive state and thus can be conserved for long periods.

- In fact, cryopreservation has been successfully applied for germplasm conservation of a wide range of plant species e.g. rice, wheat, peanut, cassava, sugarcane, strawberry, coconut.

- Several plants can be regenerated from cells, meristems and embryos stored in cryopreservation.

Mechanism of Cryopreservation:

- The technique of freeze preservation is based on the transfer of water present in the cells from a liquid to a solid state.
- Due to the presence of salts and organic molecules in the cells, the cell water requires much more lower temperature to freeze (even up to -68°C) compared to the freezing point of pure water (around 0°C).
- When stored at low temperature, the metabolic processes and biological deteriorations in the cells/tissues almost come to a standstill.

- **Limitations for Successful Cryopreservation:** Good technical and theoretical knowledge of living plant cells and as well as cryopreservation technique are essential.

- **Precautions (the limitations that should be overcome) for successful cryopreservation are :**
  - Formation ice crystals inside the cells should be prevented as they cause injury to the organelles and the cell.
  - High intracellular concentration of solutes may also damage cells.
  - Sometimes, certain solutes from the cell may leak out during freezing.
  - Cryoprotectants also affect the viability of cells.
  - The physiological status of the plant material is also important.

Technique of Cryopreservation:

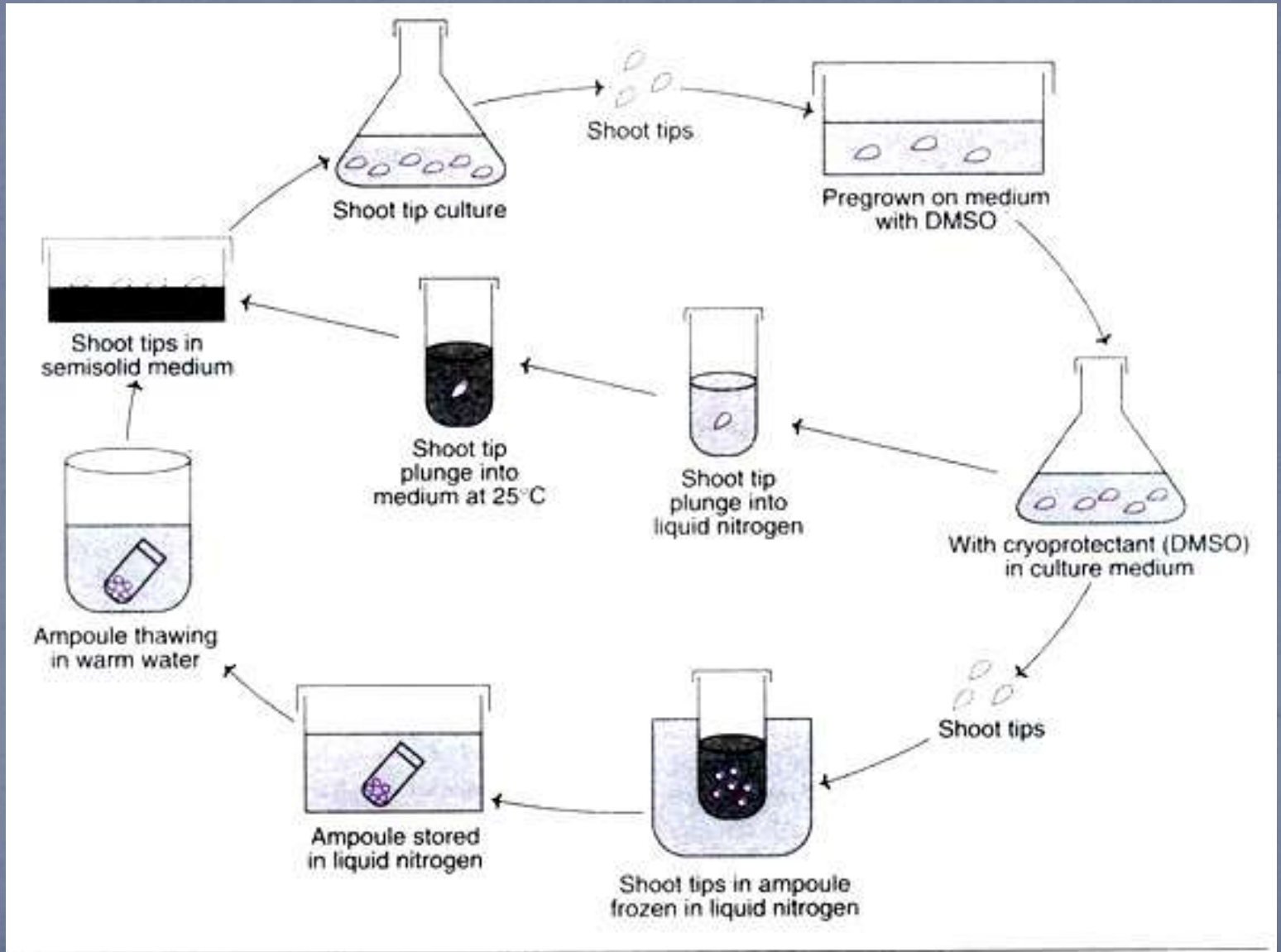


Fig. 48.1 : An outline of the protocol for cryopreservation of shoot tip (DMSO–Dimethyl sulfoxide).

Steps of cryopreservation

- 1. Development of sterile tissue cultures: culture in the late lag phase or log phase are most suitable.
- 2. Addition of cryoprotectants and pretreatment: Cryoprotectants are the compounds that can prevent the damage caused to cells by freezing or thawing. The freezing point and super-cooling point of water are reduced by the presence of cryoprotectants. As a result, the ice crystal formation is retarded during the process of cryopreservation.
  - There are several cryoprotectants which include dimethyl sulfoxide (DMSO), glycerol, ethylene, propylene, sucrose, mannose, glucose, proline and acetamide. Among these, DMSO, sucrose and glycerol are most widely used. Generally, a mixture of cryoprotectants instead of a single one is used for more effective cryopreservation without damage to cells/tissues.

- 3. Freezing
  - **1. Slow-freezing method:**
    - The tissue or the requisite plant material is slowly frozen at a slow cooling rates of 0.5-5°C/min from 0°C to -100°C→transferred to liquid nitrogen.
    - The advantage of slow-freezing method is that some amount of water flows from the cells to the outside.
    - promotes extracellular ice formation rather than intracellular freezing→the plant cells are partially dehydrated and survive better.
  - **2. Rapid freezing method:**
    - is quite simple and involves plunging of the vial containing plant material into liquid nitrogen.
    - a decrease in temperature -300° to -1000°C/min.
    - small ice crystals are formed within the cells and also the growth of intracellular ice crystals is minimal.
    - is used for the cryopreservation of shoot tips and somatic embryos.

- **3. Stepwise freezing method:**
  - This is a combination of slow and rapid freezing
  - The plant material is first cooled to an intermediate temperature and maintained there for about 30 minutes →rapidly cooled by plunging it into liquid nitrogen.
  - Stepwise freezing method has been successfully used for cryopreservation of suspension cultures, shoot apices and buds.
- **4. Dry freezing method: N**on-germinated dry seeds can survive freezing at very low temperature in contrast to water-imbibing seeds which are susceptible to cryogenic injuries. In a similar fashion, dehydrated cells are found to have a better survival rate after cryopreservation.

- 4. Storage: at temperatures in the range of -70 to -196°C. However, with temperatures above -130°C, ice crystal growth may occur inside the cells which reduces viability of cells. Storage is ideally done in liquid nitrogen refrigerator -150°C in the vapour phase, or at -196°C in the liquid phase.
- 5. Thawing: by plunging the frozen samples in ampoules into a warm water (temperature 37-45°C) bath with vigorous swirling.
  - rapid thawing (at the rate of 500- 750°C min^{-1}) occurs, and this protects the cells from the damaging effects ice crystal formation.
  - Ampules are quickly transferred to a water bath at temperature 20-25°C.
- 6. Re-culture: washing several times with water to remove ?
- 7. Measurement of survival/viability:triphenyl tetrazolium chloride (TTC), Evan's blue and fluorescein diacetate (FDA)
- 8. Plant regeneration.

Advantages

- Large quantities of materials can be preserved in small space.
- The germplasm preserved can be maintained in an environment, free from pathogens.
- It can be protected against the nature's hazards.
- From the germplasm stock, large number of plants can be obtained whenever needed.
- Obstacles for their transport through national and international borders are minimal (since the germplasm is maintained under aspectic conditions).

TABLE 48.1 A selected list of plants in various forms that are successfully cryopreserved

Plant material	*Plant species*
Cell suspensions	*Oryza sativa*
	Glycine max
	Zea mays
	Nicotiana tabacum
	Capsicum annum
Callus	*Oryza sativa*
	Capsicum annum
	Saccharum sp
Protoplast	*Zea mays*
	Nicotiana tabacum
Meristems	*Solanum tuberosum*
	Cicer arietinum
Zygotic embryos	*Zea mays*
	Hordeum vulgare
	Manihot esculenta
Somatic embryos	*Citrus sinensis*
	Daucus carota
	Coffea arabica
Pollen embryos	*Nicotiana tabacum*
	Citrus sp
	Atropa belladona

Cold Storage:

- germplasm conservation at a low and non-freezing temperatures (1-9°C)
- The growth of the plant material is slowed down in cold storage in contrast to complete stoppage in cryopreservation.
- Hence, slow growth germplasm conservation method.
- no cryogenic injuries.
- Long-term cold storage is simple, cost-effective and yields germplasm with good survival rate.
- e.g. grape plants, strawberry plants.
- Virus- free strawberry plants could be preserved at 10°C for about 6 years, with the addition of a few drops of medium periodically (once in 2-3 months).
- Several grape plants have been stored for over 15 years by cold storage (at around 9°C) by transferring them yearly to a fresh medium.

Low-Pressure and Low-Oxygen Storage:

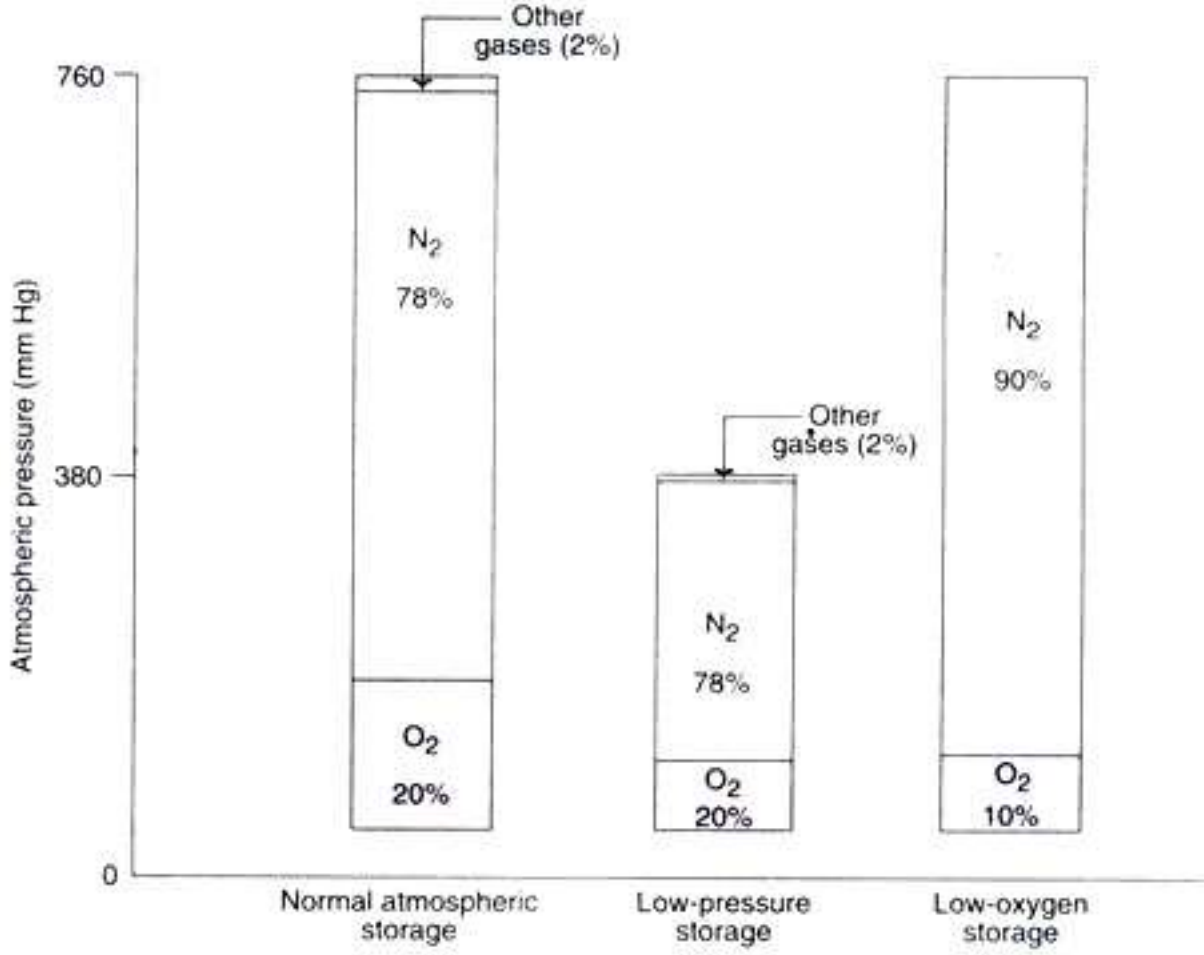


Fig. 48.2 : *A graphic representation of tissue culture storage under normal atmospheric pressure, low-pressure, and low-oxygen.*

Low-Pressure Storage

- the atmospheric pressure surrounding the plant material is reduced→partial decrease of the pressure exerted by the gases around the germplasm→ reduced in vitro growth of plants (of organized or unorganized tissues).
- The short-term storage is particularly useful to increase the shelf life of many plant materials e.g. fruits, vegetables, cut flowers, plant cuttings.
- The germplasm grown in cultures can be stored for long term under low pressure.
- LPS reduces the activity of pathogenic organisms and inhibits spore germination in the plant culture systems.

Low-Oxygen Storage

- In the low-oxygen storage, the oxygen concentration is reduced, but the atmospheric pressure (260 mm Hg) is maintained by the addition of inert gases (particularly nitrogen).
- The partial pressure of oxygen below 50 mm Hg reduces plant tissue growth (organized or unorganized tissue).
- This is due to the fact that with reduced availability of O_2, the production of CO_2 is low→ the photosynthetic activity is reduced, thereby inhibiting the plant tissue growth and dimension.

Applications of Germplasm Storage

- 1. Maintenance of stock cultures
- 2. Cryopreservation is an ideal method for long term conservation of cell cultures which produce secondary metabolites (e.g. medicines).
- 3. Disease (pathogen)-free plant materials can be frozen, and propagated whenever required.
- 4. Recalcitrant seeds can be maintained for long.
- 5. Conservation of somaclonal and gametoclonal variations in cultures.
- 6. Plant materials from endangered species can be conserved.
- 7. Conservation of pollen for enhancing longevity.
- 8. Rare germplasms developed through somatic hybridization and other genetic manipulations can be stored.
- 9. Cryopreservation is a good method for the selection of cold resistant mutant cell lines which could develop into frost resistant plants.
- 10. Establishment of germplasm banks for exchange of information at the international level.

Limitations of Germplasm Storage:

- Expensive equipment and the trained personnel.
- It may, however, be possible in the near future to develop low cost technology for cryopreservation of plant materials.


FINALLY,
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